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Wound management and healing in space

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Published in
Frontiers in Bioengineering and Biotechnology

ISSN 1664-8714
ISBN 978-2-83250-983-8
DOI 10.3389/978-2-83250-983-8

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Wound management and healing in space

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Citation

Monici, M., van Loon, J. J. W. A., Chouker, A., Iorio, C. S., eds. (2022). *Wound management and healing in space*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-983-8

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Editorial
PUBLISHED 23 November 2022
DOI 10.3389/fbioe.2022.1078986

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

RECEIVED 24 October 2022
ACCEPTED 31 October 2022
PUBLISHED 23 November 2022

CITATION
Monici M, van Loon JJWA, Choukér A and Iorio CS (2022), Editorial: Wound management and healing in space. *Front. Bioeng. Biotechnol.* 10:1078986. doi: 10.3389/fbioe.2022.1078986

Editorial: Wound management and healing in space

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KEYWORDS

wound healing, tissue repair and regeneration, space, space exploration, space exposome

Editorial on the Research Topic
Wound management and healing in space

Life without wound healing (WH) is impossible. To repair injured tissues is a biological process of crucial importance because it restores the tissue´s homeostasis and hereby the integrity of the body. WH is an extremely complex process. It consists of a succession of events that have as protagonists different cell populations whose behavior is strictly regulated qualitatively, quantitatively and temporally by multiple biochemical and biophysical factors.

Despite the large amount of studies carried out and the remarkable progress recently made in understanding the mechanisms underlying WH, there are still many knowledge gaps to be filled in order to progress in the development of effective therapeutic strategies, which aim to promote tissue regeneration instead of scarring.

This Special Issue on "Wound Management and Healing in Space" aims to address all the factors that could affect WH in this exposome of the hostile environment, as characterized by microgravity, radiation, conditions of isolation and confinement. In Space the human body undergoes an adaptation process that is characterized by pathophysiological alterations that could modify the resilience of the organism and its ability to respond to injuries. Furthermore, according to our present knowledge, the behavior of cell populations involved in WH is altered in Space. Very likely, these changes will have an impact on WH in space exploration.

In future space exploration missions beyond low Earth orbit (LEO), already small wounds can affect the mission performance and can last longer than expected. The management of serious wounds however, both traumatic and surgical, ulcers, and burns could be particularly challenging and mission ciritical due to the impossibility of carrying out a rapid medical evacuation to Earth. Also the delay in communications limits the remote assisted procedures and compromises the ability to guide the crew efficiently. Therefore, Space Agencies have included

WH in the context of spaceflight among the potentially critical problems to be approached for planning future deep space missions.

A perspective paper (Puhl et al.) included in this collection of manuscripts reports the approach of the European Space Agency (ESA), that started with the setup of a specific Topical Team (TT) of experts to identify potential concerns about WH in Space, draw up recommendations and suggest countermeasures. Currently, ESA is supporting research projects focused on specific aspects of WH and tissue regeneration in Space, and, in future perspective, it is building a 3D bioprinting system and a bioreactor for the maturation of tissue-constructs on the ISS. These facilities are a crucial starting point to enable advanced strategies for tissue regeneration and development of personalized grafts for crew members if serious injuries were to happen during long-term deep space exploration missions.

An important topic connected to WH and hemorrhage is hemostasis and platelet function, which also contributes to other biological processes through the release of growth factors and many other molecules. The paper by Locatelli et al. reviewed the very few studies on platelet function in Space. The results of these studies demonstrated that microgravity affects platelet's number and function, thus increasing the risk of hemorrhages and contributing to delay WH. However, Platelet Rich Plasma (PRP), although in simulated microgravity it proved less effective than in normogravity, could be evaluated as a countermeasure to prevent WH delay.

After hemostasis, the process of WH proper begins, classically divided into the inflammatory, proliferative, and remodelling phases. Fibroblasts have a significant role in all the three phases and they are the main protagonists of the remodelling phase. Moreover, they orchestrate the whole healing process through cross talk with immune cells, endothelial cells, and keratinocytes. Due to this central role in the process, the studies on the effects induced by real and modeled microgravity on fibroblast functions involved in WH have been reviewed by Cialdai et al. to define the gaps of knowledge about fibroblast function in Space and also to provide cues for developing adequate countermeasures. Interestingly, some microgravity-induced alterations of fibroblast function are similar to fibroblast dysfunctions observed in impaired WH on Earth.

Also research on the behavior of endothelial cells in weightlessness has been reviewed, as endothelial cells are responsible for neoangiogenesis during the proliferative phase of WH (Morbidelli et al). Angiogenesis output comes from a finely regulated balance between pro- and antiangiogenic factors, in order to avoid insufficient or excessive nonreparative neovascularization. The understanding of the factors and mechanisms that control angiogenesis and their changes in unloading conditions can help to design countermeasures to optimize neoangiogenesis in case of traumatic injury or surgical wounds during missions.

A mini-review (Bacci and Bani) on the effects of unloading conditions on epidermis and keratinocytes, that are responsible for re-epithelialization, shows that epidermal stem cells cultured in simulated microgravity undergo enhanced proliferation and viability and reduced terminal differentiation than under normal gravity. In the meantime, microgravity also triggers epithelial-mesenchymal transition of keratinocytes, promoting a migratory behavior. However the cross-talk between fibroblasts and keratinocytes is impaired and epidermal repair is delayed. These results confirm that WH is an "ensemble" that needs a strict regulation as regards timing and players, respectively.

One of the most important mechanisms in WH regulation is apoptosis, or programmed cell death. Apoptosis enables orchestrated cell removal in wounded or infected tissues. A dedicated review (Riwaldt et al.) provides an overview of alterations in the behavior of cutaneous cell lineages under microgravity, in regard to the impact of apoptosis in WH. Moreover, the current knowledge about WH in Space and simulated microgravity with respect to apoptosis and available therapeutic strategies is discussed, and the opportunity to use microgravity to obtain new insights into the role of apoptosis in WH is considered.

Correct WH evolution is also affected by systemic conditions. The association of insulin resistance and WH impairment may be hypothesized from some dysmetabolic conditions, like the metabolic syndrome, type 2 diabetes mellitus and abdominal/visceral obesity, where derangement of glucose and lipid metabolism, greater low-grade inflammation, altered adipokine secretion and adipocyte dysfunction converge to produce systemic effects that also negatively involve WH. Interestingly, chronic low-grade inflammation and insulin resistance appear to be pivotal events linking many of the pathophysiological alterations induced by spaceflight. Based on these considerations, one of the papers of this collection is devoted to discuss the pathophysiological links between microgravity-associated insulin resistance and impaired WH (Strollo et al.).

The microbial populations settled on skin, space modules, and in space suits can also play a significant role in WH. A paper of this special issue (Marvasi et al.) discusses a perspective that includes four domines for applying skin microbiota to WH in Space: 1) the natural antimicrobial properties of the skin microbiota, 2) the cross-talk between skin microbiota and immune system during WH, 3) the contribution of the microbiota in precision medicine, and 4) the role of gut-skin and gut-brain axes. A stronger understanding of the connections among bacteria, fungi, host immune system, and host metabolism could help improving WH in Space and on Earth.

Two main objectives of the studies on WH are: 1) find strategies leading to tissue regeneration; 2) create tissue substitutes to replace damaged tissues and support their functions. The achievement of these objectives is particularly important to manage WH in Space. Three-dimensional (3D) bioprinting (BP) might offer a solution, providing 3D tissue constructs, which can serve as models in basic research as well as in the development of transplantable skin grafts. The perspective paper dealing with this topic provides an overview of the state of the art of skin BP and approaches to establish this additive manufacturing technology in Space. In addition, the several advantages of BP for utilization in future manned space missions are highlighted (Cubo-Mateo and Gelinsky).

A new autologous micrografting (AMG) technology is described in a manuscript (Aliberti et al.) that shows how its use can remove the limitations associated with autograft transplants (e.g., risk of infections, secondary diseases, and low compliance for the patient). Among several re-epithelialization technologies developed to establish a physiological WH process, it has been demonstrated that the AMG technology is able to respond to the principal limitations of the current gold standard approach to autologous grafting. This includes the need to use large quantities of tissues, long sample preparation time, and long-term hospitalization. The proposed AMG technology plays a key role in the reepithelialization stage by modulating the genes responsible for angiogenesis and cell migration, including the migration of fibroblasts.

Temporary storage of nasal tissues and nasal cell sheets is an important issue in regenerative medicine. One of the presented papers (Kasai et al.) reports a study that investigated the preservation of chilled and frozen nasal tissues and expiry dates of ready-to-use nasal cell sheets. The results show that nasal tissues can be stored temporarily in refrigerators or deep freezers, and Hank's balanced salt solution (HBSS) can be used for preservation of ready-to-use cell sheets for a few days. *In vitro* cell sheet grafting assays demonstrated that cell sheets stored in HBSS for 2 days adhered to collagen gel and expanded normally.

An interesting original research by Leyi Xue et al., which is included in this paper collection, focuses on the development of Artemisia argyi plant extract (AE) loaded composite hydrogel scaffold based on methacrylate gelatin (GelMA)/methacrylate hyaluronic acid (HAMA) and mesoporous silica nanoparticle (MSN) as sustained-release drug carrier vehicles for the treatment of chronic wounds. In *vitro* and *in vivo* (animal model) experiments the AE loaded hydrogel showed stable rheological properties, suitable mechanical properties, appropriate biodegradability and biocompatibility, swelling, sustained capacity to release AE, which confers significant antibacterial and anti-inflammatory effects. Moreover, the AE-loaded hydrogel was able to induce in macrophages the transition from M1 to M2 phenotype and promote WH modulating the expression of pro- and anti-inflammatory cytokines.

Distinct physical factors existing in the cellular microenvironment are crucial to the biological homeostasis of stem cells. While substrate stiffness and orientation are known to regulate the mechanical remodelling and fate decision of mesenchymal stem cells (MSCs) separately, it remains unclear how the two factors are combined to manipulate their mechanical stability under gravity vector. An original paper studied these combined effects and showed that: i) in the different conditions, MSC mechanical stability is reached through changes in the cytoskeletal networks of actin and vimentin; ii) cell morphology and focal adhesion are mainly affected by substrate stiffness; iii) mechanistically, in the different stiffness conditions, the cell tends to be stabilized *via* β1 integrin–focal adhesion complexes–actin mechanosensitive axis (Zhang et al.).

In remote environments, such as deep space, where diagnostic tools and medical surveillance are scarce, the monitoring of clinical parameters could become crucial to timely detect life-threatening conditions. Nowadays, some signals can be measured using wearable technology. However, often the low quality of the recordings leads to wrong conclusions. Therefore, it is important to determine which parts of the signal are of sufficient quality. The results of a study by Rozo et al. on the quality assessment of respiratory signals obtained from wearable sensors show that with pre-trained machine learning classifiers in conjunction with data augmentation and transfer learning, it is possible to properly identify clean and noisy respiratory bioimpedance signals. These findings could be beneficial for the steps of data processing and connection with decision support systems when designing new bio-monitoring devices for space exploration.

Pantalone et al. described accordingly the possibility, even if remote, that serious traumatic events occur or there is a need for surgical treatments aboard a spacecraft. In this case, hemorrhage can be a life-threatening condition. Although the consequences of a haemorrhage during space flight are quite difficult to predict, the different aspects of hemorrage in Space and possible countermeasures are reviewed.

Wound management requires the development of reliable wound monitoring systems to facilitate the assessment and proper care of wounds in isolated environments, such as Space. An original study by Miskovic et al., which aims to develop a device for real-time *in-situ* wound temperature monitoring, provides a full characterization of a sensing element composed of thermotropic liquid crystals arrays embedded between two elastomer layers, and discusses how such a system compares against infrared thermography (non-local measurements), a technique commonly used to measure temperature distribution at the wound site.

The collection of papers in this special issue derives largely from the activities carried out within the ESA-TT "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration" and the ESA supported-Microgravity Application Program (MAP) WHISPER Project "Wound Healing in Space: Problems and Perspectives for Tissue Regeneration and Engineering".

The guest editors thank all the authors who contributed to the Special Issue on "Wound Management and Healing in Space" with their very interesting manuscripts and we hope the readers will share this view and future studies will contribute to a better understanding of wound behavior, and hence wound treatment, in human space exploration missions.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

This special issue has been supported by the ESA Topical Team on "Tissue healing in space: techniques for promoting and

monitoring tissue. repair and regeneration" (ESA-CIP-PSE-PA-pt-LE-2021-452 4000123985/18/NL/PG), the ESA-MAP Project WHISPER (SciSpacE Microgravity Application Promotion Programme. ESA Contract Number 4000130928/20/NL/PG/pt), and the ESA-MAP Project WHISKIES (WHISPER (SciSpacE Microgravity Application Promotion Programme. ESA Contract Number 4000129503/20/NL/PG/pt).

Acknowledgments

The achievements presented in this special issue would not have been obtained without the help and support of Jason Hatton, Angelique van Ombergen, Christiane Hahn, Christopher Puhl, who promoted and accompanied the MAP Whisper and Whiskies, and the Topical Team "Tissue Healing in Space: Techniques for promoting and monitoring tissue repair and regeneration". We did our best to reward these supports with high quality science and technology fostering the ESA leadership in the future human space exploration.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Bioengineering and Biotechnology

REVIEW
published: 17 June 2021
doi: 10.3389/fbioe.2021.679650

Role of Apoptosis in Wound Healing and Apoptosis Alterations in Microgravity

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OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *12 March 2021*
Accepted: *19 May 2021*
Published: *17 June 2021*

Citation:
Riwaldt S, Corydon TJ, Pantalone D, Sahana J, Wise P, Wehland M, Krüger M, Melnik D, Kopp S, Infanger M and Grimm D (2021) Role of Apoptosis in Wound Healing and Apoptosis Alterations in Microgravity. Front. Bioeng. Biotechnol. 9:679650. doi: 10.3389/fbioe.2021.679650

Functioning as the outermost self-renewing protective layer of the human organism, skin protects against a multitude of harmful biological and physical stimuli. Consisting of ectodermal, mesenchymal, and neural crest-derived cell lineages, tissue homeostasis, and signal transduction are finely tuned through the interplay of various pathways. A health problem of astronauts in space is skin deterioration. Until today, wound healing has not been considered as a severe health concern for crew members. This can change with deep space exploration missions and commercial spaceflights together with space tourism. Albeit the molecular process of wound healing is not fully elucidated yet, there have been established significant conceptual gains and new scientific methods. Apoptosis, e.g., programmed cell death, enables orchestrated development and cell removal in wounded or infected tissue. Experimental designs utilizing microgravity allow new insights into the role of apoptosis in wound healing. Furthermore, impaired wound healing in unloading conditions would depict a significant challenge in human-crewed exploration space missions. In this review, we provide an overview of alterations in the behavior of cutaneous cell lineages under microgravity in regard to the impact of apoptosis in wound healing. We discuss the current knowledge about wound healing in space and simulated microgravity with respect to apoptosis and available therapeutic strategies.

Keywords: skin, wound healing, apoptosis, microgravity, surgery, spaceflight, space exploration

INTRODUCTION

Programmed cell death, also known as apoptosis, is a biological process occurring in multicellular organisms (Riwaldt et al., 2017). Apoptosis is necessary to maintain the body balance and to remove cells during the organ development phase. It is involved in morphogenesis, the elimination of neoplastic cells or virus-infected cells. Furthermore, it plays a key role in myocardial infarction, cerebral ischemia, infections, neurodegenerative disorders, organ and bone marrow transplant

rejection, autoimmune diseases and, among others, in cancer chemotherapy or irradiation (Schoenberger et al., 2008).

In addition, apoptosis is an important process involved in the early phases of wound healing (Greenhalgh, 1998; Wu and Chen, 2014). In normal wound healing, programmed cell death is necessary for removing inflammatory cells and afterwards for scar formation. This removal occurs without tissue damage or inflammation.

Furthermore, conditions of real and simulated microgravity can influence cell survival and increase the amount of apoptosis in different cell types and tissues (Zhao et al., 2018; Jiang et al., 2020; Mao et al., 2020; Pan et al., 2020; Sokolovskaya et al., 2020). The conquest of space is very important to us humans. In the course of the expanded exploration of space, combined with the settlement of the Moon, the installation of new Space Stations as well as planned trips to Mars and other planets, more thought must also be given to the treatment of space travelers (Patel et al., 2020). There will be an increase in crew members and space travelers on board together with more on-board and extravehicular activities. Injuries and burns are a very important focal point, an important aspect in surviving a trauma in space is the sufficient wound healing. Therefore, we need profound knowledge of the healing process and suture behavior in space. Moreover, medical evacuation times to Earth are very lengthy or not possible at all so that medical care in space has to be improved with respect to emergency surgery, treatment of acute wounds and burns on spacecrafts or space stations.

The changes of wound healing in space and the influence of microgravity on wounds need to be clarified, and the influence of apoptosis on wound healing in space is of particular interest. We will discuss the current knowledge about (1) wound healing in space; (2) alterations in healing mechanisms affected by microgravity with respect to apoptosis; and (3) possible therapeutic strategies of wound healing in space.

This review provides an overview of real and simulated microgravity research platforms. Models to study wound repair under microgravity conditions will be discussed. It summarizes the current knowledge of the impact of microgravity on the different cell types involved in wound healing with respect to programmed cell death. We will review recent findings in keratinocytes, lymphocytes, macrophages, endothelial cells, dermal fibroblasts and adipocytes. Finally, this comprehensive review reports the influence of the space environment on the biological process of apoptosis in wound healing and evaluates the new therapies to improve wound repair onboard.

WOUND HEALING

Wounds are a break in the continuity of the skin, a "disruption of normal anatomic structures and functions" (Lazarus et al., 1994; Robson et al., 2001; Chhabra et al., 2017). The skin is a complex, multi-functional organ: it prevents fluid loss, stabilizes body temperature, and relays sensory inputs. In addition, it harbors a highly specialized immunological niche crucial for the maintenance of tissue homeostasis, defense, and repair (Nguyen and Soulika, 2019). Wound healing is a complex and dynamic process that is affected by wound environment and the general health and immune status of the host (Chhabra et al., 2017). Wounds can be classified in a number of different ways, depending on the type of treatment setting. For example, in a trauma setting wounds are defined based on the following factors:

Forces Causing the Wound

Based on the different trigger factors, wounds are defined as: *blunt force wound, penetrating forces*, the latter further subdivided into *sharp forces* and *firearms force group* (Chhabra et al., 2017).

Blunt forces causes: Abrasion, lacerations, contusions and "lacerocontusum" wounds (combination of bruise and laceration) (Chhabra et al., 2017).

Penetrating force causes: incisions/cuts, stab and puncture, firearms injuries. The latter varies depending on type of projectile, muzzle velocity, distance, angle of fire and affected body area (Chhabra et al., 2017).

Wound Depth

Superficial: the wound involves only the epidermis (for example, abrasions caused by frictional scraping forces) (Leaper and Harding, 2006).

Partial-thickness: the wound partially extends from the epidermis into the dermis (Chhabra et al., 2017).

Full-thickness: the wound may penetrate into the underlying tissue and involve adipose tissue, muscles, tendons, or bones. Wound healing occurs by granulation and contraction that requires more body resources and time (Chhabra et al., 2017).

Deep wounds: a wound that enters the main body cavities, thorax and abdomen or reaches other deep areas of the body (Oneyekwelu et al., 2017).

Another classification that is important for clinical treatment takes in account the possibility/presence of contamination.

Wounds can be also classified into surgical incisions, traumatic and accidental wounds. Surgical incisions are mainly performed and sutured in a sterile, aseptic environment, using antiseptic techniques. The use of appropriate instruments and bleeding control reduces the risk of infection. Vice versa, traumatic, accidental wounds and surgical wounds in acute care surgery can present some degree of contamination, inflammation or even infection that require specific protocols (Leaper and Harding, 2006; Oneyekwelu et al., 2017).

Wound Healing and Clinical Practice

The different steps of tissue repair are strictly regulated by a multitude of biochemical and physical factors, including gravitational/mechanical forces acting at cellular and tissue level. Interruption, failure or alteration in one or more phases of the repair process can lead to the formation of non-healing chronic wounds or fibrotic scars. Defective healing is caused by alterations in mechanisms underlying repair, such as dysregulated immune function, chronic inflammation, impaired fibroblast function, defective ECM deposition, altered endothelial function, dysregulated apoptosis, etc (Mekhail et al., 2016).

Several factors related to patient's conditions, emergency and care procedures affect the efficiency of repair mechanisms, rate and quality of healing and the onset of wound complications: age, gender, excess weight, serious diseases (e.g., diabetes, venous or arterial diseases, infections, metabolic deficiencies, etc.), wound contamination, wound overstraining, non-physiological environment, urgency, emergency care, wound care, choice of suturing materials and techniques. Some of these factors cannot be changed, but some others can be controlled and improved in order to obtain the best therapeutic result, the healing process, and can be summarized in the following stages:

Stage 1: Vascular Response

Traumatized tissues induce activation of coagulation cascades leading to the formation of a fibrin mesh to fill tissue gaps (Mekhail et al., 2016). Platelets secrete several chemokines that help to stabilize the wound through clot formation and also attract and activate macrophages and fibroblasts. The latter are the protagonists of the stromal activation that leads to important downstream effects, such as epithelial cell proliferation and neoangiogenesis. This process usually lasts up to 3 d.

Stage 2: Inflammatory Response

Vasodilation and increased permeability of the adjacent blood vessels are present (Mekhail et al., 2016). As a result of inflammatory mediators being released by mast cells, the clinical presentation is characterized by redness, swelling, localized heat, pain, and functional limitations. This presentation might be confused with wound infection-increased capillary permeability leading to the production of exudates and essential growth factors, nutrients, and enzymes that are necessary for wound healing and that exert anti-microbial properties.

Stage 3: Proliferative/Granulation Phase

This stage is characterized by epithelialization, angiogenesis, granulation tissue formation, and collagen deposition (Mekhail et al., 2016). Epithelialization starts within hours after injury. Neovascularization, regulated by many factors, is needed to deliver nutrients and maintain the granulation tissue bed. Granulation tissue begins to invade the wound space about 4 days after injury. Macrophages, transforming growth factor (TGF) and tumor necrosis factor (TNF) are the promoters of angiogenesis. Collagen and other extra-cellular materials form scaffolds for the growth of new capillaries. Meanwhile, macrophages continue to supply growth factors, promoting further angiogenesis and stimulating fibroblast to produce and remodel extracellular matrix (ECM) through the synthesis of collagen, fibronectin, laminin and metalloproteases, providing strength and substance to the wound (Flanagan, 2000). The contractile activity of fibroblasts is also responsible for wound contraction and the decrease in wound size (Mekhail et al., 2016). The ability of fibroblasts to transdifferentiate in myofibroblasts strongly affects the healing evolution: myofibroblasts regulate connective tissue remodeling and wound closure by combining their own capacity of ECM biosynthesis with cytoskeletal characteristics of contractile smooth muscle cells. Finally, the process of wound re-epithelialization process takes place across the wound.

Stage 4: Remodeling/Maturation Phase

This final stage of wound healing may last up to 2 y from the trauma event. The scar becomes less raised and reddish, more flat, smooth and turned white due to a decreased blood supply. Mature scars have no hair, no vascularization and no sweat or sebaceous glands. Remodeling of collagen fibers to maximize tensile strength is also present (Mekhail et al., 2016).

Wound Healing Classification

Wounds heal in three different ways: by primary intention, secondary intention, tertiary intention (Sinno and Prakash, 2013; Kumar and Reddy, 2014; Chhabra et al., 2017).

Primary intention healing occurs when the edges of the wound are closely re-approximated with minimal edema, minimal granulation tissue formation and no bacterial contamination. The wound heals in a short amount of time (usually 1 or 2 weeks), with no separation of the wound edges, and with minimal scar formation.

Healing by secondary intention occurs when there is significant tissue loss and/or bacterial contamination (Chhabra et al., 2017; Oneyekwelu et al., 2017). Wounds are usually left open to heal by granulation and contraction. Granulation tissue, rich in blood capillaries, replaces the lost tissue and wound contraction is due to myofibroblasts. This is a longer process, taking weeks or even months to complete: the proliferative phase, granulation in particular, lasts longer than in primary intention, and scars are irregular, retracting and/or hypertrophic.

Tertiary intention is the intentional delay of a wound closure (Sinno and Prakash, 2013). Wounds are left open and covered with a sterile dressing to slacken infection or inflammation. The choice of a delayed closure is intended to allow a complete cleaning of the wound and a reduction in size to close it with stitches or staples as in primary intention healing. This is a safe method of repair of contaminated, as well as dirty and infected traumatic wounds with extensive tissue loss and a high risk of contamination. These wounds are usually treated by debridement of non-viable tissue and left open until the repairing tissue gains sufficient resistance to infection to permit an uncomplicated closure (Oneyekwelu et al., 2017). Usually this takes place within 4–6 d post-injury. When closure is undertaken, skin edges and underlying tissue must be accurately and securely approximated (Kumar and Reddy, 2014; Chhabra et al., 2017; Oneyekwelu et al., 2017).

A wound can be further described by various attributes, including blood flow, oxygenation, infection, oedema, inflammation, repetitive trauma and/or insult, innervation, wound metabolism, nutrition, previous injury handling, and systemic factors (Mekhail et al., 2016). All these attributes can provide evidence of the origin, pathophysiology and condition of a wound. A major factor affecting wound healing is the general health of the host and his/her nutritional status, which can systemically affect the patient, and thereby may lead to an increased risk of infections and/or delayed recovery (Sinno and Prakash, 2013; Chhabra et al., 2017).

In conclusion, wounds remain an ongoing challenge, due to early and late complications that may possibly arise and develop toward an increase in morbidity and mortality. Considering the dynamic evolution of the healing process, this shall require a constant, systematic and consistent evaluation with continuous reassessment of their extent, type and severity.

APOPTOSIS

Programmed cell death or apoptosis is a biological process responsible for normal cell turnover in various organs. An example is the apoptosis of lymphocytes in the tonsil. In case of a chronic tonsillitis, the relationship between apoptosis and proliferation of lymphocytes in tonsillar follicles can be disturbed (Avramović et al., 2015). Inappropriate apoptosis is a key factor in various diseases including cancer, ischemia of the heart and brain, autoimmune or neurodegenerative diseases (Elmore, 2007; Schoenberger et al., 2008). In addition, programmed cell death has important functions on growth, differentiation, morphogenesis, organ development and tissue homeostasis. This type of cell death is able to modulate life and death of cells and exhibits a known therapeutic potential in cancer (Schoenberger et al., 2008; Grimm et al., 2011).

Apoptosis shows characteristic morphological cellular changes such as cell shrinkage, chromatin condensation, membrane blebbing and apoptotic bodies without inflammation (Kerr et al., 1972; Böhm and Schild, 2003; Kossmehl et al., 2003). The morphological changes of programmed cell death were first described by Kerr et al. (1972). These changes occur as follows: The first step is cell shrinkage, followed by a stop of adhesion to the neighboring cells. The cell membranes and the organelles of the apoptotic cells remain intact. This step is followed by chromatin condensation, which is also called pyknosis. Membrane blebbing on the cellular membrane occurs and the nucleus ruptures into fragments, a process called karyorrhexis (Kossmehl et al., 2003). Cytochrome c is released after rupture of the mitochondrial membrane, which activates a cascade of degradation reactions resulting in the secretion of lysosomal enzymes. The result of the enzymatic activity is the formation of small cell particles completely enclosed by cell membranes containing cell fragments. These particles are called apoptotic bodies. The cellular fragments are cleared quickly by phagocytosis without an inflammatory reaction (Taylor et al., 2008).

Apoptosis comprises a complex mechanism with specifically interacting pro-apoptotic and anti-apoptotic factors. It can be initiated by external signals via death receptors. This very well-defined pathway is called extrinsic pathway of apoptosis (EPA). These death receptors comprise FAS/CD95 and the receptors for tumor necrosis factors (TNF-R) (Ashkenazi and Dixit, 1998). The ligand-receptor binding results in the initiation of the cell death machinery. The canonical pathway of the EPA starts with the binding of the tumor necrosis factor receptor superfamily (TNFRSF) members to cognate trimeric ligands of the tumor necrosis factor superfamily (TNFSF). These ligands are either soluble or expressed on the cell surface of another cell.

FASL binding to FAS results in the trimerization of FAS. This process is followed by recruiting the initiator caspase-8 via the FAS-associated death domain protein (FADD). Then the plasma membrane-associated death-inducing signaling complex (DISC) converts procaspase-8 into the active form of caspase-8 (Yang, 2015; Siegmund et al., 2017). The next step is the activation of the effector caspase-3 (Siegmund et al., 2017). Caspase-3 is a cysteine–aspartic acid protease, which is known to cleave various targets and to initiate cell death.

Activated caspase-8 induces apoptosis first by the activation of caspase-3 and second by cleavage of BH3 interacting-domain death agonist (BID), a pro-apoptotic Bcl-2 family protein. The cleaved BID now termed truncated Bid (tBID) translocates to the mitochondria. Afterwards it induces the release of cytochrome c, which is a key protein that initiates the intrinsic pathway of apoptosis (IPA). Cytochrome c is normally localized in the compartment between the inner and outer mitochondrial membranes and its release results in the activation of caspase-9 and−3 (Taylor et al., 2008). The apoptotic protease activating factor 1 (Apaf-1) is involved in the cytochrome-c-dependent activation of caspase-3 (IPA). Mitochondrial cytochrome c is released into the cytosol and binds to Apaf-1 and thus forms the apoptosome. Apaf-1 subsequently activates the caspase cascade. Afterwards, the apoptosome recruits and activates the inactive pro-caspase-9. Active caspase-9 is an initiator caspase and activates effector caspases, which results in the activation of several steps leading to apoptosis.

Cysteine aspartases (caspases) are enzymes involved in the process of apoptosis. EPA and IPA merge at the activation of a procaspase. Apaf-1 activates procaspase-9, while Fas-associated protein with death domain (FADD) activates procaspase-8. The caspases-2,−8,−9, and−10 initiate and activate other caspases, while the caspases-3,−6, and−7 execute the process and cleave proteins at the site of an aspartate. Intracellular type I keratin and other intermediate filament destruction by these enzymes results in the characteristic morphological signs of apoptosis (Oshima, 2002).

NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) is a protein complex involved in DNA transcription, cell survival and cytokine production (Taniguchi and Karin, 2018). NF-κB exhibits various transcriptional regulatory functions and is a key player in apoptosis (Ghobrial et al., 2005) and it regulates the immune response to infections (Hayden et al., 2006). Incorrect regulation of NF-κB is among others associated with cancer and infections (Siebenlist et al., 1994), which might be important for wound healing processes in space. NF-κB is inactivated by binding to IκB (inhibitor of NF-κB). Moreover, the IκB degradation induces the translocation of NF-κB into the nucleus, where it changes transcription (Song et al., 2012) and triggers various processes like survival or detachment and aggregation of cells to three-dimensional aggregates (Kopp et al., 2018).

The transcription factor p65 (nuclear factor NF-κB p65 subunit) is a protein which is encoded by the *RELA* gene. The presence of the transcription factor p65 in the skin (epidermis) inhibits epithelial cell death induced by Fas ligand, tumor necrosis factor-κ, and microbes (Seitz et al., 2000).

The inhibitor of apoptosis protein (IAP) family regulates the activity of caspases, survival, and proliferation by binding to the baculovirus IAP repeat (BIR) domains (Cossu et al., 2019). Moreover, IAPs also control various pathways. The levels of X-linked IAP and other IAPs are often associated with progressive cancer and correlate with prognosis (Cossu et al., 2019). Therefore, compounds targeting the IAPs are of interest in cancer therapy (Saleem, 2013).

Today most of the key players in cellular apoptosis regulation are identified and can be targeted by therapeutic strategies. Examples are drugs targeting the EPA (FAS, tumor necrosis factor-α and tumor necrosis factor related apoptosis-inducing ligand), or compounds binding to factors of the IPA (Bcl-2 family) or Poly (ADP-ribose) polymerase (PARP) inhibitors.

AUTOPHAGY

Autophagy is a physiological process, which serves to degrade misfolded or damaged proteins or dysfunctional organelles such as the endoplasmic reticulum or mitochondria in a catabolic fashion and subsequently reuse some of the degradation products (Mizushima, 2007; Glick et al., 2010). Besides its function in cell maintenance, differentiation and survival, autophagy has also been implicated in the development of diseases (reviewed in Levine and Kroemer, 2008). Interestingly, it has also been shown, that elevated autophagy activity in the skin can lead to impaired cutaneous wound healing (Guo et al., 2016).

Only few studies so far have investigated the influence of microgravity on autophagy. Osteoclasts and endothelial cells are the most widely used cells types. When preosteoclasts were exposed to simulated microgravity on an RCCS for 24 h, the cells showed an elevated autophagy activity, which was linked to an increased syncytin-A expression (Ethiraj et al., 2018) and increased differentiation into osteoclasts (Sambandam et al., 2014) reported similar findings. The process of simulated microgravity-induced formation of osteoclasts could be suppressed by melatonin (Yoo et al., 2016) as well as 4-acetylantroquinonol B (Wu et al., 2020) by inhibiting the autophagy pathway. It can be speculated that counteracting autophagy in microgravity might be a suitable countermeasure for bone loss in space.

Several studies conducted on vascular endothelial cells reported an upregulation of autophagy under simulated microgravity. In contrast to the findings in osteoclasts, however, the results hinted toward a protective effect of autophagy against microgravity-induced stress (Wang et al., 2013; Li et al., 2018, 2019). Recently, it was also found that after 10 d of simulated microgravity on a rotating wall vessel, a disorganization of the actin cytoskeleton triggered autophagy/mitophagy and led to a reduction of mitochondrial content, oxygen consumption, and maximal respiratory capacity in human primary endothelial cells (Locatelli et al., 2020).

Similarly, it could be demonstrated, that simulated microgravity induces autophagy in TCam-2 seminoma cells (Ferranti et al., 2014) as well as human Hodgkin's lymphoma cells (Jeong et al., 2018), pointing toward a possible new target for anti-cancer therapy.

PLATFORMS FOR EXPERIMENTS IN REAL MICROGRAVITY

The term microgravity in general refers to the still existing residual accelerations. When gravitation is the only force acting on an object then the object is in free fall, and hence, it will experience microgravity. As outlined in **Figure 1**, there are several opportunities to expose objects to real microgravity. Most importantly, these differ in the duration of microgravity (seconds to months), but several other parameters, like quality of microgravity (10^{-2}-10^{-6} g), availability, experiment series (daily to 1 per several years), preparation time (month to years), cost, hardware accessibility (hours to months), masses of payload, and degree of automatization change dramatically between the platforms (Sabbatini, 2014; Hemmersbach et al., 2018; Amselem, 2019; Prasad et al., 2020a,b). On this basis, the available microgravity platforms will be described briefly in this chapter.

The most common way to conduct experiments in real microgravity is to use drop towers (or shaft towers or drop wells), which are ground-based research facilities. The duration of the microgravity time provided by operational drop towers is relatively short—3.5–4.7 s depending on the height of the tower and technical facilities. In the case of the 146 m tall ZARM drop tower in Bremen, Germany (see **Figure 1a**), the experimental time can be extended to 9.3 s due to a clever catapult system. The biggest microgravity drop well facility in the world, JAMIC, is 710 m deep (underground) and located in Japan. It can provide microgravity for the duration of 10 s, although it is currently not operational. The NASA Glenn shaft and Beijing drop tower provide 5.2 s and 3.6 s of microgravity, respectively (Zhang, 2005; Könemann et al., 2015). The experimental setup is installed into an airtight capsule which is released in a tube within the tower. To eliminate the effect of friction forces and drag most drop tower experiments are performed in an air free (vacuum) tube. After the free fall the capsule is exposed to substantial deceleration phases during the dampened impact. To double the microgravity time the capsule can be catapulted upward from the bottom of the tube, by which the experiment is exposed to a high acceleration event, followed by a free fall. Among the available drop towers, the facility at ZARM provides the best microgravity conditions of $\sim 10^{-6}$ g (Selig et al., 2010; Corydon et al., 2016). Drop towers provide several advantages including low-cost access to research in microgravity conditions, high flexibility, high payload masses (up to 265 kg), good availability, hardware accessibility a few hours before and after drop, short experiment planning phase, fast turn-around time, they allow the execution of a series of experiments within a few days with direct intervention by research teams to introduce modifications between drops, and vibrations during microgravity are very low. As a consequence

FIGURE 1 | Images of platforms for research experiments in real and simulated microgravity conditions. **(a)** ZARM Drop Tower, Bremen Germany. Ground-based facility providing installation of the experimental set up in an airtight capsule which is released in a tube inside the tower. High payload masses and up to 10 s of real microgravity can be provided (credit ZARM Drop Tower Operation and Service Company). **(b)** Airbus A310 AirZeroG parabolic aircraft operated by Novespace, Bordeaux, France. Repeated periods of ∼22 s of real microgravity can be obtained during parabolic flight maneuvers. **(c)** Experimental area inside the AirZeroG aircraft with different experiment racks. An enormous advantage of parabolic flights is that experimenters have the opportunity to access hardware during microgravity exposure. **(d)** Desktop Random Positioning Machine invented and constructed by Airbus, Defense, and Space, Leiden, NL. This ground-based instrument accommodates large sample sizes and the samples are rotated around two axes in order generated multidirectional *g*-force thereby canceling the cumulative gravity vector at the center of the device. **(e)** Payload of a TEXUS-type sounding rocket (SSC, ESRANGE, Kiruna, Sweden). **(f)** Launch of a TEXUS-51 sounding rocket from SSC, ESRANGE, Kiruna, Sweden, which empowers microgravity for ∼6 min. **(g)** SpaceX CRS-8 rocket on the launch pad, Kennedy Space Center (KFC), FL, USA providing resupply and experiments to ISS. Currently, the ISS is the only option that provides long-time exposure to microgravity (months or longer).

of the short microgravity time and the setup of drop towers, experiment packages must work fully autonomously.

Parabolic flights can be considered as another stepping stone to space (Sabbatini, 2014). Parabolic flights are conducted in a customized aircraft and provide 22 s of microgravity time (**Figures 1b,c**). To produce a free fall period (microgravity) the aircraft is flying parabolic trajectories consisting of three phases: From steady flight, the aircraft climbs at a gradually steeper angle until 50° is reached. In this first phase, the experiments as well as the personal onboard the aircraft will experience hypergravity in the range of 1.8–2 *g* (Ma et al., 2014; Pletser et al., 2016). Next, the pilot reduces the thrust to compensate for drag and the aircraft will follow a ballistic trajectory, also known as a parabola. In this second phase, the aircraft is in free fall and the experiments will experience microgravity in the range of $\sim 10^{-2}$ *g* (Corydon et al., 2016). At the apex of the parabola, the aircraft nosedives until a decline angle of ∼42° is reached, thereby entering the third and last phase of the parabola, in which the airplane again experiences hypergravity (1.8–2 *g*). Finally, the pilot levels out the aircraft for steady flight. The free fall phase persists for 22 s, whereas the two hypergravity phases before and after the free fall phase each last for 20 s. A parabolic flight campaign provided by Novespace, Bordeaux-Merignac, France, has a duration of 3–4 d with 31 parabolas on each flight day. A huge benefit of parabolic flights is that the scientists have the opportunity to be onboard the aircraft during flights, thereby allowing them to direct hardware access during microgravity exposure. Hence, full automatization is not required. In addition, experiments involving microgravity effects on human physiology can be performed. The availability is good, half a dozen flight campaigns are annually conducted by DLR, CNES and ESA in Europe. Typically, the hardware is handed over for installation in the aircraft a few days before the first flight day, and experiments are transferred as soon as a few hours before take-off. High payload masses up to 200 kg can be accommodated by parabolic flights and only few months of experiment planning are needed. The consecutive flight days allow conduction of a series of experiments with modifications between the flights. Hypergravity and vibrations are unavoidable events of parabolic flights, which need to be taken into consideration when evaluating the impact of microgravity (Corydon et al., 2016).

A longer microgravity-exposure demands a visit to space, and suborbital spaceflights in terms of sounding rockets can be an option. For the TEXUS-type sounding rocket (DLR) the payload is in free fall for a period of 6 min (**Figures 1e,f**), and time spans as long as 14 min of microgravity-exposure are delivered by the MAXUS platform (ESA/DLR) (Sabbatini, 2014). In both cases microgravity below 10^{-4} *g* is obtained. The sounding rockets follow a parabolic trajectory with an apex of ∼250 km (TEXUS) or 750 km (MAXUS) above the Earth's surface. The hardware is typically installed in the rocket a few days before lift-off, whereas the experiments (e.g., late access modules) are handed over 1–2 h prior to launch. Inherently, experiment packages must be fully autonomous. The payload will be subjected to hypergravity and vibrations during lift-off and landing which need to be taken into consideration (Corydon et al., 2016). Otherwise, residual accelerations are very low. Recovery of the payload after landing takes a few hours, bad weather conditions might double or triple this period. Operating costs are considerably higher compared to the previously discussed platforms.

Building a road to space is not only an endeavor for public institutions but has also, step by step, become a strategy for

private companies. Blue Origin and Virgin Galactic are noticeable examples of suborbital spaceflight options delivering up to 4–6 min of microgravity (Vanderploeg et al., 2011). Key to these companies is to make access to space less expensive and more reliable through reusable launch vehicles, including development of the so-called VTVL technology (vertical take-off and vertical landing). All experiments must be fully autonomous.

Exposure that lasts up to 5 days can be obtained onboard a spacecraft during a resupply mission to the International Space Station (ISS). However, only a few studies have been reported using this rare option (Horn et al., 2007). An unmanned spacecraft can increase the duration of microgravity time. These are usually satellites, equipped with return vehicles designed to descent back to Earth (e.g., Russian Bion-M- and Foton-M 4-type or Chinese FSW-type) which can provide exposure that lasts a few weeks. These options are likewise very rare: The first Bion-M and Foton-M4 missions were launched in 2013 and 2014, respectively, and relaunches are scheduled in 2023. Experiment packages must be fully autonomous, and years of preparation time should be anticipated. Payload masses are typically low.

As China became the third country sending humans to space in 2003, scientific experiments were conducted on board the Shenzhou spaceships also. Shenzhou missions are launched on the Long March rocket 2F from the Jiuquan Satellite Launch Center.

In order to gain long-term access to real microgravity, we took advantage of the opportunity to participate in the Shenzhou-8 Spaceflight mission, accomplished by a joint endeavor of the German Space Agency and the Chinese Manned Space Engineering Office (CMSEO). Shenzhou-8 was launched by means of a Long March rocket 2F in the Cosmodrome Jiuquan on November 1, 2011 (Pietsch et al., 2013). SIMBOX (Science in Microgravity BOX) contained 17 experiments (https://www.dlr.de/rd/en/desktopdefault.aspx/tabid-2283/3420_read-32536/). Currently, manned and unmanned Chinese spacecrafts are available for scientific endeavors.

The ISS is currently the only option that provides exposure to microgravity for months or longer. It is however, also the most expensive flight option. On the other hand, there are many flight opportunities (e.g., NASA/ESA missions operated by SpaceX or Russian Soyuz missions, **Figure 1g**). A microgravity quality of 10^{-4} g is obtained. In general, experiment packages must be fully autonomous as crew members have a tight schedule. The hardware is typically handed over 1–2 d before lift-off. Experiments should be designed in a way that allows for a 3–5 day delay of the experiment start due to the flight time to the ISS. Accelerations are low during lift-off and the flight. Access to experimental data is possible during or after the experiment has been completed. Hand-over of the hardware is possible several days after return to the Earth.

Moreover, China is presently constructing a Chinese Space Station (CSS) named Tiangong, which will be approximately one-fifth the mass of the ISS. The CSS should consist of multiple modules allowing scientific experiments to be carry out onboard, and it is anticipated to be operational by 2022.

DEVICES TO STUDY ALTERED GRAVITY CONDITIONS ON EARTH

Access to space is expensive, limited and a logistic challenge. On top of this, repetitions of the experiments as well as the number of samples are severely limited (Brungs et al., 2016). To simulate microgravity on Earth a variety of ground-based alternatives have therefore been developed (Herranz et al., 2013; Brungs et al., 2016). One of the simplest devices used for small samples, like cells, is the rotating wall vessel (RWV). In this device simulated microgravity is obtained by placing the cells in a horizontal vessel rotating with a speed that cancels sedimentation (Schwarz et al., 1992). Ideally, the RWV device produces a low shear fluid environment that is optimized for tissue growth and suspension cultures (Klaus, 2001).

In an attempt to reproduce the quiescent, unstirred fluid conditions achievable in orbit, clinostats have been developed for cell culture systems. The so-called clinorotation provided by the device also seeks to simulate microgravity by canceling sedimentation. In clinostats this is accomplished by subjecting the cells to rotations, either constantly or through directional alterations. In the former case the classical two-dimensional (2D)-clinostat rotates a sample perpendicular to the gravity vector (Hemmersbach et al., 2006; Herranz et al., 2013). If samples do not exceed a radius of 1.5 mm around the rotation axis, and the speed of rotation is limited to <60 rpm, a theoretical g-force in the range of 10^{-2}-10^{-3} g can be obtained (Hemmersbach et al., 2006). Even though the sample size of the 2D-clinostat platform is limited, shear forces and fluid disturbances are low (Hemmersbach et al., 2006). The 3D-clinostat uses a comparable principle but can add another dimension. Hence, the sample rotates around two axes in order to provide a status of "vector-averaged gravity," and the generated multidirectional g-force thereby cancels the cumulative gravity vector at the center of the device (Hoson et al., 1997; Schwarzenberg et al., 1999). The quality of g-force provided by a 2D-clinostat is comparable to that of 3D-clinostats. In the latter case, randomized directional changes are delivered by a random positioning machine (RPM) (**Figure 1d**). During operation, the movement of the sample describes a sphere, and studies have demonstrated that at 70 mm around the rotation point the g-force is in the range of 10^{-3}-10^{-4} g (Van Loon, 2007). Compared to the 2D clinostat the RPM clearly provides a larger sample size. However, shear forces arise, especially on the edges of the culture flasks, which need to be taken into consideration (Wuest et al., 2015, 2017).

For larger samples, e.g., mice or rats, the rodent hindlimb suspension (HS) model has been used to study various aspects of musculoskeletal loading (Simske et al., 1994; Morey-Holton and Globus, 2002). In this model the hindlimbs are elevated, unloading them from any force, to produce a 30 degrees head-down tilt. Besides simulating reduced forces on the skeleton and muscles of the hindquarters the model also results in a cephalad fluid shift (Morey-Holton and Globus, 2002). The typical duration of a HS experiments is in the range of days or a few weeks.

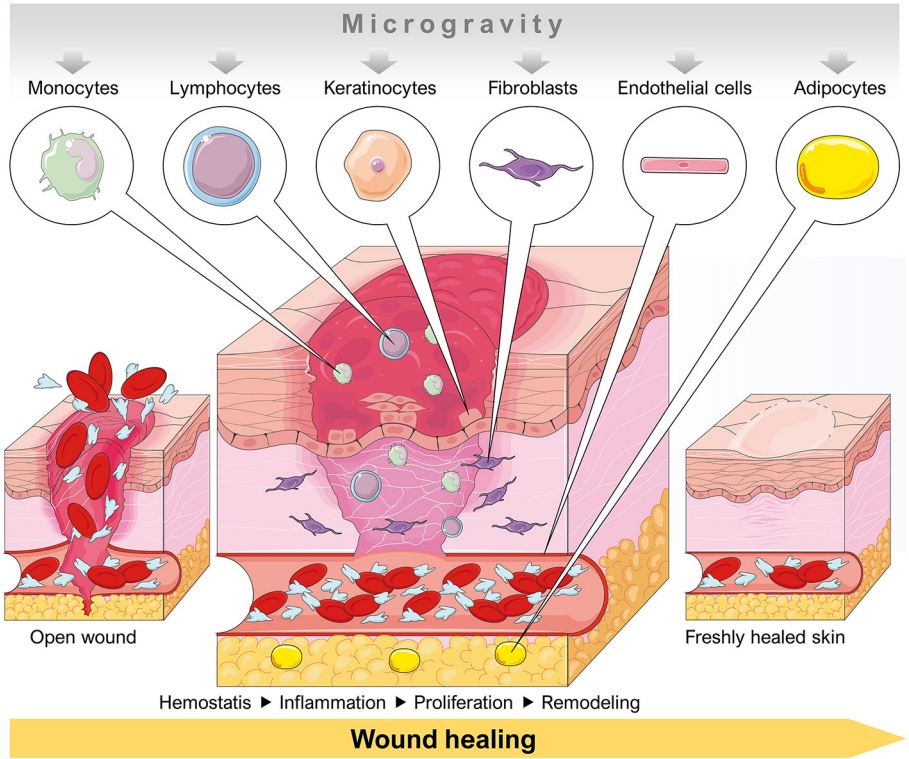

FIGURE 2 | Schematic overview of a dermal wound healing process involving cell types that are influenced by a microgravity environment. Parts of the figure are drawn using pictures from Servier Medical Art (https://smart.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0).

To simulate microgravity effects in humans the head-down bed rest (HDBR) model can be applied. In this model the subjects adhered to a strict 6° head-down tilt bed rest, and several studies have demonstrated that it is replicating the effects of spaceflights (Pavy-Le Traon et al., 2007). The HDBR model induces several physiological changes, including a cephalad fluid shift, promotes movement of abdominal organs toward the chest, reduces the hydrostatic gradient in the cardiovascular system, and results in reduced loading on the musculoskeletal system (Fortney et al., 2011). Notably, in all cases it is important to compare the results from experiments conducted under simulated microgravity with data obtained from experiments performed under real microgravity.

IMPACT OF MICROGRAVITY ON APOPTOSIS IN DIFFERENT DERMAL CELL TYPES

Several dermal cell types had been investigated in space and under conditions of simulated microgravity. Various studies focused on the influence of microgravity on programmed cell death (**Figure 2**). A detailed summary of these publications in respect to apoptosis detection in different dermal cell types is given in **Table 1**.

Keratinocytes

Keratinocytes (KC) are the most abundant and specialized epithelial cell type in the epidermis and apoptosis of KC presents a vital pattern in the modulation of proliferation and maintenance of epidermal thickness and removal of premalignant cells. The data regarding KC apoptosis under simulated and real microgravity is very limited. Ranieri et al. demonstrated that apoptosis of the keratinocytes is not affected by the exposure to the simulated microgravity (Ranieri et al., 2017). In another study Clement et al. stated that ground-based simulated microgravity conditions, by using high aspect ratio vessel (HARV) bioreactors, results in decreased cell death of 3 and 4 day basal-like type of immortalized human epidermal keratinocytes (Clement et al., 2008). Earlier on, Ohnishi et al. reported a 4-fold increase in the content of p53 in skin keratinocyte cells of rats that were taken into space on SLS-2 (Columbia, STS-58) for 14 d, which could protect the damaged cell allowing for recovery and resumption of cell proliferation and reduced apoptosis process (Ohnishi et al., 1996). Despite the limited knowledge the obtained findings suggest that real and simulated microgravity influence the apoptosis process in keratinocytes.

Lymphocytes

Apoptosis plays a vital role in maintaining epidermal structure and homeostasis in the skin. Keratinocytes are the major cell

type in the epidermis. Apoptotic cell death is critical for balancing of keratinocyte proliferation as well as for formation of the stratum corneum (Raj et al., 2006). Apoptosis of keratinocytes occurs not only during normal keratinization but also in response to various intracellular or extracellular stimuli such as genetic defects, UV radiation, exposure to real and simulated microgravity. The keratinocytes act as the first line of innate immune defense against infection through direct activation of primed T lymphocytes and NK cells through major histocompatibility complex I (MHC-I). Adaptive immunity requires the production of specific T lymphocytes to identify an antigen with precision help of B cells to produce specific antibodies that bind to the microbes in a "lock-and-key" fashion.

There is evidence that exposure of lymphocytes to spaceflight as well as to simulated altered gravity conditions created by microgravity-simulating devices results in elevated apoptosis of immune cells (Prasad et al., 2020a). Lymphocytes cultured in simulated microgravity conditions exhibited elevated apoptosis and reduced cell proliferation (Girardi et al., 2014). Previous studies revealed that the rate of apoptosis in Jurkat T lymphocytes was increased in microgravity conditions (Lewis et al., 1998; Cubano and Lewis, 2000, Battista et al., 2012), which was reflected in time-dependent release of apoptosis-related factors like Fas/APO1 in the culture medium during exposure of 2 d real microgravity aboard different space shuttle flights conditions (Lewis et al., 1998, Cubano and Lewis, 2000). Furthermore, microgravity led to increased DNA fragmentation, poly (ADP-ribose) polymerase (PARP) protein expression and p53 and calpain mRNA. These changes were paralleled by an early increase of 5-lipoxygenase (5-LOX) activity (Battista et al., 2012). During the 28th Parabolic Flight Campaign, the European Space Agency has demonstrated that microgravity directly enhances catalytic efficiency of pure lipoxygenase, up to ∼4-fold over the ground (1 *g*) controls. The lymphocytes exhibited increases in apoptosis induced by simulated microgravity created by an RPM (Maccarrone et al., 2003). Programmed cell death in lymphocytes is caused by a mechanism based on calcium-dependent 5-LOX activation, damage of the mitochondrial membrane, the release of cytochrome c and caspase activation (Maccarrone et al., 2003). Nine years later, Battista et al. (2012) confirmed the results with the help of the microgravity simulator ROALD experiment in real microgravity on the ISS as part of the BIO-4 mission of the ESA.

On the other hand, there are also some studies reporting about reduced or inhibition of apoptotic cell death in human lymphocytes cultured in a modeled microgravity condition where the RWV culture system was used (Risin and Pellis, 2001). Another research group demonstrated a reduced expression of cell-cycle genes and downregulation of pro-apoptotic genes in lymphocytes exposed to the RWV, where authors suggested that extended exposure to simulated microgravity may result in a reduction of the cells' ability to undergo apoptosis (Kumari et al., 2009).

Even though the number of studies is limited, these findings provided a molecular background for the regulated function of lymphocytes under altered microgravity conditions.

Macrophages

Phagocytosing of apoptotic cells by macrophages is a major key phenomenon related to active tissue restoration from wound inflammation. Macrophages constantly monitor the skin microenvironment for signals that indicate cell stress, tissue injury or infection (Murray and Wynn, 2011).

Shi et al. (2020) demonstrated that both spaceflight and simulated microgravity significantly reduced macrophage differentiation, decreased macrophage quantity and functional polarization. They also demonstrated that microgravity upregulated the expression of p53 via decreasing the expression of Mdm2 and increased p53 expression reduced apoptosis, enhanced DNA repair, and DNA damage prevention.

Yang et al. (2019) mentioned that unlike lymphocytes, U937 cells (a human macrophage cell line) cultured under simulated microgravity did not undergo apoptosis (Maccarrone et al., 2003; Maier, 2006), where lack of 5-LOX expression might protect U937 cells from apoptosis under microgravity (Maccarrone et al., 2003). In addition, the major stress protein Hsp70 (the 70 kDa heat shock protein) was shown to be up-regulated in U937 cells under simulated microgravity, which could also protect the cells from apoptosis (Maier, 2006). Furthermore, the ICAM-1 expression in the macrophage-like differentiated human U937 cells was up-regulated by simulated microgravity (Paulsen et al., 2015). The impaired PKC signaling under real microgravity (Hatton et al., 1999, 2002) might also contribute to the decreased cell motility.

An international group of researchers explored the rate at which rat macrophages adapt to weightless environments in the ISS's BioLab and reported that the production of ROS in NR8383 rat alveolar macrophages was shown to be significantly reduced by changes in real microgravity (Thiel et al., 2017). Similar results in parabolic flight and 2D clinostats showed significantly diminished ROS production during the oxidative burst in macrophages upon zymosan, curdlan, and lipopolysaccharide stimulation (Brungs et al., 2015). In another study stimulation of mouse macrophages with LPS and exposure to simulated microgravity via Rotary Cell Culture System (RCCS)-1 for 24 h resulted in significant depression TNF-α expression (Wang et al., 2014), which might lead to tumor necrosis factor-related apoptosis. Collectively, these findings provide evidence that microphage apoptosis is influenced by altered gravity conditions.

Microvascular Endothelial Cells

The endothelium is probably one of the tissues most affected by gravitational changes regulating basic homeostatic responses such as vascular tone, angiogenesis and inflammation (Davies, 2009; Bryan et al., 2014; De Cesari et al., 2020). Endothelial cells (ECs) are known to react to microgravity by changing their proliferation rate, nitric oxide (NO) production, and cytoskeletal organization (Balsamo et al., 2014; Maier et al., 2015). The microgravity-induced impact on EC proliferation, survival, and apoptosis is probably the main causes of endothelium dysfunction and cardiovascular deconditioning that occurs in astronauts returning from space (Delp et al., 2016). Particularly

TABLE 1 | Summary of selected articles addressing research on primary cells and specialized differentiated cells *in vitro* cultured under real or simulated microgravity, ordered by cell type.

Cell line	Cell type	Device and exposure duration	Findings in microgravity (μ*g*)	References
HaCat	Keratinocytes, human	RPM (6, 24, 60 h); 1 *g*-samples	Triggers EMT	Ranieri et al., 2017
HEK001	Epidermal keratinocytes, human	HARV (3, 4, 4 d + recovery, 9 d + recovery, 10 d + recovery)	Gene expression profiling, reduced cell death	Clement et al., 2008
–	Epidermal keratinocytes, rats	Space Shuttle Columbia during the STS-58 mission (SLS-2)	Accumulation of cellular p53	Ohnishi et al., 1996
Primary lymphocytes	Lymphocytes (PBLs), human	RWV (24, 48, 72 h); 1 *g*-samples	Increased frequency of apoptosis and decreased cell proliferation	Girardi et al., 2014
Jurkat T	Lymphocytes	Space Shuttle flight STS-80 (Columbia) and STS-95 (Discovery) (75 h); 1 *g*-samples	Increased rate of apoptosis	Lewis et al., 1998
Primary lymphocytes	Lymphocytes (PBMCs), human	RPM (72 h); 1 *g*-samples	Increased apoptosis Calcium-dependent 5-LOX activation	Maccarrone et al., 2003
Primary lymphocytes	Lymphocytes (PBMCs), human	ISS (2 d); 1 *g*-samples	Increased rate of apoptosis Increased DNA fragmentation and 5-LOX activity	Battista et al., 2012
Primary lymphocytes	Lymphocytes (PBMCs), human	RCCS (18–24 h); 1 *g*-samples	Reduced apoptotic cell death	Risin and Pellis, 2001
Lymphocytes	Human B-lymphocytes Human T-lymphocytes	Clinostat (4, 72 h, 7 d); 1 *g*-samples	Decreased DNA repair capacity Reduced expression of cell-cycle genes Downregulation of pro-apoptotic genes	Kumari et al., 2009
U937	Macrophage, human	RWV (24, 72 h); 1 *g*-samples	Reduce cell growth, no sign of apoptosis induction	Maier, 2006
U937	Macrophage, human	2D clinostat; 1 *g*-samples	Regulation of ICAM-1	Paulsen et al., 2015
U937	Macrophage, human	Space Shuttle Atlantis during the STS-81 mission	Modified translocation of protein kinase C isoform	Hatton et al., 2002
NR8383	Macrophages, rat	Spaceflight to the ISS (up to 500 min); 1 *g*-samples	Rapid adaptation to reduced gravity	Thiel et al., 2017
NR8383	Macrophages, rat	2D PMT-clinostat, parabolic flight (22 s); and 1 *g*-samples	ROS production in macrophages is a gravisensitive process	Adrian et al., 2013; Brungs et al., 2015
Differentiated HPCs (Lin^-)	Macrophage, mouse	Tianzhou-1 cargo spaceflight, SJ-10 satellite (12 d), and RCCS (12 d)	Suppressed macrophage development	Shi et al., 2020
Primary macrophages	Macrophage, mouse	RCCS (28 h); 1 *g*-samples	Tumor necrosis factor-related apoptosis	Wang et al., 2014
RAW264.7	Macrophage, mouse	RCCS (28 h); 1 *g*-samples	Tumor necrosis factor-related apoptosis	Wang et al., 2014
EA.hy926	Endothelial cells, human	3D clinostat (4, 12, 24, 48 and 72 h), VEGF (10 ng/ml), 1 *g*-samples	Caspase-3, Bax, Fas, and 85-kDa apoptosis-related cleavage fragments increased Anti-apoptotic effects by VEGF	Infanger et al., 2006
EA.hy926	Endothelial cells, human	3D clinostat (up to 10 d), 1 *g* samples	Caspase-3, Bax, and Bcl-2 protein content elevated	Infanger et al., 2007
PAEC	Porcine aortic endothelial cells (PAEC) overexpressing VEGFR2	RPM (72 h), 1 *g* samples	Proapoptotic signals increased, Anti-apoptotic and proliferation/survival genes were down-regulated	Morbidelli et al., 2005
HPMECs	Human pulmonary microvascular endothelial cells	Clinostat (72 h), 1 *g* samples	TUNEL: elevated apoptosis in Clinostat-exposed cells Increased expression of NF-κB	Kang et al., 2011
HMEC-1	Endothelial cells, human	Ground experiment, 1 *g*-samples	Determination of the biological and engineering requirements that will allow retrieval of suitable samples after culturing, fixing and storing ECs in space	Balsamo et al., 2014

(Continued)

TABLE 1 | Continued

Cell line	Cell type	Device and exposure duration	Findings in microgravity (μ*g*)	References
HPMECs	Human pulmonary microvascular endothelial cells	Clinostat (72 h); 1 *g*-samples	miR-503-5p induced apoptosis and decreased Bcl-2	Tang et al., 2019
HUVEC	Human umbilical vein endothelial cells	RWV (48 h)	miR-27b-5p could protect vascular endothelial cells from apoptosis partially via regulating the expression of ZHX1	Pan et al., 2020
HUVEC	Human umbilical vein endothelial cells	2D-Clinostat	Apoptosis, pro-inflammatory cytokine production, nuclear factor kappa B (NF-κB)/IκB signaling	Jiang et al., 2020
CVEC	Choroidal vascular endothelial cells, human	RCCS (24 h, 72 h), 1 *g* samples	Activated Bcl-2 apoptosis pathway and PI3K/AKT pathway Elevated Bax, Caspase3, and Cytochrome C	Zhao et al., 2021
HUVEC	Human umbilical vein endothelial cells	RWV (4 d, 10 d)	HSP70 up-regulation as adaptive response to RWV exposure	Cazzaniga et al., 2019
CF	Cardiac fibroblasts, porcine	RPM (24 h) bFGF, VEGF, 1 *g* samples	Increase in apoptosis in RPM samples, VEGF and bFGF reduced the amount of apoptosis	Ulbrich et al., 2010
WI-38	Quiescent normal human fibroblasts, derived from fetal lung	Space Shuttle, STS-93 mission (5 day spaceflight); ground controls	Changes in gene expression associated with cellular stress signaling, directing cells to either apoptotic death or premature senescence	Liu and Wang, 2008
STO	Mouse fetal fibroblast cells	RPM, 24 h, 1 *g* controls, irradiation (0, 0.5, 1, or 4 Gy)	Decrease in apoptosis at all doses as measured by caspase-3 activity	Beck et al., 2012
NIH3T3	Fibroblasts, NIH Swiss mouse embryo	RPM, 72 h, 1 *g* samples	Reduction in cell number	Cialdai et al., 2020
Primary cells	Adipocytes (ADSCs), human	Clinostat, 1, 3, 7 d; 1 *g*-samples	Altered gene expression of ECM and adhesion molecules, which potential may facilitate wound healing	Ebnerasuly et al., 2018

2D, two-dimensional; 3D, three-dimensional; ADSCs, adipose-derived stem cells; CF, cardiac fibroblast; CVECs, choroidal vascular endothelial cells; EMT, epithelial-mesenchymal transition; HARV, high aspect ratio vessel; HMEC, human microvascular endothelial cell; HPC, Hematopoietic progenitor cell; HPMEC, human pulmonary microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; ISS, International Space Station; NIH, National Institutes of Health; PAEC, porcine aortic endothelial cell; PBL, Peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; PMT, photomultiplier; RCCS, rotary cell culture system; ROS, reactive oxygen species; RPM, random positioning machine; RWV, rotating wall vessel; r-μg, real microgravity; s-μg, simulated microgravity; SLS, space launch system; STS, space transportation system. VEGFR2, vascular endothelial growth factor receptor 2.

microvascular ECs (HMECs) reduced proliferation or were directly induced to apoptosis when exposed to simulated microgravity (Cotrupi et al., 2005; Mariotti and Maier, 2008). In pulmonary HMECs, this behavior can be explained by increased NF-κB expression, downregulation of the PI3K/Akt pathway, and F-actin depolymerization (Kang et al., 2011). Tang et al. (2019) recently reported the involvement of miR-503-5p in the induction of apoptosis, at least in part, by inhibiting the expression of the anti-apoptotic Bcl-2 protein under simulated microgravity conditions. On the contrary, no apoptosis was observed when dermal HMECs were cultured in the RWV or on the RPM (Mariotti and Maier, 2008). This could be explained on the one hand due to HMEC heterogeneity and different experimental conditions. On the other hand, a fast induction of heat shock protein 70 (Hsp70), could protect HMECs from apoptotic stimuli by acting downstream of cytochrome c and upstream of caspase-3 (Carlsson et al., 2003; Cotrupi and Maier, 2004; Cazzaniga et al., 2019).

Fibroblasts

ISS astronauts showed decreased skin elasticity after a 6-month space mission (Tronnier et al., 2008). Major physical stress factors responsible for this health problem are cosmic radiation and microgravity. In addition, this finding suggests possible morphological and structural alterations of the skin of humans exposed to real microgravity in space. A recent study showed that juvenile normal human dermal fibroblasts (NHDF) exposed to the RPM exhibited changes in the cytoskeleton, focal adhesion molecules, extracellular matrix, and growth (Buken et al., 2019). The RPM-exposed cells grew as adherent monolayer and as 3D aggregates revealing no dead cells. A similar result was observed previously (Beck et al., 2012). RPM-exposure and irradiation of mouse fetal fibroblasts for 24 h resulted in a decrease in apoptosis (Beck et al., 2012). **Figure 3** shows the results obtained from NHDF exposed for 24 h exposed to an RPM. We used the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect DNA fragmentation by labeling the 3′-hydroxyl termini in the double-strand DNA breaks characteristic

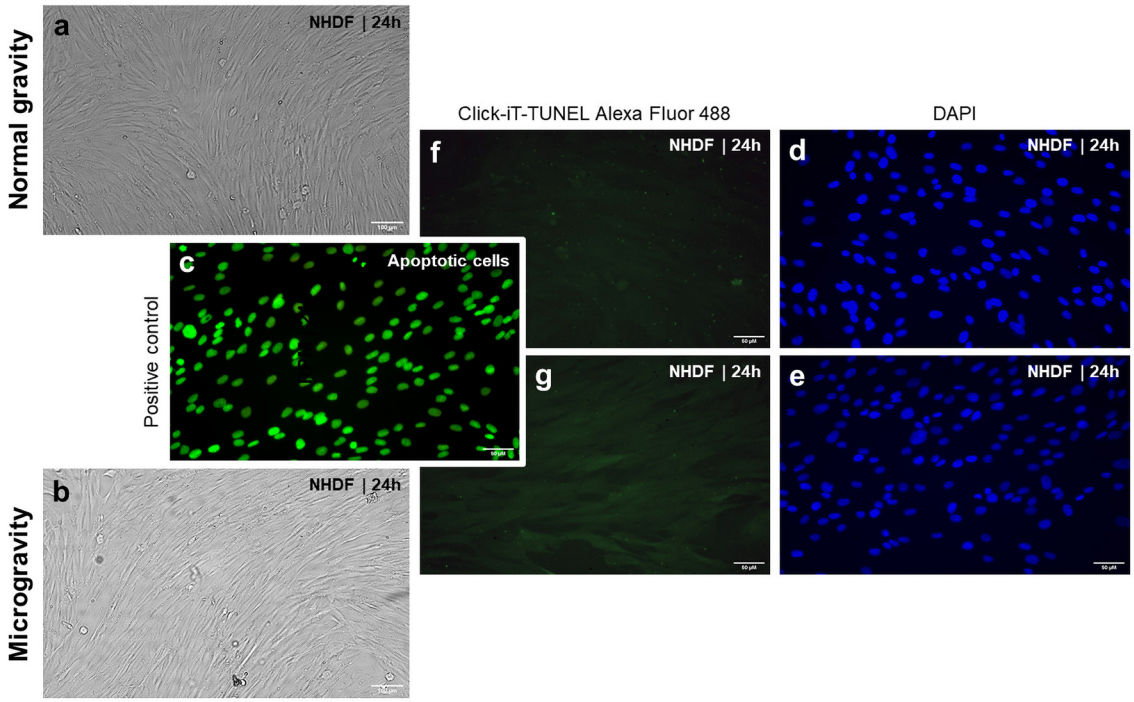

FIGURE 3 | Phase contrast microscopy of normal human dermal fibroblasts (NHDF, catalog number C-12300; PromoCell GmbH, Heidelberg, Germany): **(a)** NHDF cultured under static 1 *g* conditions and **(b)** NHDF exposed to the RPM for 24 h. Click-IT terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay [(Thermo FisherScientific, Waltham, Massachusetts, USA; Click-iT TUNEL Alexa Fluor 488 (cat# C10245)] performed on NHDF exposed to 1 *g* **(f,d)**, and the random positioning machine (RPM) **(g,e)**. Green staining indicates free fluorophores in the cytoplasm in all images with the exception of the positive control **(c)**. In the positive control, samples have been pretreated with DNase to induce DNA fragmentation, which is visualized by an enrichment of the fluorophores in the nucleus. Blue staining (DAPI) highlights the cells' nuclei **(d,e)**. Green stained nuclei present apoptotic cells as shown in **(c)**. None of the applied experimental approaches (1 *g* and RPM) had induced apoptosis in the cells **(f,g)**. The evaluation was done using a Leica DM 2000 microscope equipped with an objective with a calibrated magnification of x400 and connected to an external light source, Leica EL 6000 (Leica Microsystems GmbH, Wetzlar, Germany).

for apoptosis. The fibroblasts remained healthy and no signs of apoptosis was detectable.

Another study focused on the effects of an RPM-exposure on porcine cardiac fibroblasts which exhibited an increase in apoptosis as determined by terminal deoxynucleotidyl transferase-mediated dUTP digoxigenin nick end labeling (TUNEL) analysis, 4′, 6-diamidino-2-phenylindole (DAPI) staining, and caspase-3 detection (Ulbrich et al., 2010). Vascular Endothelial Growth factor (VEGF) and basic fibroblast growth factor (bFGF) application attenuated the number of apoptotic fibroblasts.

Fibroblasts are strongly involved in wound healing. Thus, activity changes can strongly compromise the repair process. NIH3T3 fibroblasts exposed to the RPM for 72 h showed a decrease in cell number, which might be due to apoptosis or necrosis or a decreased proliferation of these cells (Cialdai et al., 2020).

Only few data exist about the behavior of fibroblasts during spaceflight. An ISS experiment investigated possible changes in gene and microRNA (miRNA) expression profiles in confluent human fibroblast cells (Zhang et al., 2016). These were fixed after 3 and 14 d. The cell densities were greater for fibroblasts on day 14 compared with cells on day 3. Flown fibroblasts were also denser than the ground control cells on day 14. This supports the findings of Beck et al. (2012), which showed an increased cell growth of fibroblasts in space aboard the ISS. After 3 d, both the flown and ground cells were still proliferating slowly (Ki-67(+) positive cells). Moreover, gene and miRNA expression data revealed an activation of NF-κB and other growth-related pathways (Zhang et al., 2016).

Another paper reports about quiescent normal human WI-38 fibroblasts flown on the STS-93 space shuttle mission (Liu and Wang, 2008). They identified, among other differentially displayed genes, an activation of three pro-apoptotic genes and a repression of six anti-apoptotic genes. Three apoptotic genes, encoding the RNA binding protein NCL, osteopetrosis-associated transmembrane protein (*OSTM1*), and calcium-dependent phospholipid-binding protein annexin A5 (*ANXA5*) were up-regulated by spaceflight in WI-38 cells. Furthermore, six anti-apoptotic genes such as nuclear pore membrane glycoprotein (*POM121*), a small G-protein (*APMCF1*), the inhibitor of calpain calpastatin (*CAST*), a TNF receptor-associated factor binding protein (*T2BP*) a myeloid leukemia-associated SET translocation (SET), and ferritin heavy chain 1 (*FTH1*) were all downregulated (Liu and Wang, 2008). The authors suggested that the fibroblasts are triaged in space to either

premature replicative senescence or apoptosis. A further study focused on fibroblasts exposed to simulated microgravity. The fibroblasts exhibited alterations in the microtubules and alpha-SMA bundles together with an impaired adherence, migration, and response to chemoattractants (Cialdai et al., 2017).

Taken together, wound healing is of enormous importance for the survival of organisms. In regard to increased space exploration adventures, we need to determine the exact influence of microgravity on dermal cells involved in the wound repair process and to research drugs with the ability to improve wound healing in space. Unfortunately, the behavior of fibroblasts in microgravity is not yet clarified. Therefore, new research projects studying human dermal fibroblasts in space have to be performed in the near future.

Adipocytes

Adipose-derived stem cells (ADSCs) have been tested in multiple preclinical and clinical trials and have been found to enhance cutaneous wound healing through a variety of wound-healing pathways (Atala et al., 2010; Gimble et al., 2012; Shingyochi et al., 2015; Bertozzi et al., 2017). ADSCs have major potential to release angiogenic, vasculogenic, and other factors and they can stimulate their surrounding cells through the paracrine angiogenic and vasculogenic effects and accelerate wound treatment (Goodarzi et al., 2018). Nie et al. showed in their preclinical study that wound closure in normal diabetic rats can be accelerated by ADSCs, via increased epithelialization and granulation tissue deposition (Nie et al., 2011). Rigotti et al. have reported that injection of ADSCs can be effectively applied for treatment of patients with progressive wounds following radiation therapy (Rigotti et al., 2007). In cell-assisted lipotransfer (CAL) applications of ADSCs have become one of the novel stem cells transplantation strategies specifically in field of skin reconstruction (Yoshimura et al., 2008). In the CAL as an autologous tissue transfer method, fat derived ADSCs are attached to the aspirating fat which acts as living scaffolds to provide optimized condition for grafting.

The literature on ADSCs apoptosis induced by microgravity and how they behave in wound healing under altered gravity condition is very scarce. However, Ebnerasuly et al. recently demonstrated that when ADSCs were exposed to simulated microgravity by using a clinostat for 1, 3 and 7 d no significant changes in the viability or rate of apoptosis were observed. Interestingly, the research group also found increased ECM expression of *ITGB1*, *COL3*, *MMP1*, and *CD44* and declined expression of *FBN1* and *VIM* genes (Ebnerasuly et al., 2018). Hence, simulated microgravity in ADCS cells may increase their differentiation capacity toward fibroblastic cells to facilitate the wound healing process.

Stem Cells and Wound Healing

Real microgravity during a spaceflight changes the migration behavior of stem cell-derived keratinocytes which can impair wound healing (Finkelstein et al., 2011).

Epidermal stem cells (EpSCs) play an important role in the renewal and repair of the epidermis, and are considered to have the ability to divide and differentiate into different cell types. Li and team (Li et al., 2020) have demonstrated that when EpSCs are cultured on a rotary cell culture system (RCCS) for 3 d, the amino acid metabolism pathway, lipid metabolism pathway, membrane transport pathway, cell growth and death pathways were changed by the influence of simulated microgravity.

Blaber and coworkers (Blaber et al., 2015) have studied the influence of microgravity (STS-131 STL spaceflight) on early lineage commitment in mouse embryonic stem cells (mESCs). Exposure to real microgravity for 15 d inhibited the mESC differentiation and expression of terminal germ layer lineage markers in embryoid bodies. Mechanical unloading of cells and tissues during a spaceflight revealed an inhibition of the proliferation and differentiation potential of stem cells.

Another group had shown that mesenchymal stem cells (MSCs) exposed to simulated microgravity for 72 h underwent adipogenic differentiation (Xue et al., 2017). Ratushnyy and team had found that conditioned medium from RPM-exposed adipose-derived MSCs stimulated the formation of a vessel network *in vivo*, an endothelial cell (EC) capillary-like network, and non-directed EC migration *in vitro* where an elevated expression of angiogenic regulators serpin E1, serpin F1, IGFBP, VEGF, and IL-8 was detected (Ratushnyy et al., 2018).

Beneficial effects of adipose-derived stem cells on wound healing have been reported. A further study focused on the influence of simulated microgravity using a clinostat on the gene expression of extracellular matrix (ECM) proteins and adhesion molecules in human ADSCs (Ebnerasuly et al., 2018). There were no significant changes in the cell viability detectable when the cells were exposed to a clinostat. An increase in ECM components was found and is known as one of the fibroblast markers. The authors suggest that pretreatment of adipose-derived stem cells by clinorotation may increase their differentiation capacity toward a fibroblastic phenotype (Ebnerasuly et al., 2018).

A recent study reported that it is safe to culture MSCs on the ISS. These ISS-grown MSCs exhibited an MSC-characteristic morphology and phenotype, a normal proliferative and differentiation potential. In addition, these cells were more potent in their immunosuppressive abilities compared to ground control MSCs (Huang et al., 2020). This data shows that MSCs grown in space might be useful for possible future clinical applications. It is necessary to increase the number of studies in this field to validate these results.

WOUND HEALING IN SPACE

Soon, increased commercial spaceflights and new space exploration programs to, among others, Moon and Mars will expand the amount and duration of space missions, the number of humans in space together with on board activities and elevated extravehicular work. These activities will increase the possibility to be injured and will heighten the amount of emergency surgeries in orbit. Therefore, to improve wound healing in space is currently a hot topic.

Drudi et al. reported in 2012 about the "state of the art" in-space surgery including wound healing and sutures in space

(Drudi et al., 2012). As already reviewed and discussed earlier, wound healing is a well-known long-term process starting with the injury, together with tissue damage and can last months. Effective wound repair ensures the integrity of the human body and our survival.

A journey to the Mars comprises about 50 million km and thus, a cargo resupply from Earth is not possible (Thirsk, 2020). Therefore, it is mandatory to organize the onboard facilities and supplies for wound surgery in case of accidents, burns or injuries of the space crew from a diagnostic and therapeutic point of view. Additionally, long-term exposure of space travelers to cosmic radiation can negatively influence wound repair and result in degenerative tissue diseases (Thirsk, 2020).

Onboard the spacecraft medical resources such as laboratory analysis equipment, imaging, surgery, and emergency care is mandatory (Thirsk, 2020). It is of fundamental importance, that a well-trained physician with experience in remote medicine is on board as crew member, due to the communication lag it is currently not possible to guide the medical doctor remotely. In respect to the planned Mars mission, training countermeasures need to be developed in the near future. Astronaut training courses to manage in-flight accidents on long-term missions are absolutely necessary (Robertson et al., 2020).

A study investigated wound healing and mucosal immunity during a short MARS analog mission and found that stress can have significant consequences for wound healing (Rai et al., 2012). The authors concluded that the effects of stress on wound repair can influence the recovery from surgery (Rai et al., 2012).

In general, little is known about wound healing in space, because stays on the ISS or in the past on Space Shuttles and MIR were not problematic for wound repair and could be easily managed.

Interestingly, endothelial cell culture experiments performed in real microgravity revealed alterations in endothelial cell function, changes in ECM production, and 3D growth (Versari et al., 2013; Pietsch et al., 2017; Krüger et al., 2019).

Activated T cells investigated in space on board the ISS showed an activation of genes (cell cycle check-point, oxidative stress response, heat shock proteins) or by repressing genes involved in antigen recognition (Pippia et al., 2011). Battista et al. demonstrated 5-Lipoxygenase-dependent programmed cell death of human lymphocytes in space onboard the ISS (ROALD experiment) (Battista et al., 2012). This space experiment confirmed earlier data obtained with the RPM (Maccarrone et al., 2003).

Astronauts staying for a prolonged time in space exhibit an impaired immune function. The development of acquired immunity and immune responses are disturbed (Akiyama et al., 2020). Acquired immune responses are also influenced by gravitational fluctuation, as well as stressors together with cosmic radiation (Akiyama et al., 2020). A long-term spaceflight triggered a sustained stress-dependent release of endocannabinoids. Stress is stimulating inflammation-related diseases in people at risk (Buchheim et al., 2019). These health problems are resembling some features of systemic diseases which can impair wound healing on Earth. Stress, diabetes and others can negatively influence the body's response to injury and wound healing. Therefore, it is important to increase our knowledge in this field, and to design studies focusing on wound healing in space.

The international experiment of principal investigator Professor Monica Monici and co-investigators entitled "Wound Healing and Sutures in Unloading Conditions," selected by ESA (ILSRA-2014-0043), has the objective to investigate the behavior of *in vitro* sutured wound models in real microgravity on the ISS (**Figure 4**). Preparatory investigations and a detailed characterization of the wound healing model had been published earlier (Riwaldt et al., 2017). Importantly, no signs of apoptosis were found in the skin samples after different culture conditions and culture duration times. The findings of the project ILSRA-2014-0043 are expected to increase our knowledge on wound healing and sutures in space. The project will help to develop novel strategies for tissue healing and management of defective healing in space and on Earth. The performed techniques and newly defined analytical protocols demonstrated to be applicable for post-flight studies on skin samples after return of the future space mission (Riwaldt et al., 2017). The principal aim of this project is to enlarge our current knowledge in wound healing in space leading to novel strategies for wound repair, tissue regeneration and engineering not only for humans in space but also for patients on Earth.

WOUND HEALING AND APOPTOSIS IN SPACE

Wound healing is a continuous process over an extended stretch of time, a complicated and multifaceted repair procedure involving various changing cell types throughout the progression of the process. To recap from chapter 2.3, wound healing is categorized into four phases: the homeostasis and coagulation phase starts immediately after injury, followed promptly by the inflammation phase. Thereafter, the migration and proliferation phase sets in until lastly, new skin is developed during the remodeling phase (Hunt et al., 2000; Guo and Dipietro, 2010; Reinke and Sorg, 2012).

During the first phase, the wound healing cascades are instigated, with vascular constriction and fibrin clotting to stop bleeding and form a provisional wound matrix triggered by platelets, with support from leukocytes, keratinocytes, fibroblasts and endothelial cells. During this process, platelets and leukocytes deposit pro-inflammatory cytokines and growth factors (Reinke and Sorg, 2012).

While these pro-inflammatory factors attract immune cells as neutrophils and macrophages, growth factors TGF-β1, platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) stimulate tissue growth (Gosain and DiPietro, 2004; Broughton et al., 2006). Neutrophils amplify the inflammatory response, degrade necrotic cells and prevent bacterial infection (Eming et al., 2007). Macrophages are responsible for the host defense by clearing the site of pathogens and apoptotic cells within the wound, additionally they contribute to the initiation of proliferation and angiogenesis (Mosser and Edwards, 2008; Koh and DiPietro, 2011).

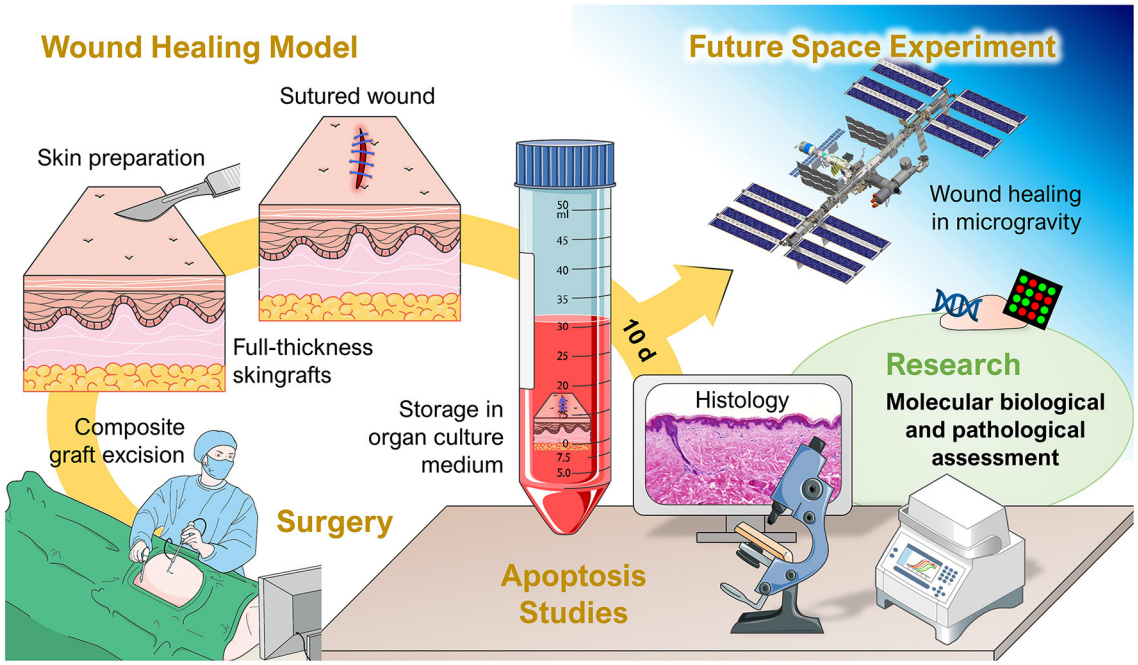

FIGURE 4 | Overview of the project "Wound Healing and Sutures in Unloading Conditions." Parts of the figure are drawn using pictures from Servier Medical Art (https://smart.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0).

The most prominent cell types during the proliferative phase are fibroblasts and epithelial cells. Within the wound, fibroblasts immigrate along the fibrin network and produce extracellular matrix components like collagen, proteoglycans, glycosamyloglycans, to name a few, and so promote vascularization and filling of the wound cavity (DiPietro and Polverini, 1993; Gosain and DiPietro, 2004). The formation of new vessel in the wound filling is a complex order of events, initiated by growth factors like VEGF, PDGF, and then serine protease thrombin. These factors activate the endothelial cells of existing vessels and initiate the re-vascularization via sprouting (Reinke and Sorg, 2012).

During the last process of wound healing, the proliferation and remodeling phase, a flurry of various cell types are recruited to the close the wound. The main drivers of this stage are fibroblasts who produce the majority of materials necessary such as e.g., collagen end extracellular matrix components (hyaluronic acid, proteoglycans, glycosaminoglycans, fibronectin). In this way they form the scaffolding for cell adhesion during the tissue repair which organizes the growth and movement of the involved cells (Eckes et al., 2010; Barker, 2011). However, it should be mentioned that during the transition between the different repair phases and in order to finalize the process, cells from previous stages that are not required any longer are consistently eliminated via apoptosis throughout the entire process (Greenhalgh, 1998; Cialdai et al., 2017). An investigation in preparation to wound healing experiments during spaceflight revealed that in skin tissue cultures the healing process did not result in excessive apoptosis or necrosis after 10 d of incubation under 1 *g* conditions (Riwaldt et al., 2017).

The space environment subjects the human organism to drastic gravitational changes compared to terrestrial conditions. The lack of mechanical forces in microgravity settings causes widespread impact on the human body. Spaceflight instigates not only muscle and bone loss, immune system impairment and cardiac problems, but also negatively affects normal regenerative tissue repair and wound healing (Blaber et al., 2014; Paulsen et al., 2015; Cialdai et al., 2017). The underlying physiological changes can be described on an organic level as well as changes in protein and gene expression in the respective cell types (Blaber et al., 2014).

Cialdai et al. showed in detail how cell behavior adapts to simulated microgravity, particularly in would healing (Cialdai et al., 2017). Since fibroblasts are drivers of tissue regeneration, the focus on this study was on put on the changes of these cells after exposure to modeled microgravity. Due to a thorough rearrangement in the cytoskeleton, the adherence and migration ability of fibroblasts in wounds was significantly impaired. These findings are supported by the investigation of Davidson et al. who examined wound healing in rats sent in orbit. The animals exposed to real microgravity form 60% less collagen in an injury site compared to ground controls, hinting that wound healing in orbit is delayed (Davidson et al., 1999). Similarly, Infanger et al. showed that simulated microgravity resulted in an increase in ECM components and alteration of cytoskeletal structures in human EA.hy926 cells, moreover apoptosis increased after only 4 h of microgravity conditions (Infanger et al., 2006). In addition

to the effects of the change in mechanical forces in microgravity, space radiation is another influencing factor. DNA damage due to ionizing radiation leads to increased DNA repair, cell cycle arrest and apoptosis in various cell types (Prasad et al., 2020a).

Under normal gravity, the deposition of ECM is mainly found during the proliferation phase of wound healing when fibroblast and endothelial cell proliferation peaks. Endothelial cells though were found to exhibit increased apoptosis during simulated as well as real microgravity conditions. After only 72 h of simulated microgravity conditions downregulation of the PI3K/Akt pathway and increased expression of NF-κB could be demonstrated, which may explain another aspect of the delay in healing (Kang et al., 2011).

In another *in vivo* experiment Fisher 344 rats were investigated to test wound healing responses in the orbiting Space Shuttle for 10 d (Davidson et al., 1999). The results of this study suggested that the spaceflight retarded the wound healing process and the response to exogenous stimuli.

GROUND-BASED WOUND HEALING STUDIES USING MICROGRAVITY SIMULATORS

This chapter reports about the current knowledge about wound healing studies using microgravity simulators like the RPM, 3D clinostat or the RCCS.

Microgravity may influence the process of wound healing and its progression both by changing the tissue response, apoptosis or the suture behavior. In the past, several publications showed that conditions of microgravity impaired and delayed wound repair. Until today the underlying mechanisms for an imperfect wound repair with scarring in space are not clear.

In chapter 6, we had summarized the current knowledge about various cell types active in wound repair but when exposed to microgravity several of them can become apoptotic which might result in a delay and an imperfect wound repair.

Human lymphocytes were activated and proliferate in space and on the 3D clinostat (Cogoli and Cogoli-Greuter, 1997). In free-floating cells (lymphocytes) the mitogenic response is depressed by 90% in microgravity. Maccarrone et al. showed that human lymphocytes exposed to a clinostat exhibited apoptosis by 5-lipoxygenase-mediated mitochondrial uncoupling and cytochrome c release (Maccarrone et al., 2003). These findings might influence the early phases of wound healing in space.

Another cell type of the dermis which is affected by microgravity are endothelial cells. There are several reports that endothelial cells exposed to simulated microgravity can become apoptotic and show upregulations of apoptosis signaling, downregulation of the PI3K/Akt pathway, increased expression of NF-κB, depolymerization of F-actin and that miR-503-5p can induce apoptosis of human pulmonary microvascular endothelial cells through, at least in part, inhibiting the expression of Bcl-2 (Morbidelli et al., 2005; Infanger et al., 2006; Kang et al., 2011; Maier et al., 2015; Xu et al., 2018; Tang et al., 2019; Zhao et al., 2021). In addition, clinorotation for 48 h activated autophagy in vascular endothelial cells (Wang et al., 2013).

Fibroblasts are key players in wound repair and are mechanosensitive cells (Gabbiani, 2003) and are able to produce extracellular matrix proteins (Buken et al., 2019). It is well-known that cells exposed to simulated microgravity created by an RPM show changes in the cytoskeleton and the extracellular matrix (Buken et al., 2019). Earlier studies (Morbidelli et al., 2005; Monici et al., 2011) showed that cells exposed to simulated microgravity reveal changes in extracellular matrix proteins and endothelial function, which might affect the edema behavior and tissue stiffness in wound healing. Fibroblasts exposed to a RCCS showed changes in the cytoskeleton, a reduced VEGF and elevation of COX2, responsible for an inflammatory response (Cialdai et al., 2017). Fibroblasts exposed to both, real and simulated microgravity revealed changes in their gene expression profile (Liu and Wang, 2008; Beck et al., 2012), thus suggesting the hypothesis that these alterations in fibroblast behavior, increased stress, inflammatory signals and altered gene expression pattern may be involved in disturbed wound healing in space. The findings are supported by Liu and Wang who studied normal human WI-38 fibroblasts flown on the STS-93 space shuttle mission. They reported altered key genes related to oxidative stress, DNA repair, and fatty acid oxidation in spaceflight samples (Liu and Wang, 2008). An important finding was the up-regulation of pro-apoptotic genes (Liu and Wang, 2008). The data show spaceflight induced changes in gene expression associated with cellular stress signaling, directing cells to either apoptotic death or premature senescence, which might impact wound repair and promote scarring.

Little is known about skin tissue samples exposed to simulated microgravity. One group cultured living skin equivalents (LSEs) in a microgravity environment (NASA-designed bioreactor) for 3 days (Doolin et al., 1999). Microgravity-exposed LSEs showed nuclear and cellular hypertrophy. Finally, a study of three mice living for 91 days on the ISS reported about abnormalities in the animals' skin. The spaceflight induced skin atrophy, deregulation of their hair follicle cycle, and markedly affected the transcriptomic repertoire of the cutaneous striated muscle panniculus carnosus (Neutelings et al., 2015).

The rat HS model of microgravity mimics physical inactivity by removing weight-bearing loads from the hindlimbs and producing a systemic cephalic fluid shift. Using this *in vivo* microgravity model Radek and team (Radek et al., 2008) demonstrated a delay in wound healing and wound closure in cutaneous tissue with retarded epithelial cell migration across the wound bed in the tail-suspended hindlimb-unloaded rats. The authors suggested an impaired keratinocyte and endothelial cell function during the wound healing process under simulated microgravity in the rat (Radek et al., 2008).

NEW THERAPIES TO IMPROVE WOUND REPAIR IN SPACE

The principal goal is to develop novel strategies (including drugs and biochemical factors, devices, protocols and techniques) which allow a long-term stay in orbit or on other planets. This approach should promote wound repair and tissue

regeneration in space. Furthermore, these strategies should be transferable to clinical applications for regenerative medicine and tissue engineering.

In this chapter, we focus on drugs and growth factors as well as biofabrication to improve wound repair.

Examples for countermeasures toward a defective neoangiogenesis are proangiogenic factors like VEGF, PDGF, and other endothelial protectives which can favor healing by modulating the neoangiogenesis process (Öhnstedt et al., 2019). Several papers reported about a reduction of VEGF in skin cells exposed to microgravity. Application of VEGF164 in rat wound healing models on Earth has demonstrated cell-protective beneficial effects on reendothelialization and vascular healing after microsurgery (Infanger et al., 2005). In addition, VEGF164 application to endothelial cells exposed to modeled microgravity exerted anti-apoptotic and cell-protective on the cells (Infanger et al., 2007).

Therefore, VEGF seems to be a promising candidate to be tested in skin wound healing models. Another option to be tested is PDGF which is approved by the FDA for non-healing wounds. Becaplerim, a gel containing recombinant PDGF (the BB isoform) is a treatment availability. Furthermore, granulocyte macrophage colony stimulating factor (GM-CSF) as injection, cream or gel can be tested.

Another growth factor with beneficial effects is epidermal growth factor (EGF). rhEGF is available as a spray and was tested in a phase III double-blind, randomized, placebo-controlled trial (Park et al., 2018). As published by Park et al. "*167 adult patients with chronic diabetic foot ulcers at six medical centers were randomized to receive routine wound care plus either topical spray treatment with 0.005% rhEGF (n = 82) or an equivalent volume of saline spray (n = 85) twice a day until ulcer healing or for up to 12 weeks.*" The study showed that application of spray-applied rhEGF in the patients induced a faster healing velocity and higher complete healing rate regardless of HbA1c levels (Park et al., 2018).

Recently, Cialdai et al. studied the influence of gravitational unloading on wound healing and the effectiveness of platelet rich plasma (PRP) as a countermeasure (Cialdai et al., 2020).

They used a new *in vivo* sutured wound healing model in the leech (*Hirudo medicinalis*) to evaluate the effect of microgravity on the healing process and the effects of PRP. The authors found a healing delay and structural alterations in the repair tissue, which were prevented by PRP treatment. In addition, PRP was able to counteract the microgravity-induced impairment in fibroblast migration to the wound site. In addition, it contains various growth factors. This would explain the beneficial effects of PRP in wound healing *in vivo*. Therefore, it can be assumed that PRP is also effective to promote healing in space. In respect to PRP application in space, on the ISS, its effectiveness, stability and storage conditions in microgravity have to be evaluated. Moreover, requirements to automate direct PRP preparation during spaceflight will be evaluated.

3D bioprinting technology or biofabrication of skin using endothelial cell, dermal fibroblast, and multilayered keratinocyte layers for skin tissue are currently a hot topic (Haldar et al., 2019; Barros et al., 2020).

Furthermore, biofabrication of new tissues in space on the ISS is possible, which will help humans traveling to the Moon or Mars and prosper there for years. In collaboration with ESA, OHB System AG and Blue Horizon, a research team from Dresden Technical University has developed a 3D bioprinting method for use in space, creating new skin and bone tissue from resources that might be available to astronauts, taikonauts and cosmonauts as well as space tourists traveling to the outer universe (https://www.esa.int/Enabling_Support/Space_Engineering_Technology/Upside-down_3D-printed_skin_and_bone_for_humans_to_Mars).

Taken together, several therapeutic strategies are available for humans in space on a deep exploration mission.

CONCLUSIONS AND OUTLOOK

In the near future, increased commercial spaceflights and space exploration programs will elevate the number of space missions. With the Artemis program, NASA will land the first woman and next man on the Moon in 2024. In addition, Russia and PR China will build a Moon station together. It is envisioned as "*a complex of experimental research facilities created on the surface and/or in the orbit of the moon*." Innovative technologies for the exploration of the lunar surface will start. Afterwards, the next goal is to send astronauts to Mars.

These new activities also mean an increased number of crew members and space tourists in space and on the Moon, which is accompanied by a higher risk of traumatic events or unexpected emergencies.

In case of emergency, ISS crew members will be transported to Earth, which is not possible in outer space. There is a continuous resupply with resources and medical equipment to the ISS. In outer space, guidance of the crew members remotely is not possible. Therefore, in this respect the spacecraft has to be self-sufficient. The crew members must be capable to manage severe situations like emergency surgery, acute trauma, wounds and burns. For these reasons, the spacecraft, space stations, Moon or Mars bases should host cutting-edge medical technology like 3D bioprinting equipment, regenerative medicine capacities, surgical instruments, functioning laboratories as well as amongst other medical equipment, freezers for blood samples, cells and tissues.

An important aspect is the behavior of the wound and/or suture and its healing. In the microgravity environment in space on the ISS are wound/suture behavior and repair processes completely unknown. Apoptosis plays an important role in normal wound healing without pathologic scarring. Several cells involved in wound repair have demonstrated an increase in apoptosis, when exposed to simulated or real microgravity. Especially lymphocytes and endothelial cells cultured in space and on microgravity simulators exhibited an increased number of apoptotic cells (Maccarrone et al., 2003; Infanger et al., 2006). In contrast, dermal fibroblasts exposed to the RPM did not reveal increases in programmed cell death (Buken et al., 2019), which implicates that mainly the early phases of wound repair might be influenced by microgravity.

In vitro experiments focusing on processes involved in wound repair demonstrated abnormal migration behavior, collagen formation, enhanced inflammation and reduced cellular organization (Morbidelli et al., 2005; Monici et al., 2006; Cialdai et al., 2017). Cells exposed to real microgravity revealed changes in the gene expression pattern with pro-apoptotic signaling (Liu and Wang, 2008). Mice flown for 91 d in space exhibited signs of skin senescence with skin atrophy (Neutelings et al., 2015).

Taken these findings together, it can be assumed that wound healing in space might be delayed and defective, which needs to be proven. Therefore, space missions to the ISS with focus on wound healing models are necessary.

The spaceflight proposal to the ISS with the number ILSRA-2014-0043 (PI Professor Monica Monici, Florence, Italy) with the title "Wound healing and sutures in unloading conditions" was selected by ESA. The spaceflight experiment focuses on the influence of gravity on the behavior of sutures and wound healing on the ISS. It will clarify how suturing materials and techniques can be adapted in space and it will develop strategies to improve wound healing in space. Furthermore, the project aims to improve suturing methods and wound healing avoiding scarring on Earth.

AUTHOR CONTRIBUTIONS

TC compiled the **Table 1** and **Figure 1**. DM performed the NHDF RPM experiment and the TUNEL assay. MK provided **Figures 2–4**. All authors reviewed and evaluated the literature, wrote the article, contributed to the article, and approved the submitted version.

FUNDING

This research was funded by Deutsches Zentrum für Luft- und Raumfahrt (DLR), BMWi projects 50WB1524 and 50WB1924 (DG).

ACKNOWLEDGMENTS

We like to thank Viviann Sandt for culturing the NHDF cells for **Figure 3**.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Bioengineering and Biotechnology

BRIEF RESEARCH REPORT
published: 21 July 2021
doi: 10.3389/fbioe.2021.687946

Temporary Storage of the Human Nasal Tissue and Cell Sheet for Wound Repair

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Temporary storage of nasal tissues and nasal cell sheets, which entails transportation between hospitals and cell culture facilities, is an important issue in regenerative medicine. Herein, we investigated the preservation of chilled and frozen nasal tissues and expiry dates of ready-to-use nasal cell sheets. Although the cell number in preserved tissues was lower than that in fresh tissue, nasal cell sheets could be fabricated from tissues that had been refrigerated for 5 days and frozen–thawed over 5 days. Moreover, the nasal mucosal cell sheets were preserved in a non-hazardous buffer. The cell number, viability, and structure were not maintained in saline containing E-cadherin for 2 days; however, these were maintained in Hank's balanced salt solution for 2 days, but not for 5 days. To assess the proliferation capacity of cells in the stored cell sheets, we performed cell sheet grafting assays *in vitro*. Cell sheets stored in Hank's balanced salt solution for 2 days adhered to collagen gel and expanded normally. Our results show that nasal tissues can be stored temporarily in refrigerators or deep freezers, and Hank's balanced salt solution can be used for preservation of ready-to-use cell sheets for a few days.

Keywords: human nasal tissue, cell sheet, preservation, cryopreservation, ready-to-use, wound healing

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *30 March 2021*
Accepted: *16 June 2021*
Published: *21 July 2021*

Citation:
Kasai Y, Morino T, Dobashi I, Mori E, Yamamoto K and Kojima H (2021) Temporary Storage of the Human Nasal Tissue and Cell Sheet for Wound Repair. Front. Bioeng. Biotechnol. 9:687946. doi: 10.3389/fbioe.2021.687946

INTRODUCTION

Intractable otitis media, cholesteatoma, and adhesive otitis media were successfully treated using autologous nasal mucosal cell sheet as a regenerative medicine in a clinical study (Yamamoto et al., 2017). To develop new regenerative medicines using autologous somatic cells, the expiration date of the final product should be defined (Hayakawa et al., 2015; Knoepfler, 2015; Lopez-Beas et al., 2020). We previously showed that both nasal tissue and nasal mucosal cell sheets can be transported for 3 h without a decrease in quality (Kasai et al., 2019). However, the expiration dates for both nasal tissue and its cell sheet are unknown. In a study on preservation of the lung tissue, considerable apoptosis of cells stored for 5 days at 4°C was observed (Abe et al., 2006), indicating that the expiration date for the lung tissue preserved in refrigerator could be 5 days. Moreover, cells from cryopreserved umbilical cord or adipose tissues have the ability to grow and differentiate (Shimazu et al., 2015; Arutyunyan et al., 2018; Zanata et al., 2018); not only suspended cells, native tissues can also be stored in a deep freezer. In this context, we hypothesized that the

Abbreviations: CFE, colony-forming efficiency; HBSS, Hank's balanced salt solution; HE staining, hematoxylin-eosin staining; KCM, keratinocyte culture medium; PARP, poly (ADP-ribose) polymerase.

nasal mucosal tissue could be preserved in refrigerator for 5 days and can be potentially preserved in deep freezer. Because the medium used for preservation of the cell sheet (the final product) contains some adventitious agents, it must be completely washed off. Hank's balanced salt solution (HBSS)-based buffers reportedly keep the morphology of oral keratinocyte sheet intact for 7 days (Katori et al., 2016). We hypothesized that the nasal mucosal cell sheet could be preserved in HBSS for at least a few days.

In this study, we evaluated the expiration date of nasal mucosal tissue in keratinocyte culture medium (KCM), in which nasal tissue can be preserved for at least 3 h without contamination, as well as the expiration date of nasal cell sheets in non-hazardous buffer, namely HBSS and normal saline. We analyzed the cell number, viability, and the proliferative capacity of nasal mucosal cells in the cell sheets using previously established *in vitro* assays (Kasai et al., 2020) to compare various parameters before and after preservation.

METHODS

Preparation and Preservation of Nasal Mucosal Tissue

All the experiments were performed in accordance with the Declaration of Helsinki and Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan. This study was approved by the Institutional Review Board of the Jikei University, Tokyo, Japan. All 13 patients, who were scheduled to undergo endoscopic sinus surgery, provided informed consent. Nasal mucosal tissue was collected from inferior nasal turbinate mucosa during endoscopic sinus surgery. The collected nasal mucosal tissue was disinfected using povidone-iodine. Depending on the experiment, tissues were maintained in KCM at 4°C or in freezing medium at −80°C (Stem-Cellbanker, GMP-grade, Nippon Zenyaku Kogyo, Fukushima, Japan). None of the volunteers was infected with human immunodeficiency virus, syphilis, or hepatitis B and C virus.

Cell Expansion

KCM containing 10 μM Rho-associated kinase inhibitor (Y-27632, Wako Pure Chemical) was prepared, and explant culture was performed, as previously described by us (Kasai et al., 2020). Briefly, washed nasal mucosal tissue samples were cut into cubes (1.5 mm^3); 32 cubes were placed on eight cell culture dishes (60 mm Primaria dish, Corning, Inc., Corning, NY, United States) and incubated at 37°C in an atmosphere of 5% CO_2. Following 13 days of culture, the cells were collected by treatment with trypsin EDTA. In subculture, 3T3-J2 cells irradiated with X-ray from a J-TEC (Aichi, Japan) were seeded at a density of 4.0×10^4 cells/cm^2 as the feeder layer. Epithelial cells were seeded at a density of $1.0–3.0 \times 10^4$ cells/cm^2 on the 3T3-J2 cells. Following 4–7 days of culture, the cells were collected and stored in freezing medium.

Cell Sheet Culture and Preservation

The 3T3-J2 cells were seeded into temperature-responsive cell culture substrate (CellSeed, Tokyo, Japan) in KCM with 10 μM Y-27632 and incubated for over 2 h. Expanded nasal mucosal epithelial cells were thawed and seeded at a density of 3.0×10^4 cells/cm^2 over the 3T3-J2 cell layer. The medium in the dishes was replaced with KCM on days 3, 5, and 7. The cells were detached as a cell sheet on day 8. For the preservation test, confluent cells were submerged in HBSS or saline.

Histological Analysis

Histological analysis was performed as described in our previous article (Kasai et al., 2019). All sections were fixed in 4% paraformaldehyde (Wako Pure Chemical), embedded in paraffin, and sliced into 4 μm-thick sections. The sections were deparaffinized and stained with hematoxylin and eosin (HE; both from Wako Pure Chemical Industries, Osaka, Japan). For immunohistology, antigens were activated by autoclaving (121°C, 10 min) with citrate buffer (Histo-VT One, Nacalai Tesque, Kyoto, Japan). Non-specific reactions were prevented by peroxidase blocking (Dako, Carpinteria, CA, United States) and protein blocking (Nacalai Tesque). The sections were incubated overnight with the primary antibodies, namely anti-Ki-67 monoclonal antibody (1:100; M7240; Dako), anti-cleaved PARP (Asp214) monoclonal antibody (1:50; clone: D64E10, #5625; Cell Signaling Technology, Danvers, MA, United States), or anti-E-cadherin monoclonal antibody (1:100; M3612; Dako) at 4°C. After washing the sections with PBS, they were incubated with horseradish peroxidase-tagged secondary antibodies (REAL EnVision™ Detection System, Dako) at room temperature for 1 h. Thereafter, the sections were treated with the peroxidase substrate, 3,3′-diaminobenzidine (K5007; Dako). Nuclei were stained with hematoxylin.

Viable Cell Count and Colony-Forming Assay

To analyze the proliferative potential of the cells in the sheets, viable cells were counted and a colony-forming assay was performed as described in our previous article (Morino et al., 2018). A total of 2,000 live cells from cell sheets were seeded on mitomycin-treated 3T3 cells in KCM containing 1 μM Y-27632. The medium was refreshed on days 5 and 10. After 12 days, the cells were fixed using 4% paraformaldehyde, and stained with crystal violet solution (Merck, Darmstadt, Germany).

In vitro Grafting Assay

An *in vitro* grafting assay was performed as previously reported (Kasai et al., 2017, 2020). Briefly, a harvested nasal cell sheet was attached onto type I collagen gel in a 60 mm dish, and cell cultivation was continued for 7 days in KCM containing 1 μM Y-27632.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 7.0 software (GraphPad, Inc., La Jolla, CA, United States). The mean values in a two-sample comparison were determined by Student's *t*-test and expressed as *P*-values. The comparison of mean values

of multiple sample groups was done by Bonferroni and Tukey–Kramer multiple comparison tests after one-way analysis of variance. $P < 0.05$ were considered significant.

RESULTS

Evaluation of Proliferative Ability of Nasal Tissue After Preservation

Results of HE staining show the presence of cilia, goblet, and basal epithelial cells in the pre-storage tissue, while some upper layer cells were peeled off from the refrigerated and cryopreserved tissue samples (**Figure 1A**). To detect the proliferation activity of the cells in fresh tissue stored at 4°C for 5 days (refrigerated tissue) and at −80°C for 19–70 days (cryopreserved tissue), the expression of proliferation marker, Ki-67, and apoptosis marker, poly (ADP-ribose) polymerase (PARP), was examined by immunohistological analysis. Although some upper layer cells were desquamated from the refrigerated tissue, the expression of Ki-67 was observed in all the specimens. PARP was expressed in the refrigerated tissue, suggesting the initiation of apoptosis. For long-term preservation, we also investigated applicability of the cryopreservation method. Although PARP was expressed in cryopreserved tissue, Ki-67 was also expressed. These results indicate that the cell proliferation ability was retained in all tissues.

In the explant culture, cellular outgrowths were observed irrespective of whether preservation was performed. After 13 days of culture, $15.3 \pm 3.4 \times 10^6$ epithelial cells were collected from fresh tissue samples (**Figure 1B**, $n = 6$). The difference in the cell number was not significant compared with that obtained from refrigerated tissue samples ($10.4 \pm 3.4 \times 10^6$ cells, $n = 4$; $P > 0.05$; **Figure 1B**). The cell number in the case of cryopreserved tissue samples was significantly lower than that in the case of fresh tissue ($7.1 \pm 2.4 \times 10^6$ cells, $n = 6$; $P < 0.01$; **Figure 1B**) but was sufficient for fabricating more than 10 cell sheets.

Preservation and Its Limits for the Fabrication of Nasal Mucosal Cell Sheets

Feeder supported nasal epithelial cells, derived from the pre-storage tissue samples, formed cell sheets with no defects, contained over 1 million cells ($15.8 \pm 0.2 \times 10^5$ cells, $n = 7$; **Figures 2A,B**), and exhibited high viability ($89.5 \pm 2.0\%$, $n = 5$; **Figure 2C**). After the cell sheet was preserved in HBSS for 2 days, no significant difference in the cell number ($14.6 \pm 1.8 \times 10^5$ cells, $n = 5$, $P > 0.05$; **Figure 2B**) or cell viability ($87.9 \pm 3.5\%$, $n = 5$, $P > 0.05$; **Figure 2C**) of the pre-storage cell sheet was observed. In contrast, the shape of cell sheets could not be maintained for 2 days in saline (**Figure 2A**). In addition, the cell number ($6.0 \pm 0.6 \times 10^5$, $n = 7$, $P < 0.05$; **Figure 2B**) and cell viability ($38.0 \pm 9.2\%$, $n = 5$, $P < 0.05$; **Figure 2C**) upon storage in saline were significantly lower than the values during pre-storage. After preserving the cell sheet for 5 days in HBSS, it was impossible or difficult to detach an intact cell sheet (**Figure 2A**). Cell viability in the post-storage cell sheet in HBSS for 5 days tended to be lower than that during pre-storage ($70.7 \pm 22.3\%$, $n = 7$, $P > 0.05$; **Figure 2C**). Although the difference was not significant, the cell numbers in the cell sheet stored in HBSS for 5 days were significantly lower than those during pre-storage ($6.3 \pm 1.3 \times 10^5$ cells, $n = 7$, $P < 0.05$; **Figure 2B**). These results indicate that HBSS is suitable for preserving nasal mucosal cell sheets for 2 days.

As observed for cells derived from either refrigerated or cryopreserved tissue samples, the cell sheet could be detached in an intact condition (**Supplementary Figures 1A,D**). Although the cell number and viability were not significantly changed in pre- and post-storage samples (**Supplementary Figures 1B,C,E,F**), it was not possible to detach the cell sheet without any defect after 2 days in saline or after 5 days in HBSS. These results are similar, as the cell sheets were derived from fresh tissues.

Histological Evaluation of Nasal Mucosal Cell Sheets

We performed HE staining and immunohistological analysis of cell sheets (**Figure 2D**). HE staining showed that pre-storage cell sheets were composed of approximately 2–5 layers of squamous epithelial cells; however, no cilia or secreting cells were observed. Moreover, the structure of cell sheet was maintained for 2 days in HBSS even if the cell sheets cells were derived from refrigerated or cryopreserved tissue samples (**Figure 2D** and **Supplementary Figures 2A,B**). In contrast, the cell sheet could barely be sampled for 2 days in saline derived from pre-preserved tissue (**Figure 2D**), it was impossible to sample for 2 days in saline derived from post-preserved tissues¯there were no data in **Supplementary Figures 2A,B**. E-cadherin was expressed in almost all cells within normal cell sheets and in cell sheets preserved for 2 days in HBSS, whereas its expression was not strong in cell sheets preserved in saline for 2 days. Based on the analysis of expression of Ki-67, cells in the sheets could proliferate for 2 days in HBSS. Ki-67 was expressed in the cell sheets at 5 days after preservation in HBSS. We believe that the upper layer cells, rather than the basal proliferating cells, were peeled off as dead cells. These results support the notion that HBSS can maintain the quality of cell sheets for 2 days.

Proliferative Ability of Nasal Mucosal Cell Sheets Is Maintained for a Few Days

To assess the proliferation potential of cell sheets before and after preservation, we performed an *in vitro* cell sheet grafting assay. Because samples of cell sheets preserved for 2 days in saline or for 5 days in HBSS could not be detached intact, they were not amenable to *in vitro* assays. All samples of normal cell sheet and cell sheets preserved for 2 days in HBSS successfully adhered and migrated to the collagen gel (**Figure 3A**). The expansion rate at 7 days after grafting was not significantly different from that of pre-storage cell sheets ($193 \pm 67\%$, $n = 4$) and of cell sheets stored in HBSS ($92 \pm 31\%$, $n = 4$, $P > 0.05$; **Figure 3B**). Moreover, the expansion rate of the cell sheet derived from refrigerated tissue samples was $342 \pm 33\%$ ($n = 4$) and for the cell sheet stored in HBSS was $405 \pm 82\%$ ($n = 4$, $P > 0.05$;

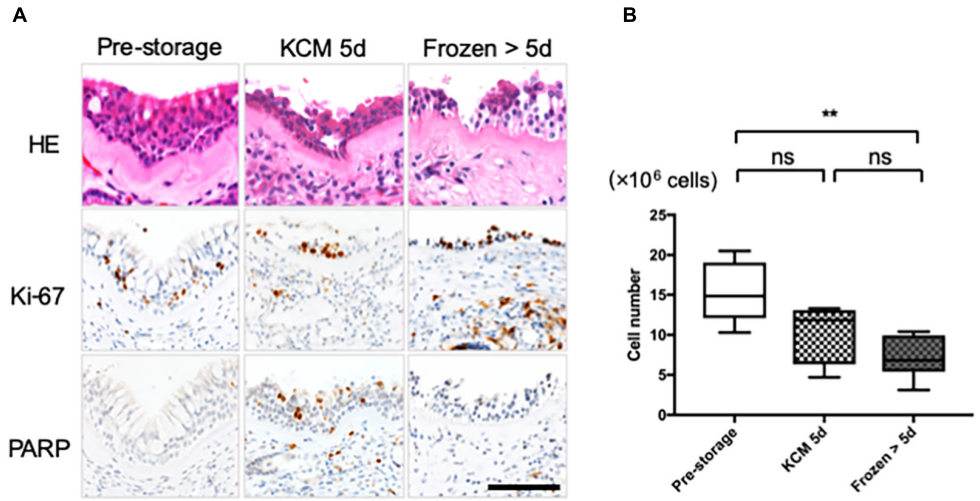

FIGURE 1 | Nasal cells expanded from tissue refrigerated for 5 days and frozen–thawed tissue. **(A)** Hematoxylin and eosin (HE) staining and immunohistological evaluation of the expression of Ki-67 and PARP in pre- and post-storage nasal tissue. **(B)** Cell number was determined for cells cultured from each explant in eight dishes under each condition. Scale bar = 100 μm. $^{**}P < 0.01$; ns, not significant.

Supplementary Figures 2A,B); the expansion rate of the cell sheet derived from frozen tissue samples at 7 days after grafting was 303 ± 27% (n = 4) and for the cell sheet stored in HBSS was 314 ± 64% (n = 4, $P > 0.05$; **Supplementary Figures 2C,D**). We performed HE staining and immunohistological analysis of cell sheets derived from pre-storage (**Figure 3C**), refrigerated (**Supplementary Figure 3E**), and cryopreserved (**Supplementary Figure 3F**) tissue samples at 7 days after grafting. HE staining showed that the pre-storage cell sheets and those stored in HBSS adhered to the collagen gel. Immunohistological analysis showed that E-cadherin connected each cell, enabling the collective migration as observed in epithelial wound healing. Moreover, Ki-67 was still expressed in the grafted cell sheets. These findings indicate that the proliferation ability and wound healing potential of nasal mucosal cell sheets stored in HBSS was retained for 2 days.

Colony-forming assays (**Figure 3C**) showed that the colony-forming efficiency (CFE) of a normal cell sheet was 3.1 ± 1.4% (n = 4; **Figure 3D**). The CFE score of cell sheets at 2 days after preservation in HBSS showed no significant difference (3.9 ± 2.3%, n = 4, $P > 0.05$; **Figure 3D**). The CFE of the cell sheet at 2 days after preservation in saline was 0.9 ± 0.6% (n = 4; **Figure 3D**) and at 5 days after preservation in HBSS was 1.5 ± 0.8% (n = 4; **Figure 3D**); the value tended to be lower than that at pre-storage, although the difference was not significant. In addition, the CFE scores of cell sheets derived from tissue samples refrigerated for 5 days and from frozen tissue samples were similar to those obtained for cell sheets derived from fresh tissue samples (**Supplementary Figure 3**).

DISCUSSION

Tissue preservation has important effects on precise analysis, cell culture, and tissue transportation in regenerative medicine (Takagi et al., 2015; Mizuno et al., 2017). We found that nasal cells cultured from refrigerated and frozen–thawed tissues formed nasal mucosal cell sheets. In the refrigeration method, KCM maintained the cell proliferation ability for 5 days, which is sufficient for short-term preservation and domestic transportation (**Figure 4**).

Two-stage operation is an effective technique for treating middle ear cholesteatoma and is performed 6–12 months after the first operation (Ho and Kveton, 2003; Kojima et al., 2006). If the nasal mucosal tissue can be preserved longitudinally, it would be useable for the second surgery. According to Shimazu et al., Stem-Cellbanker® is the best medium for cryopreservation of umbilical cord tissue (Shimazu et al., 2015). Based on their study, we used Stem-Cellbanker® to cryopreserve nasal mucosal tissue and succeeded in fabricating cell sheets (**Supplementary Figures 1D–F**), although the cell number was lower than that in cells sheets derived from fresh tissue on day 13 (**Figure 1B**). Therefore, cryopreservation may be used for the two-stage operation. Moreover, because part of the nasal mucosal tissue is discarded during nasal surgery, the cryopreservation technique may be useful for further basic research.

HBSS used for preservation of cell sheets contains inorganic ions (i.e., calcium ions) and glucose, which contribute to calcium-dependent adhesion via E-cadherin (Takeichi, 1977). The cell number and viability of cells in the cell sheet were maintained for 2 days (**Figures 3A–C**). Because calcium ions are not present in saline, calcium dependent cell–cell adhesion was not retained in cell sheets preserved in saline (**Figure 2D**). In other studies, a retinal pigment cell sheet was preserved for 5 h (Hori et al., 2019) and an oral mucosal cell sheet was preserved for 12 h (Oie et al., 2014). Therefore, cell sheet quality can be maintained in HBSS for 2 days, which is long enough for stability studies and for transport throughout Japan. This time period is longer than that reported in our previous study (3 h) (Kasai et al., 2019).

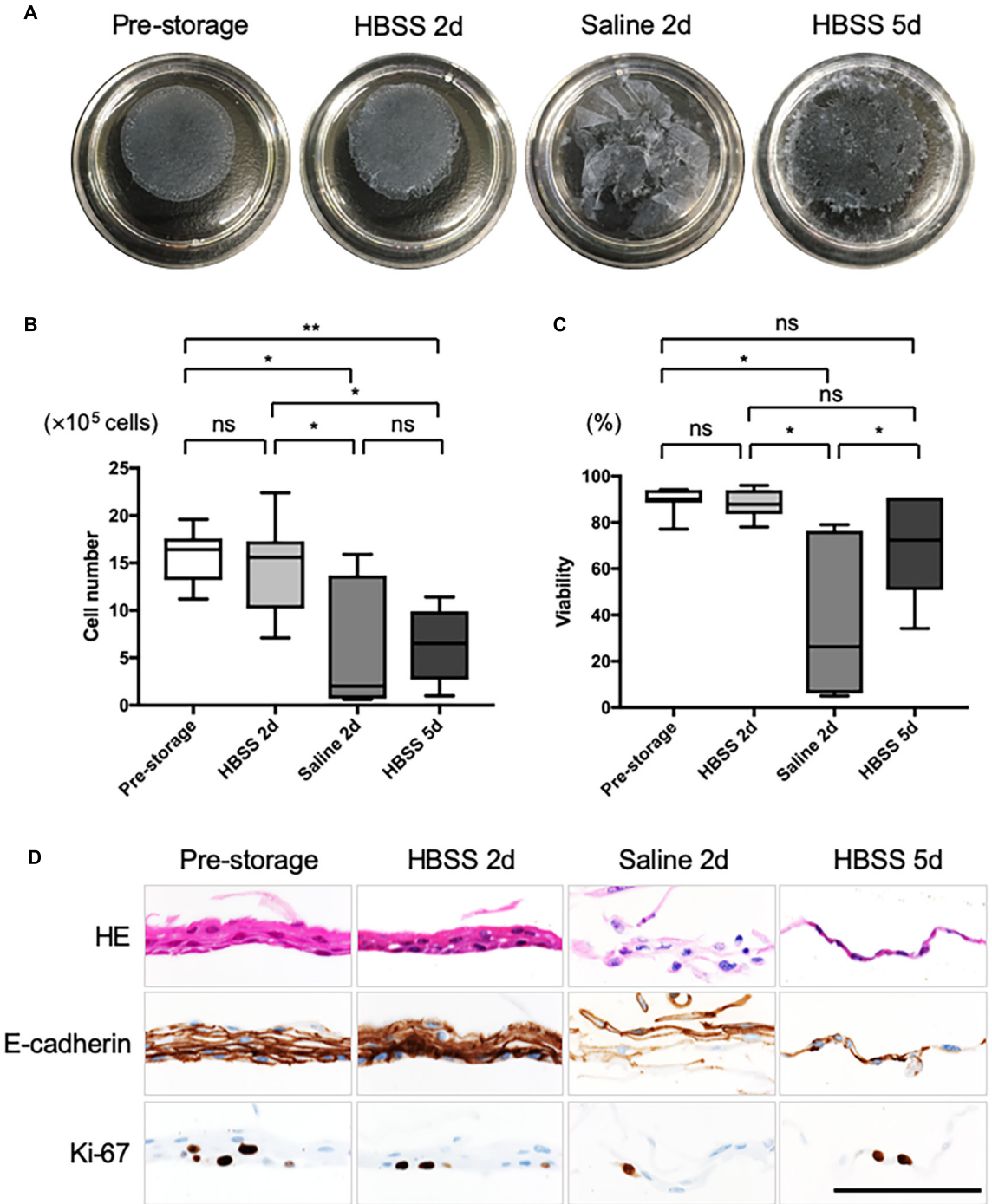

FIGURE 2 | Quality evaluation of post-storage cell sheet. **(A)** Representative images of the cell sheet before and after preservation. **(B)** Cell number was determined from a harvested cell sheet derived from nasal mucosal tissues (n = 5) under each condition. **(C)** Cell viability was determined from a harvested cell sheet obtained from nasal mucosal tissue (n = 5) under each condition. **(D)** Hematoxylin and eosin (HE) staining and immunohistological evaluation of the expression of E-cadherin and Ki-67 in pre- and post-storage nasal tissues. Scale bar = 100 μm. *P < 0.05; **P < 0.01; ns, not significant.

It is important that a cell sheet maintains its proliferative potential in a manner similar to that observed for other regenerative medicines (Rama et al., 2010; Butler et al., 2016; Islam et al., 2017). Epithelial cell sheet is known to be applicable for wound healing (Gallico et al., 1984; Nishida et al., 2004). The wound healing-like behavior of cell sheet has been observed using *in vitro* grafting assay (Kasai et al., 2017). Similarly, cell sheet preserved for 2 days in HBSS expressed Ki-67 after grafting and

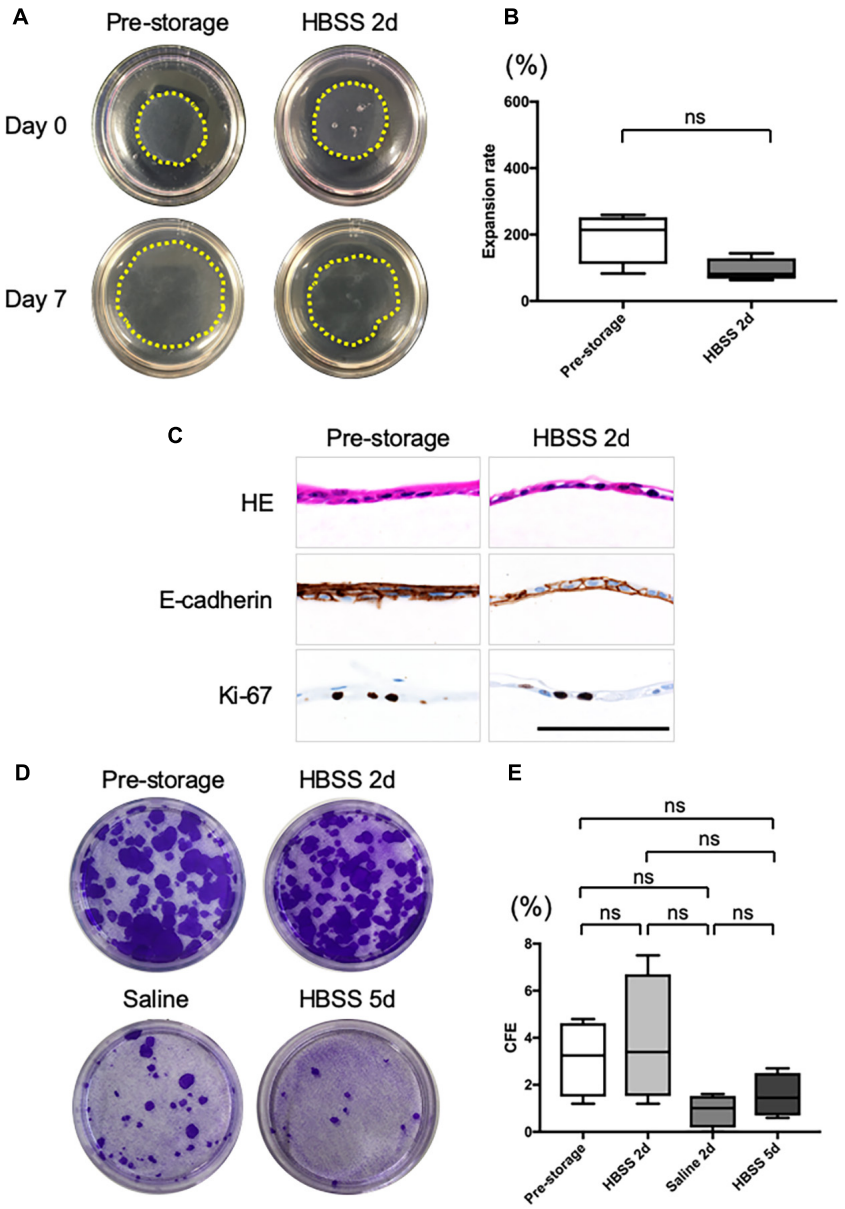

FIGURE 3 | *In vitro* evaluation of the cell sheet after grafting. **(A)** Representative images of pre- and post-storage cell sheets, 0 and 7 days after grafting on type I collagen gels in 60 mm dishes. The yellow dotted line shows the edge of grafted cell sheets. **(B)** Expansion rate of cell sheet size from day 0 to day 7 after grafting (n = 6). **(C)** Hematoxylin and eosin (HE) staining and immunohistological evaluation of the expression of E-cadherin and Ki-67 in cell sheet 7 days after grafting on collagen gel. **(D)** Representative images of colony-forming assays for pre- and post-storage cell sheets under each condition. **(E)** Colony-forming efficiency (CFE). Values are expressed as the mean ± SEM (n = 6) values. ns, not significant.

showed wound healing-like behavior, indicating its proliferation on the collagen gel (**Figure 3**). Thus, our results show the wound healing potential of cell sheets preserved for 2 days in HBSS.

There are some limitations to this study. First, we preserved the cell sheets only at room temperature. Hori et al. preserved retinal pigment epithelium cell sheets at 37°C (Hori et al., 2019), and Kawazoe et al. preserved skin grafts at 4°C (Kawazoe et al., 2008). Therefore, optimization of temperature may be one of the key factors for long-term preservation of cell sheets. Second, considering mass production, we used foreign substances, including gamma-irradiated fetal bovine serum and X-ray-irradiated 3T3-J2 cells. Although these agents have been used for the development of JACE® as a regenerative medicine product, which has an excellent safety record, the residues in the final product must be analyzed. Third, we did not precisely characterize the components of various cell types in the cell sheets before and after grafting. Because nasal mucosal tissue contains different cell types, including basal epithelial cells, goblet cells,

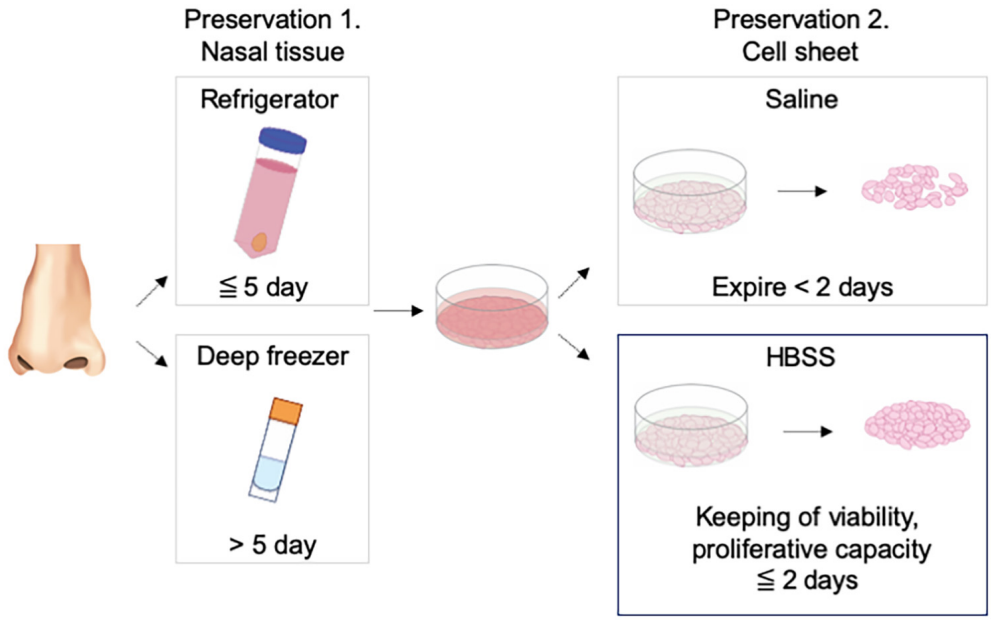

FIGURE 4 | Schematic diagram for the study. Nasal mucosal cell sheet could be fabricated from nasal mucosal tissue stored in a refrigerator or deep freezer and stored in Hank's balanced salt solution for 2 days in a ready-to-use condition.

and ciliated cells, precise mRNA and protein assays are important to understand the safety and effectiveness of the nasal mucosal cell sheet. Finally, although the wound healing potential was evaluated *in vitro*, an assessment of the effect of nasal mucosal cell sheet after *in vivo* grafting is still a challenging issue for this regenerative medicine. Further studies are needed to address these limitations.

CONCLUSION

Nasal tissue cells maintained their proliferative ability in a cell sheet when stored in a refrigerator for 5 days or in a deep freezer for more than 5 days. Nasal mucosal cell sheets can retain their wound healing potential for 2 days in HBSS, which can be used as a ready-to-use preservative. Our findings may facilitate the fabrication of stable cell grafts for use in regenerative medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of the Jikei University, Tokyo, Japan (approval number: 26-359). Informed consent was obtained from all volunteers. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YK, TM, KY, and HK contributed to conception and design of the study. YK, ID, and EM performed the experiments. YK wrote the first draft of the manuscript. TM, ID, and KY revised the manuscript. HK supervised this project. All authors approved the original and revised versions of the manuscript.

FUNDING

This work was funded by JSPS KAKENHI (Grant nos. 18K16907, 19H03806, 18K09332, 21K09640, and 21K16848), AMED (Grant nos. 18bk0104051, 19bk0104086, 20bk0104086, and 21bk0104086), and The Jikei University Strategic Prioritizing Research Fund.

ACKNOWLEDGMENTS

We appreciate the volunteers who donated nasal mucosal tissues. We wish to thank Hsu Hung-I for providing cell culture-related support. We wish to thank Japan Tissue Engineering Co., Ltd., for providing 3T3-J2 cells and advising us regarding this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.687946/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Bioengineering and Biotechnology

REVIEW
published: 22 September 2021
doi: 10.3389/fbioe.2021.720091

Effect of Microgravity on Endothelial Cell Function, Angiogenesis, and Vessel Remodeling During Wound Healing

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Wound healing is a complex phenomenon that involves different cell types with various functions, i.e., keratinocytes, fibroblasts, and endothelial cells, all influenced by the action of soluble mediators and rearrangement of the extracellular matrix (ECM). Physiological angiogenesis occurs in the granulation tissue during wound healing to allow oxygen and nutrient supply and waste product removal. Angiogenesis output comes from a balance between pro- and antiangiogenic factors, which is finely regulated in a spatial and time-dependent manner, in order to avoid insufficient or excessive nonreparative neovascularization. The understanding of the factors and mechanisms that control angiogenesis and their change following unloading conditions (in a real or simulated space environment) will allow to optimize the tissue response in case of traumatic injury or medical intervention. The potential countermeasures under development to optimize the reparative angiogenesis that contributes to tissue healing on Earth will be discussed in relation to their exploitability in space.

Keywords: wound healing, angiogenesis, angiogenic factors, endothelial cells, microgravity, drugs, cell therapy, physical therapy

OPEN ACCESS

Edited by:
Jack J. W. A. van Loon, Vrije Universiteit Amsterdam, Netherlands

Reviewed by:
Livia Visai, University of Pavia, Italy
Ngan F. Huang, Stanford University, United States

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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *03 June 2021*
Accepted: *03 August 2021*
Published: *22 September 2021*

Citation:
Morbidelli L, Genah S and Cialdai F (2021) Effect of Microgravity on Endothelial Cell Function, Angiogenesis, and Vessel Remodeling During Wound Healing. Front. Bioeng. Biotechnol. 9:720091. doi: 10.3389/fbioe.2021.720091

INTRODUCTION

The skin is the largest organ by surface area in the human body. Its structure is highly organized with different cell types (epithelial, stromal, and endothelial cells-ECs), which finely cooperate in order to guarantee a constant functioning and structural homeostasis of the organ. Indeed, the skin is the first defensive barrier of our body that protects internal tissues from microbial infections, mechanical damages, UV radiations, dangerous substances, and high temperatures. Therefore, the maintenance of its integrity is fundamental for our survival and skin repair, following a mechanical or physical injury, implying very complex and delicate processes to recover its integrity and barrier function (Sorg et al., 2017; Rodrigues et al., 2019). Here we report the state-of-the art of tissue wound healing phases and factors, the role of angiogenesis and the potential pharmacological, cellular, and physical countermeasures acting on ECs. The aim of the article is to understand how to apply all these findings in the space environment as the one that astronauts face during long duration missions. Indeed during space travels the possibility for astronauts to hurt themselves during their routine activities is not excluded. The availability of countermeasures with verified effectiveness and safety in unloading conditions will guarantee the optimal tissue healing and health recovery in extreme environments without the urgency to rapidly return to Earth. The review article is based on the recent/most cited

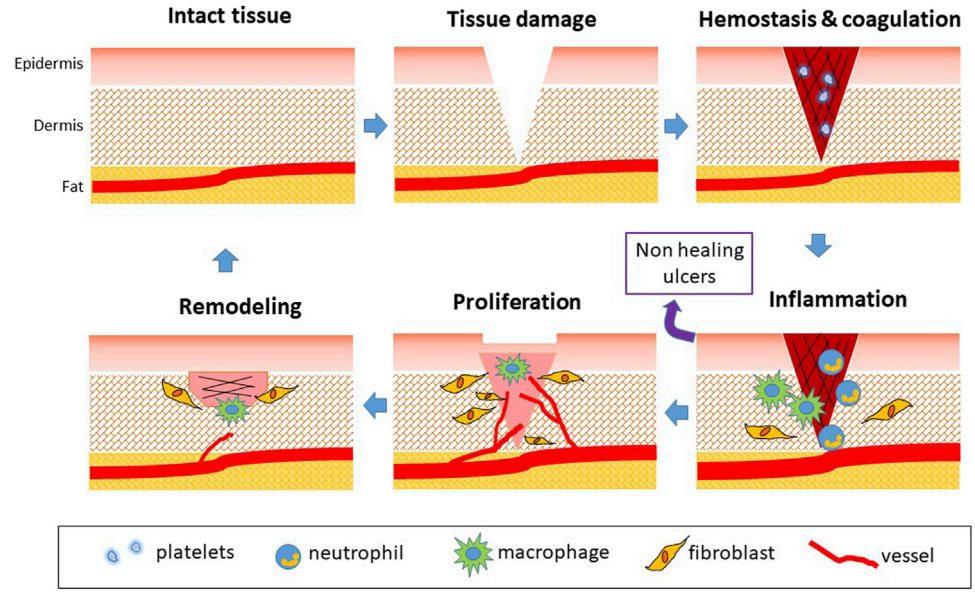

FIGURE 1 | Phases of physiological wound healing. Following tissue injury, hemostasis occurs. Coagulation is responsible for fibrin clotting and cessation of blood flowing. Platelets activate and secrete PDGF, EGF, TGF-β, CXCL4, and PF4. The following phase is represented by inflammation, also induced by tissue debris and bacteria. Vessels dilate in a NO-dependent manner. Neutrophils (0–3 days) exert antimicrobial activity, secreting MMPs and cytokines as TNF-α and IL-1/4/6. Monocytes are attracted (1–7 days) and transform in M1 macrophages involved in host defense with phagocytic activity. ROS are generated to eliminate bacteria. Then, M2 phenotype occurs with the production of growth factors involved in tissue repair. The proliferation phase is characterized by angiogenesis occurrence due to release of VEGF and FGF, EC proliferation and migration and fibroblast proliferation. Fibroblasts participate in angiogenesis through secretion of proteinases and deposition of ECM proteins. Reepithelialization starts and actively continues during the remodeling phase, characterized by collagen conversion and vascular regression. The release of TGF-β induces wound contraction and closure, and tissue remodeling, till the recovery of tissue integrity. When inflammation persists, wounds do not heal and chronic ulcers occur.

papers of PubMed and Scopus databases on the topic of reparative angiogenesis, wound healing, and related countermeasures.

WOUND HEALING PHASES

Wound healing involves a coordinated interaction of cells, proteins, growth factors, small molecules, proteases, and extracellular matrix (ECM) components aiming at restoring tissue morphology and functioning. The network of communications between stromal, endothelial, and immune cells is the key for determining the course of healing and recovery of tissue function and features (Rodrigues et al., 2019). Skin repair process can be divided into sequential phases: hemostasis, inflammation, proliferation, and remodeling (**Figure 1**). Although different growth factors, cytokines, and predominant cell types characterize each phase at different times, a considerable amount of overlap can occur (Sorg et al., 2017; Rodrigues et al., 2019). The four phases require different kinetics: 1) coagulation and hemostasis, starting immediately after injury; 2) inflammation, shortly after, lasting few days; 3) proliferation, occurring in several days; 4) wound remodeling, lasting days or even many months (**Figure 1**) (Reinke and Sorg, 2012; Velnar and Gradisnik, 2018).

The first response to a wound is constriction of the injured blood vessels, accompanied by activation of platelets and coagulation to form a fibrin clot to stop blood flow and provide a scaffold for incoming inflammatory cells. Indeed, in response to a mechanical injury, coagulation and hemostasis activate to prevent exsanguination and form a supportive matrix (including fibrin, fibronectin, vitronectin, and thrombospondins) that represents a substrate for invading cells, required later during the process. Upon injury, the microvasculature is disrupted leading to fluid accumulation, inflammation, and the development of hypoxia (Veith et al., 2019). At this stage, platelet-derived chemotactic factors released by platelet α-granules recruit leukocytes and monocytes to the area of injury to start the inflammatory phase finalized to tissue debris removal and bacteria killing (Etulain, 2018; Ridiandries et al., 2018). Leukocytes produce and release chemokines and cytokines (interleukin IL-1α, -1β, -6 and tumor necrosis factor-alfa, TNF-α), which, together with reactive oxygen species (ROS) release, amplify the inflammatory response (Reinke and Sorg, 2012; Veith et al., 2019). This second phase, often associated with edema, erythema, heat, and pain, aims to prepare the wound bed for the growth of new tissue. However, inflammation can become problematic if it is prolonged or excessive.

Once the lesion is cleaned out, the wound enters the third proliferative phase, where the aim is to fill and contract the wound edges and cover the gap. The proliferative phase is characterized by granulation tissue formation, collagen deposition, angiogenesis, and reepithelialization. Granulation tissue consists of layers of fibroblastic cells separated by a

collagenous extracellular matrix containing capillary buds and inflammatory cells. The recruited neutrophils and macrophages release growth factors, such as transforming growth factor-beta (TGF-β), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), responsible for activating resident ECs toward an angiogenic phenotype. Angiogenesis consists of EC proliferation, migration, and branching to form new blood vessels. Neovascularization, regulated predominantly by hypoxia-induced VEGF released by macrophages and fibroblasts, is needed to deliver nutrients and maintain the granulation tissue bed (Tonnesen et al., 2000, Li et al., 2003; Reinke and Sorg, 2012). Additionally, soluble factors induce endothelial precursor cells (EPC) recruitment in the granulation tissue and promote fibroblast proliferation and migration with changes in the ECM architecture. In this intermediate phase, macrophages continue to supply growth factors, promoting angiogenesis and stimulating resident fibroblasts to invade the wound and proliferate and remodel ECM through the synthesis of collagen, fibronectin, laminin, and metalloproteases, providing strength and elements to the injured tissue (Gurtner et al., 2008; Darby et al., 2014; Etulain, 2018; Ridiandries et al., 2018; Rodrigues et al., 2019). In the complex, the granulation tissue is composed by a high density of fibroblasts, granulocytes, macrophages, and capillaries (for the 60% of the mass) and loosely organized collagen bundles. It is well documented that angiogenic factors are present in wound fluid and promote repair, while antiangiogenic factors inhibit repair (Li et al., 2003; Schultz and Wysocki, 2009). During healing of the tissue defect, the edges of the wound are progressively brought together by the retraction of granulation tissue. This is due to the effect of TGF-β1 on fibroblasts which are induced to differentiate in myofibroblasts, contractile cells with stress fibers containing α-smooth muscle actin. This phenomenon, called wound contraction, is of great clinical importance in reducing the size of the wound. Reepithelialization simultaneously occurs and involves the proliferation of both unipotent epidermal stem cells from the basal layer and dedifferentiation of terminally differentiated epidermal cells (Gurtner et al., 2008; Rodrigues et al., 2019).

The final stage of the repair process, the remodeling or maturation phase, is characterized by the transition from granulation tissue to scar formation and maturation. Wound remodeling takes place when components of ECM undergo changes (e.g., replacement of collagen III by stronger collagen I) and myofibroblasts reduce the scar surface (Tomasek et al., 2002; Darby et al., 2014).

The process concludes with a decrease in cell density and a gradual arrest of angiogenesis, involving cell apoptosis and release of antiangiogenic factors (Reinke and Sorg, 2012; Sorg et al., 2018). When all these steps proceed in a regular and coordinated manner, a morphological and functional recovery of the injured tissue is obtained. However, if one or more of these mechanisms is impaired or delayed, healing is not guaranteed, and ulcer can occur with the risk of infections and chronic damage.

ANGIOGENESIS DURING WOUND HEALING

Among the different events that occur in the proliferative phase, angiogenesis is particularly important, because it forms the basis for tissue survival and recovery in the wound. Angiogenesis is defined as the formation of new vessels from preexisting ones and the microcirculation is the main site of vascular remodeling and angiogenesis. Angiogenesis appears to occur by two mechanisms, namely nonsprouting (intussusceptive) and sprouting angiogenesis (Ribatti and Crivellato, 2012). During intussusception, endothelial protrusions of opposing capillary walls extend towards each other and fuse creating an interendothelial contact (Burri et al., 2004). Sprouting angiogenesis is however the major form of neovascular growth, which requires migration, proliferation, and differentiation of ECs under the stimulation of specific glycoproteins called angiogenic factors, mainly VEGF and FGF (Hughes, 2008; Vandekeere et al., 2015). The development of blood vessels, which is a requirement for growth and regeneration, depends on a highly structured communication of ECs with their surrounding tissue. *In vivo* vascularization is based on complex cell-matrix and cell-cell interactions, where the ECM seems to play a pivotal role (Neve et al., 2014; Mongiat et al., 2016; Tracy et al., 2016).

The principal stimulus for angiogenesis occurrence is a lack of nutrients and oxygen, which characterize the hypoxic condition arising during tissue growth or following tissue injury and impaired blood flow (Ahluwalia and Tarnawski, 2012). Hypoxia is indeed able to promote hypoxia inducible factor1-α (HIF1-α) upregulation at nuclear level, which is responsible for angiogenic factor overexpression and vasoactive molecules upregulation. Under the action of the angiogenic factors VEGF, FGF, and PDGF, ECs undergo receptor activation and modification of intracellular pathways and cytoskeleton structure, leading to cell proliferation and chemotaxis (Ahluwalia and Tarnawski, 2012).

The first step is the binding of proangiogenic factors including VEGF, FGF-1 and 2, PDGF, and angiopoietins and stromal-derived growth factor (SDF-1), to their receptors on ECs of existing vessels, triggering complex and intricate intracellular signaling cascades. Heparin sulfate proteoglycans and syndecans also play a key role in regulating the angiogenic activity of VEGF and FGF-2 (Corti et al., 2019). Activated ECs secrete matrix metalloproteinases (MMPs) to degrade the capillary basement membrane (BM) and allow their migration and proliferation outside of the original blood vessel. Therefore, new vessels are formed as capillary sprouts and are then extended and remodeled. Finally, ECs interconnect to form a loop or a tube and the recruitment of pericytes stabilizes the newly formed vessels in a mature conformation (Carmeliet, 2003; Sorg et al., 2018).

In this framework, EC migration plays an important role for vascular remodeling and is a necessary condition for angiogenesis to occur. EC migration is a coordinated process that involves changes in cell adhesion, signal transduction, and cytoskeleton

dynamic reorganization (Li et al., 2003; Velnar and Gradisnik, 2018).

The regulation of this process is achieved by three types of mechanisms: chemotaxis or migration towards a concentration gradient of soluble chemoattractants; haptotaxis or rather migration in response to a gradient of immobilized ligands; and mechanotaxis which is the migration induced by mechanical forces. Specifically, chemotaxis of ECs is driven by growth factors (VEGF and FGF-2, among the most important), haptotaxis is related to increased EC migration activated in response to integrins (e.g., αvβ3 and αvβ5), bound to ECM components, and mechanotaxis is associated to a polarization of cytoskeleton and cell-ECM interactions in the blood flow direction (Lamalice et al., 2007).

Due to the combined action of cell migration and proliferation, the nascent vessels grow in the hypoxic/ischemic wounded tissue particularly rich of angiogenic stimuli. Circulating EPCs concur to the nascent vessels, being recruited by the factors released by the hypoxic environment, primarily VEGF and SDF-1 (Zhu et al., 2016). The process arrives at its end when the bud cavitates and blood starts to flow, bringing oxygen and nutrients to the tissue and taking away CO_2 and waste catabolites.

The Complexity of Wound Angiogenesis

Sprouting angiogenesis has recently been a subject of intense research since it is a requirement for growth and regeneration. The process has many sequential hierarchical steps that require the close interaction of EC with both cellular and acellular components of the surrounding tissue (Hughes, 2008). *In vivo*, capillaries are embedded in a microenvironment that consists of the ECM and cellular components including fibroblasts as well as immune cells. The ECM is a complex, noncellular network constituted by distinct components that is found in two different locations, i.e., the interstitium as interstitial ECM and, in association with epithelial and endothelial tissues, as the BM (Neve et al., 2014).

During early phases of wound healing, capillary sprouts invade the fibrin/fibronectin-rich wound clot and within a few days organize into a microvascular network throughout the granulation tissue. Growth factors that are released from the ECM trigger sprouting angiogenesis. These include a range of angiogenic factors, the most important being VEGF-A (Henning, 2016). Angiogenic stimuli activate the ECs to migrate into the avascular tissue (Eming and Hubbell, 2011). ECs express VEGF receptor-2 (VEGFR-2) that responds to the VEGF-A gradient. Once an angiogenic stimulus occurs, MMPs break down the BM of the blood vessel, mainly near the trigger sites (Davis and Senger, 2008). During sprouting, ECs are triggered by the VEGFR-2/VEGF-A binding to temporarily transform into migrating tip cells. These cells are polarized and have well-developed filopodia that enable them to interact with the ECM via integrins. These proteins (primarily αvβ3) of the ECs filopodia surface have an adhesive function during the endothelial migration (Davis and Senger, 2008). ECM proteins are important for adhesion and migratory processes of the endothelial tip cells and therefore promote angiogenesis (Neve et al., 2014; Mongiat et al., 2016). The endothelial tip cells move into the surrounding avascular extracellular matrix towards the angiogenic stimulus (Stratman et al., 2009). To enable this process, the MMPs form tunnels in the ECM to facilitate endothelial migration (Sacharidou et al., 2012).

Membrane-type 1-matrix metalloproteinases (MT1-MMP), synthesized by the ECs themselves, are responsible for most of the proteolysis of the ECM (Stratman et al., 2009). Endothelial stalk cells follow the tip cells into the ECM where they proliferate and elongate the developing capillary sprout as well as establish its internal lumen. The tubular lumen is formed by intraendothelial vacuoles that fuse. For the development of the vacuoles, MT1-MMP and the integrins αvβ3 and α5β1 play important roles (Welch-Reardon et al., 2014). Tight and adherens cell junctions are established between the stalk cells of the newly built tube and, consequently, a new vessel arises.

Due to their roles in cell-matrix interactions and especially matrix remodeling, fibroblasts are crucial in vascular development through transmitting biochemical signals and mechanical forces that affect cell survival, shape, and orientation (Kamei et al., 2006; Costa-Almeida et al., 2015). Stalk ECs synthesize, in cooperation with surrounding fibroblasts, basement membrane proteins, namely laminin, collagen IV, perlecan, nidogen, collagen XVIII, and fibronectin. The BM envelops and stabilizes the newly developing capillary sprout, serving as an acellular barrier against the capillary microenvironment and ensuring the correct polarity of ECs (Davis and Senger, 2008). Maturation and stabilization as well as remodeling of the dynamic capillary structures follow initial angiogenesis. As tubules mature, their ECs transform into quiescent phalanx cells (Senger and Davis, 2011). When collagen accumulates in the granulation tissue to produce scar, the density of blood vessels diminishes.

In dermal wounds with robust healing, the angiogenic activity during the proliferative phase initially creates a disorganized vascular network with highly tortuous vessels pathways, often reaching higher vessel numbers than normal (DiPietro, 2016). Following this peak in neovascularization, there is increased expression of antiangiogenic factors, such as Sprouty2 and pigment epithelium-derived factor (PEDF), leading to vascular regression and pruning (Wietecha et al., 2011; Wietecha et al., 2015). Maturation and stabilization of the new vascular network require the involvement of pericytes and vascular smooth muscle cells (Bergers and Song, 2005). A key growth factor is PDGF-BB, which acts on pericyte differentiation (Hellberg et al., 2010). Additionally, there is the contribution of angiopoietin-2/Tie2 receptor in pericytes which regulate angiogenesis and maturity of vascular networks (Teichert et al., 2017).

Signaling Pathways in Endothelial Cells During Angiogenesis

ECs receive multiple stimuli from the environment that eventually induce them to progress along all the stages of angiogenesis, until new vessels are formed in the wound bed. Angiogenic signals that promote EC survival, proliferation, migration, and finally differentiation are the result of a

complex framework, involving cell-ECM and cell-cell interactions, and action of soluble mediators (Muñoz-Chápuli et al., 2004; Lamalice et al., 2007). ECs express a large number of receptors that make them responsive to several growth factors and cytokines involved in the promotion of angiogenesis, but the most important and specific for their action is VEGFR (Hofer and Schweighofer, 2007). In particular, the binding of VEGF-A, secreted by cells in the hypoxic environment, to VEGFR-2 is the major way by which EC migration is promoted (Olsson et al., 2006). Specifically, like other tyrosine kinase receptors, VEGFR-2 undergoes ligand (VEGF-A) induced dimerization and oligomerization, which activates its intrinsic tyrosine kinase activity, resulting in auto- and transphosphorylation on specific tyrosine residues in the cytoplasmic domain, ultimately being responsible for cell proliferation and migration. These pathways involve activation of the small GTPases of the Rho family, PI-3K/Akt, p38 MAPK, FAK, and ERK1/2 signaling cascades (Muñoz-Chápuli et al., 2004; Webb et al., 2004; Lamalice et al., 2007; Yang et al., 2017).

Intermediate messengers are upregulated during angiogenic cell activation as gasotransmitters nitric oxide (NO) and hydrogen sulfide (H_2S). PI-3K/Akt-activated eNOS produces NO, which is a major regulator of EC migration and angiogenesis, by inducing expansion of EC surface, after vasodilatation, associated with a more proper response of endothelium to angiogenic and promigratory stimuli (Dimmeler et al., 2000; Morbidelli, 2016b). In addition, a particular attention has been put on hydrogen sulfide (H_2S). Endothelial-associated H_2S producing enzymes are activated or upregulated by hypoxia and VEGFR-2 signaling cascades, as recently reviewed (Ciccone et al., 2021).

Endothelial Dysfunction and Impaired Healing

All the above mechanisms however are working in healthy endothelial cells. Physiological conditions such as ageing and pathologic conditions as metabolic syndrome or diabetes are characterized by endothelial impaired functions, called endothelial dysfunction, with reduced ability of ECs to release vasoactive and protective factors like NO and to undergo beneficial and sufficient angiogenesis. These disorders are indeed accompanied by altered healing, chronic ulcers, and infections that in extremis may need amputation (Brem and Tomic-Canic, 2007). Beside the inability of ECs to promote a reparative angiogenesis, we have to consider the microenvironment composition. Recent analysis of the exudate of venous nonhealing leg ulcers has found increased antiangiogenic factors, increased VEGF proteolytic products, and increased levels of soluble VEGFR-1, known to neutralize VEGF-A activity (Lauer et al., 2000; Drinkwater et al., 2002; Eming et al., 2004; Eming et al., 2010).

All these findings push scientists to identify novel biochemical targets for the development of therapies to promote endothelial proper functions during reparative angiogenesis. The research is nowadays active in characterizing novel drugs or therapies and their delivery systems to be used in pathological conditions characterized by endothelial dysfunction and impaired angiogenesis and wound healing.

PHYSICAL-CHEMICAL FACTORS CONTROLLING WOUND HEALING

The different steps of tissue repair are strictly regulated by a multitude of biochemical and physical factors, including gravitational/mechanical forces acting at cellular and tissue level. Interruption, failure, or alteration in one or more phases of the repair process can lead to the formation of nonhealing chronic wounds or fibrotic scars, accompanied by pain and inflammation (Guo and DiPietro, 2010).

In general terms, the factors that influence repair can be categorized into local and systemic. Local factors are those directly influencing the characteristics of the wound itself like oxygenation/hypoxia, infection occurrence, vascular insufficiency, or presence of foreign bodies. On the other hand, systemic factors are the overall health or disease state of the individual affecting his or her ability to heal, such as age and gender, stress, nutrition, alcoholism and smoking, immunocompromised conditions, and diseases (as diabetes) (Guo and DiPietro, 2010). Although many factors related to a patient's conditions cannot be changed, local factors can be controlled and improved in order to obtain the best therapeutic result.

Among the exogenous factors, there are mechanical stressors as pressure, vibrations, and loading. ECs are particularly sensitive to changes in gravitational forces and the mechanisms of mechanotransduction are now known (Maier et al., 2015; Li et al., 2018).

In particular, studies on unloading condition can be performed at different levels as single cell, cell cocultures or organoids, and living animals, as rodents or invertebrates (leeches), exposed to real microgravity in vehicles operating in terrestrial orbits or to terrestrial models of unloading (Cialdai et al., 2020). Animal hindlimb unloading (HLU) or bed rest experimental studies in humans are established models designed to mimic unloading conditions in space.

In Vivo Experiments of Unloading Effect on Wound Healing

Very few are the reports on the effects of mechanical unloading and physical deconditioning on wound healing *in vivo*. Several studies have indicated that spaceflight can adversely affect tissue repair in muscle and bone (Pospishilova et al., 1989; Ilyina-Kakueva and Burkovskaya, 1991; Kaplansky et al., 1991; Bolton et al., 1997; see Genah et al., 2021a for review). In relation to skin repair, Davidson et al. (1998) conducted a study to determine the effects of microgravity on wound healing in rats, in term of granulation tissue and collagen formation. They found a reduced capacity of wounds to heal, being the cells less responsive to added growth factors.

Radek et al. demonstrated that in rats exposed for 2 weeks to HLU before excisional wounding, the healing process was delayed

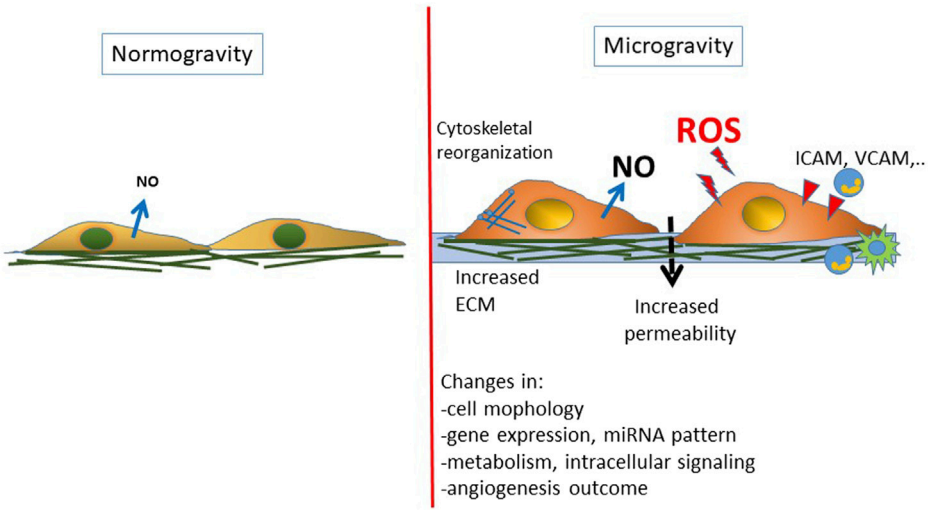

FIGURE 2 | Schematic representation of the changes reported on endothelial cells exposed to real or simulated microgravity. A general condition of endothelial dysfunction is found associated with an inflammatory/prooxidative phenotype.

on day 2 with respect to ambulatory controls. Although the levels of proangiogenic growth factors FGF-2 and VEGF were similar between the two groups, wound vascularization in HLU animals was significantly reduced at day 7. To further examine this disparity, total collagen content was assessed and found to be similar between the two groups (Radek et al., 2008). Recently, inhibition of cell proliferation and angiogenesis was verified in skeletal muscles of rats exposed to hindlimb unloading through RNA sequences analysis (Cui et al., 2020).

Taken together, these results suggest that keratinocyte and EC functions may be impaired during the wound healing process under periods of prolonged inactivity or bed rest.

Effect of Weightlessness on Endothelial Cell Transcriptome, Morphology, and Function

ECs are mechanosensitive cells undergoing morphological and functional changes in response to fluid shear stress, cyclic tensile strain, and substrate stiffness. In space their alterations contribute to cardiovascular deconditioning and immune dysfunction commonly faced by astronauts during spaceflight. Ground-based and space experimentation has provided a body of evidence about how ECs can respond to the effect of simulated and real microgravity (Byfield et al., 2009; Chancellor et al., 2010; Maier et al., 2015; Li et al., 2018). Exposure of ECs to microgravity in space can change morphology and gene expression, displaying heterogeneous cell size and shape (Kapitonova et al., 2012), 3D growth (Pietsch et al., 2017), energy and protein metabolism deficiency (Chakraborty et al., 2018), significant suppression of genes associated with host defense (Chakraborty et al., 2014), and alterations in genes involved in cell adhesion, oxidative phosphorylation, and stress responses (Versari et al., 2013) (**Figure 2**). To date, only a limited number of studies in space have been performed and the impact of real microgravity on EC functions still remains unclear.

Most of the data derive from ground-based microgravity simulators, as rotating wall vessel (RWV) (a 2D clinostat) (Goodwin et al., 1993) and random positioning machine (RPM) (a 3D clinostat) (Morbidelli et al., 2005; Wuest et al., 2017).

Specifically, markers for leukocyte adhesion and recruitment, adhesive counterreceptors and inflammatory cytokine expression pattern are altered in RWV (Wang et al., 2015), documenting a proinflammatory phenotype. Nevertheless, the outcomes are controversial in literature. For instance, a decreased expression of intercellular adhesion molecule-1 (ICAM-1) has been found in ECs cultured in RPM for 24 h (Grenon et al., 2013). However, upregulation of ICAM-1 transcription was found after 30 min clinorotation and the clustering of ICAM-1s on cell membrane was observed when ECs have been activated by TNF-α and cultured in RWV (Zhang et al., 2010).

Mechanistically, the cytoskeletal remodeling has been considered the transducer of cellular responses to microgravity (van Loon, 2009; Long et al., 2015). The reorganization of cytoskeleton proteins following microgravity exposure includes microtubule and actin filament (F-actin) upregulation (Zhang et al., 2017; Buravkova et al., 2018). Significantly downregulated amount of actin (Carlsson et al., 2003; Corydon et al., 2016), depolymerization of F-actin (Kang et al., 2011), and clustering of the stress fibers at the cellular membrane and around the nucleus (Infanger et al., 2007; Grenon et al., 2013) have been reported, and the actin rearrangement is typically RhoA dependent (Shi et al., 2017). The controversial results probably depend on the EC type used in the experiments, stimulation facility, and time of exposure.

Cytoskeletal rearrangement was accompanied by the overexpression of ECM proteins, including collagen I, fibronectin, osteopontin, and laminin (Infanger et al., 2006; Grimm et al., 2009; Buravkova et al., 2018), but again also downregulation has been described (Corydon et al., 2016).

Furthermore, intracellular signaling and cell–cell communication are crucial for EC functional alterations in microgravity. For example, NO has been reported to be upregulated by RPM and is deemed to be responsible for angiogenesis and cardiovascular deconditioning experienced by astronauts during spaceflight (Siamwala et al., 2010; Grenon et al., 2013). The increased eNOS activation found in 2D or 3D clinostat cultures in ECs seems to be due to PI-3K pathway (Shi et al., 2012), actin remodeling (Siamwala et al., 2010), and caveolin-1- (Cav-1-) mediated mechanotransduction (Shi et al., 2016).

Concerning angiogenesis outcome, in agreement with the results reported by other authors (Kang et al., 2011; Xu et al., 2018), our studies demonstrated that microgravity induces significant changes in EC behavior with reduced cell survival, induction of apoptosis, and angiogenesis impairment (Morbidelli et al., 2005; Maier et al., 2015).

Controversial results were however reported both in differentiated ECs depending on the district (micro- or macrocirculation) and in mesenchymal stem cells (MSC). Simulated microgravity promotes angiogenic output in HUVEC via RhoA-dependent rearrangement of actin and cytoskeleton (Shi et al., 2017). It has been demonstrated that microgravity could stimulate mature ECs and MSC to produce IL-8 and VEGF as well as other paracrine factors involved in angiogenesis. This is responsible for the regulation of EC functions by creating a specific microenvironment in support of EC proliferation and migration (Dittrich et al., 2018; Ratushnyy et al., 2018).

Recently, in human ECs cultured under simulated microgravity achieved with a clinostat, a total of 1,870 miRNAs were found to be differentially expressed with respect to normal gravity. The functional association of identified miRNAs targeting specific mRNAs revealed that a series of miRNAs (hsa-mir-496, hsa-mir-151a, hsa-miR-296-3p, hsa-mir-148a, hsa-miR-365b-5p, hsa-miR-3687, hsa-mir-454, hsa-miR-155-5p, and hsa-miR-145-5p) differentially regulated the genes involved in cell adhesion, angiogenesis, cell cycle, JAK-STAT signaling, MAPK signaling, NO signaling, and VEGF signaling, finally favoring angiogenesis (Kasiviswanathan et al., 2020). In a study on endothelial progenitor cells, cultured in simulated microgravity, a facilitation of functional angiogenic properties has been reported, with increased HIF-1α and eNOS/NO induced FAK/ERK1/2 pathway upregulation in HUVEC (Kong et al., 2021).

In real gravity study, cultured ECs were kept on board of the SJ-10 Recoverable Scientific Satellite for 3 and 10 days (Li et al., 2018). Space microgravity suppressed the glucose metabolism; modulated the expression of cellular adhesive molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and CD44; and depressed the secretion of proangiogenic factors and proinflammatory cytokines. Moreover, space microgravity induced the depolymerization of actin filaments and microtubules, promoted vimentin accumulation, restrained collagen I and fibronectin deposition, regulated the mechanotransduction through focal adhesion kinase and Rho GTPases, and enhanced the exosome-mediated mRNA transfer. As previously seen in simulated microgravity, neither three-dimensional growth nor enhanced NO production has been observed in real microgravity (Li et al., 2018).

Moreover, some preliminary results from *in vitro* studies in modeled microgravity indicate that the cross-talk between fibroblasts and ECs, a building block in the healing evolution, is impaired. The production of angiogenic growth factors is altered as well (Cialdai et al., 2017), with a consequent inability of ECs to form tube-like structures. The complex cross-talk between fibroblasts and ECs and its role in tissue healing are beyond the focus of the present paper.

Despite these controversial results in cultured cells, probably due to different cell source and microgravity protocols, astronauts spending a long time in International Space Station (ISS) are more vulnerable to vasculopathies, associated to endothelial dysfunction (Zhang and Hargens, 2018; Garrett-Bakelman et al., 2019; Navasiolava et al., 2020), thus strengthening the finding of defective angiogenesis and tissue repair in space environment.

PHARMACOLOGICAL, CELLULAR, AND PHYSICAL COUNTERMEASURES

In current clinical practices, a series of drugs can be employed to control symptoms related to wound healing like inflammation, oedema, pain, and steroidal or nonsteroidal anti-inflammatory drugs. Furthermore, dressings and topic products are used to create and keep a humid environment, providing the ideal conditions for a correct wound healing process (Dreifke et al., 2015). However, side effects, or even opposite effects on wound healing, such as hypertrophic scarring, contraction, and necrosis, can limit their employment, especially considering their long-term use, raising the necessity for alternative countermeasures (Dreifke et al., 2015).

Emerging skin regeneration techniques involving scaffolds activated with growth factors, bioactive molecules, and genetically modified cells are being exploited to overcome wound healing technology limitations and to implement personalized therapy design. Results are however partial and under consolidation. The following sections report the state of the art or the ultimate findings on various types of countermeasures for recovery of tissue integrity (**Table 1**). However, not all these countermeasures can be adopted in a space environment, due to their shelf life and stability in space, the necessity to be produced/manipulated in real time by highly prepared operators or in dedicated facilities, and the consideration of the specific personal need.

Pharmacological Countermeasures

As reported above, different growth factors as FGF, VEGF, EGF, and PDGF are needed to orchestrate neovascularization and the whole wound healing process and many attempts have been made to use recombinant growth factors in dermal wound healing with different approaches and formulations (see Veith et al., 2019 for a comprehensive review). To the best of our knowledge, there are only a few drugs of protein nature

TABLE 1 | Countermeasures to improve or accelerate wound healing through enhanced angiogenesis.

Type of therapies	Specific clues
Protein therapeutics	Growth factors: PDGF, EGF, FGF, and VEGF Nongrowth factor proteins/glycoproteins: insulin, erythropoietin, SDF-1, syndecans Peptides: antimicrobial peptides, vasointestinal peptide Blood-derived factors: PRP, hemoglobin
Gene and nucleic acid-based therapies	Gene therapy for growth factors microRNA
Drugs and bioactives	Statins NO donors ACE inhibitors Natural compounds (astragaloside, centelloids, and asiaticoside) Nutraceuticals
Polymers for dressing or scaffold preparations	Dextran hydrogels Hyaluronan oligosaccharides
Stem cell–based therapies (in the form of naïve or genetically modified cells, cell secretome, exosomes, and EV)	Adipose-derived stem cells Bone marrow–derived mesenchymal stem cells Induced pluripotent stem cells Endothelial precursor cells
Physical therapies	Ultrasound/low pressure shock-waves Laser therapy/photobiomodulation Electrical stimulation Hyperbaric oxygen Vacuum-assisted closure Hypergravity

For details on the state of the art of the single therapies, see Dreifke et al. (2015) and Veith et al. (2019). For hypergravity use in unloading conditions, see Physical Countermeasures *section.*

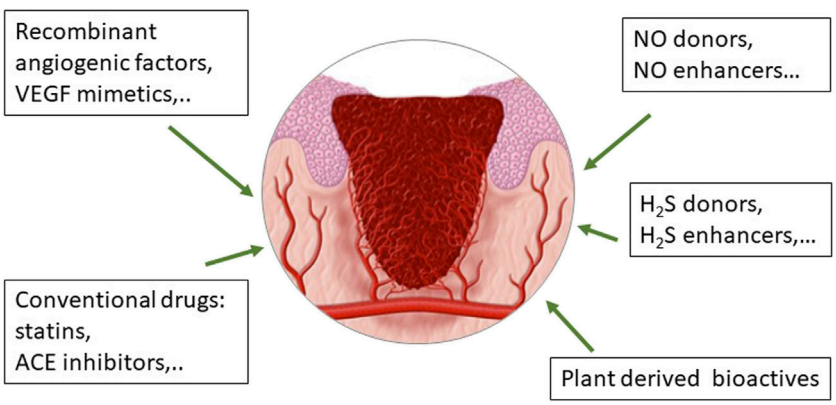

FIGURE 3 | Drugs in use or under development to improve wound healing through their effect on endothelial functioning and angiogenesis, crucial events during the formation, and evolution of the granulation tissue.

approved to promote wound healing with potential proangiogenic properties. The first is represented by recombinant PDGF prepared as a skin preparation (Regranex Gel, 0.01% becaplermin). Regranex Gel is the first and only FDA-approved recombinant PDGF therapy for use on diabetic neuropathic ulcers. Regranex contains becaplermin, a human PDGF that is indicated for the treatment of lower extremity ulcers that extend into the subcutaneous tissue or beyond and have an inadequate blood supply. PDGF initiates healing by attracting repair cells to revitalize wounds (Pierce et al., 1991). Indeed, PDGF works by stimulating fibroblast proliferation, increasing granulation tissue and the rate of reepithelialization and revascularization, and promoting collagen production (Heldin and Westermark, 1999).

Other attempts have included studies with gene therapy or recombinant growth factors administered directly in wounds or delivered with scaffolds, nanomaterials or cells (Veith et al., 2019), but the results still need to be confirmed both in clinical studies on an appropriate number of patients/pathological conditions and, on top of this, in a space environment.

Conventional drugs are small molecules that could be an optimal alternative to recombinant growth factors to be used in space, since many of them are part of the on-board pharmacy and their pharmacological and toxicological characterization is robust (**Figure 3**). Among the small molecules, particular attention has been posed to statins, widely used to lower cholesterol level due to their inhibitory activity on the liver enzyme 3-hydroy-3-methyl-glutaryl-coenzyme A reductase. Statins are molecules with pleiotropic and multitarget activity, demonstrating a high protective profile on ECs and anti-inflammatory and antioxidative properties, among others (Khaidakov et al., 2009) (**Figure 3**). Statins have been established to possess proangiogenic features, protecting the cardiovascular system against ischemic injury (Kureishi et al., 2000). Simvastatin has been characterized for its healing activity in experimental diabetic ulcers, demonstrating a proangiogenic action with NO pathway upregulation (Bitto et al., 2008). The efficacy of statins has been demonstrated through daily oral administration and by topical treatment, being able not only to produce neovascularization but also lymphangiogenesis (Asai et al., 2012). Recently, novel drug delivery systems for statin local release are under evaluation, demonstrating their efficacy and potential clinical application in chronic ulcers (Rezvanian et al., 2016; Yasasvini et al., 2017). The research on the best formulation and treatment protocols is still active and no report has explored their employment in space environment.

As stated above, NO has been identified to exert a pivotal role in wound healing. NO levels increase significantly following skin injury and then gradually decrease as healing progresses (Childress and Stechmiller, 2002). At the same time pathological conditions characterized by H_2S deficiency as diabetes are known to be associated with impairment of skin healing and progressive ulcerations (Ciccone et al., 2021). These considerations encouraged us to develop strategies with the ability to release both gasotransmitters in a tunable manner for the control of endothelial function and angiogenesis.

Considering the central role of NO in angiogenesis, we have contributed to demonstrate the beneficial effect on endothelial cell functions and angiogenesis by a series of synthetic molecules: 1) a peptidomimetic of VEGF, as QK; 2) the NO donors metal NONOates; and 3) the angiotensin converting enzyme (ACE) inhibitor zofenoprilat, which shares the common feature of releasing NO and H_2S in a controlled manner (Finetti et al., 2012; Monti et al., 2016; Monti et al., 2018) (**Figure 3**).

The peptidomimetic analogue of VEGF, QK, reproduced all the angiogenic functions of the whole molecule, including its ability to promote the eNOS pathway (Finetti et al., 2012). Being a peptidomimetic, QK could be a promising tool to be used in space, for its easy manipulation and stability.

The metal-nonoate Zn(PipNONO)Cl shows an interesting kinetic of NO release (both fast and prolonged) due to the ability to upregulate eNOS and H_2S producing enzymes in ECs, thus contributing to improve endothelial function (Monti et al., 2018). Its role in human skin tissue repair is under evaluation within space agencies programs (LM personal communication). The H_2S facilitating properties on tissue repair have been recently strengthened by the findings that H_2S improves wound healing by induction of angiogenesis (Ciccone et al., 2021) and restoration of EPC functions in type 2 diabetic mice (Liu et al., 2014).

Concerning conventional drugs used in clinics for other purposes, we have demonstrated endothelium protective, proangiogenic, and anti-inflammatory properties by the active moiety of the ACE inhibitor zofenopril, namely zofenoprilat, which through its SH group behaves as a H_2S donor and promoter (Monti et al., 2013; Monti et al., 2016) (**Figure 3**). Its indication in wound healing however has never been verified.

Beside the recombinant growth factors and conventional drugs, an interesting therapeutic approach for wound healing management is represented by ethnopharmacology and traditional remedies, which are present in the various cultures and provide preparations and active principles with anti-inflammatory, antibacterial and proproliferative/proangiogenic properties (Morbidelli, 2016a; Morbidelli et al., 2018) (**Figure 1**). Plant-derived active principles have been isolated and demonstrated to reduce scar formation as astragaloside IV from *Astragalus membranaceus* and centelloids and asiaticoside from *Centella asiatica* (Chen et al., 2012; Bylka et al., 2014). Concerning the beneficial effects of Mediterranean diet, olive oil–based diet has been demonstrated to improve cutaneous wound healing of pressure injury in mice through the reduction of inflammation and stimulation of redox equilibrium (Schanuel et al., 2019). In this scenario, we have contributed to evaluate the protective effect of extra-virgin olive oil polyphenol hydroxy-tyrosol and its metabolic derivative which positively controls angiogenesis and the endothelial-to-mesenchymal transition (Terzuoli et al., 2020). Another example of nutraceuticals which exerts antioxidant and protective effects on EC function affected by high glucose is erucin, derived from *Eruca sativa* Mill. seeds. Its endothelial positive outcomes are correlated to increased H_2S intracellular levels (Martelli et al., 2021). Studies are needed to further validate these findings in experimental models and in the clinic and to define other nutraceuticals with protective effects on ECs and promoting reparative angiogenesis.

Cell Therapies

The use of stem cells, alone or by the help of a scaffold, provides better and faster healing of burn wounds, with decreased inflammation, slow scar progression, and reduced fibrosis. Stem cell homing at the wound site results in granulation tissue formation, immunomodulation, neovascularization, apoptosis inhibition, and induction of epithelial cell proliferation with skin regeneration. While these findings are clear in animal models, the validation of their clinical use is at the beginning (Veith et al., 2019; Phua et al., 2021). Sources of MSCs in adults are bone marrow, adipose tissue, and umbilical cord blood. Additionally, induced-pluripotent-stem-cell- (iPSC-) derived MSC have been reported to be suitable for cell therapy. However, their effectiveness in human patients remains to be established (Jo et al., 2021; Mazini et al., 2021; Ullah et al., 2021). The proposal of proangiogenic paracrine secretion by stem and precursor cells cultured in microgravity has been provided (Ratushnyy et al., 2018; Kong et al., 2021).

Dermal fibroblasts are the major cell type in skin dermal layers. As said above, they actively participate in skin regeneration and these cells are becoming attractive candidates for cell-based therapies in wound healing. Due to their heterogeneity linked to variable activation by inflammatory stimuli, tissue niche of origin and different scar forming properties, their potential clinical exploitation is far (Xue et al., 2021).

Due to the plethora of secretory products involved in angiogenesis (as VEGF and PDGF), tissue remodeling, and wound healing (Pierce et al., 1991; Frechette et al., 2005; Lacci and Dardik, 2010), platelet therapy has been applied in regenerative medicine and wound healing from decades (Martinez-Zapata et al., 2016). Recently, in cultured fibroblasts and in an experimental model of dermal injury in leech exposed to clinostat induced microgravity, we have demonstrated the efficacy of platelet rich plasma (PRP) in accelerating healing by acting on fibroblast migration (Cialdai et al., 2017; Cialdai et al., 2020).

While the use of cells in a space environment seems not feasible due to the many risky procedures (self-harvesting, *in vitro* culture and checking of safety, inoculation in the wound or systemically), the use of autologous PRP appears a very promising approach in the astronauts not only for dermal repair but also for regenerative bone, tendon, and endodontic treatment, where improvement of angiogenesis is a necessary step for proper repair.

Microvesicles and Exosomes

Recently, a therapeutic role for extracellular vesicles (EV) derived from stem cells has been described in animal wound models (Dalirfardouei et al., 2021). Extracellular vesicles and in particular exosomes contain various bioactive molecules as proteins, enzymes, and nucleic acids, thus providing the wound with all the necessary stimuli to promote angiogenesis and cell proliferation and regulate inflammation and collagen remodeling, ultimately leading to tissue healing (**Table 1**). The use of stem-cell-derived exosomes seems more feasible with respect to cell therapy, without potential problems related to the use of living cells, which would make their use in space quite impossible. Additionally, they are not rejected by the immune system and have a homing effect and their dosage can be easily controlled. *In vivo* studies on animal models demonstrate the beneficial effects of EVs on accelerating wound closure and reepithelization in a dose-dependent manner. Various studies demonstrate induction of angiogenesis through the conventional mechanisms of PI-3K/Akt, MAPK/ERK1/2, and JAK/STAT pathways. Interestingly, the upregulation of TGF-β2/SMAD2 pathway involved in scar inhibition has been reported (Dalirfardouei et al., 2021). They can also act as carriers for other interventions and be combined with scaffolds. Also this innovative approach should be, however, thoroughly verified before clinical utilization in particular with respect to EV manufacturing, treatment protocols, and long-term follow-up (An et al., 2021).

Physical Countermeasures

In recent years, many therapies aiming at stimulating the healing process are in clinical use, such as ultrasound, laser therapy, and other forms of photobiomodulation, electrical stimulation, hyperbaric oxygen, and vacuum-assisted closure (Dreifke et al., 2015; Nesi-Reis et al., 2018; Priyadarshini et al., 2018; Micheli et al., 2019; Xu et al., 2021). The efficacy of physical approaches is due to the fact that all the cell types involved in wound healing (fibroblasts, keratinocytes, and ECs) are sensitive to mechanical forces at cellular and molecular level acting as mechanotransducers.

ECs in particular respond to mechanical stimuli such as shear, strain, and stretch. This property can be exploited to induce reparative angiogenesis in pathological conditions associated with insufficient angiogenesis. In cultured ECs and in a mouse model of wound healing, it has been demonstrated that low-pressure shock waves induced angiogenesis. Increased EC migration and proliferation were associated with enhanced Ca^{++} influx and PI-3K which is usually observed when ECs are exposed to stretch. Shock wave treated mice showed enhanced wound-induced angiogenesis documented by increased vascular area and vessel length. Accelerated wound closure was observed compared to control mice (Sundaram et al., 2018).

Photobiomodulation, i.e., lasers emitting red/IR radiation, promotes angiogenesis in wound healing (de Madeiros et al., 2017). In a previous experimental paper, ECs were exposed to simulated microgravity or pulsed Nd:YAG laser radiation to assess their behaviour and morphology. Increased fibronectin and laminin could be the cause for impaired ECM rebuilding and altered cell adhesion/migration in microgravity (Monici et al., 2011) in accordance with previous data on impairment of angiogenesis (Morbidelli et al., 2005). On the contrary, the exposure to Nd:YAG laser pulses induced the formation of a highly ordered array of fibronectin fibrils on EC surface and cell spreading to form a monolayer (Monici et al., 2011). Recently, we have additionally demonstrated the anti-inflammatory effect of NIR laser on dermal fibroblasts stimulated with inflammatory cytokines, through an inhibition of NF-kB transcription pathway (Genah et al., 2021b). All these results suggest a beneficial effect of photobiomodulation as an effective healing option with proangiogenic and anti-inflammatory properties. Its inflight applicability should however be verified.

Among different countermeasures implemented to minimize the effects of microgravity, a promising one could be artificial gravity. We have demonstrated that discontinuous hypergravitational stress did not significantly affect cell survival in macrovascular and microvascular ECs. In both cell populations, we found similar changes in cytoskeleton and αvβ3 integrin distribution that in microvascular ECs were combined with an increased anaerobic metabolism and cell detachment from the substratum (Monici et al., 2006; Morbidelli et al., 2009).

Exposure to artificial gravity provides protection against microgravity induced apoptosis and oxidative stress in retinal endothelial cells of rodents flown on ISS (Mao et al., 2018).

A profound rearrangement of the cytoskeleton network, dose-dependent increase of FAK phosphorylation, and Yes-associated protein 1 (YAP1) expression was found in dermal microvascular ECs exposed to hypergravity, suggesting improved motility and proangiogenic response. Transcriptome analysis showed changes in the expression of genes associated with cardiovascular homeostasis, NO production, angiogenesis, and inflammation

(De Cesari et al., 2020). These results show that adaptation to hypergravity has opposite effects compared to microgravity on the same cell type, suggesting it as a potential physical countermeasure. Its real application is however far away.

In summary, the efficacy of physical countermeasures, alone or combined with other therapies, remains to be defined in cultured cells and in integrated and innovative tissue models in order to be effective and safe in a spaceflight arrangement.

CONCLUSION AND PERSPECTIVES

While the events and mechanisms controlling wound healing are well known and characterized, the pharmacological interventions to prevent or treat healing dysfunction are few and nowadays still under evaluation and validation on Earth. The data available in relation to unloading conditions document that the impaired wound healing results from the following mechanisms: 1) persistent inflammation with neutrophil infiltration (Dovi et al., 2003; Radek et al., 2008) and overall alteration of the inflammatory phase; 2) altered blood flow with more permeable vessels (McDonald et al., 1992), presumably linked to increased NO and VEGF levels (Shi et al., 2012; Dittrich et al., 2018); 3) an altered neovascularization that may result from impaired EC proliferation and migration in response to angiogenic factors, increased EC apoptosis, and altered gene expression and signalling pathways (Morbidelli et al., 2005; Radek et al., 2008; Li et al., 2018; Kasiviswanathan et al., 2020); 4) exposure to simulated microgravity also resulting in enhanced ROS production that may contribute to unloading-induced oxidative stress. The systemic oxidative status can be derived from radiation-induced immune system alterations especially relevant in long-duration space flight (Rizzo et al., 2012).

Considering the space environment and the critical issues characterizing long duration space travels (unloading, confinement, scarce hygiene, and radiations) is mandatory to further study angiogenesis and wound healing in space, to precisely define the target for therapeutic interventions and to validate efficient and safe countermeasures and treatment protocols. The combination of various stressors needs to be characterized. Recently, a paper by Mao et al. (2019) revealed the synergistic worsening effect of combined unloading and radiation exposure on oxidative stress and dysfunction of retinal ECs, events responsible for sight loss associated to space permanence.

Additionally, for a series of countermeasures, it is difficult to imagine their exploitation in a space environment since they require freshly isolated living cells or facilities and competences to extract/cultivate cells from the injured subject. In long duration missions, to deliver in time, ad hoc cells/tissues will be not possible and proangiogenic/regenerative countermeasures should be available on shelf/on board or rapidly achieved. Therefore, the effort of national and international space agencies goes in this direction and this review reflects the state of the art on the specific phenomenon of angiogenesis contribution on wound healing and the potentiality of developing effective and safe countermeasures. Up-to-date techniques are needed both for the study of the mechanisms of angiogenesis alterations in space environment and for the validation of countermeasures to improve wound healing. Examples are tissue engineering, cocultures, 3D multicellular structures, lab-on-chip approaches (Grimm et al., 2014; Ma et al., 2014; Huang et al., 2020), which are starting to substitute experimental animals due to ethical issues. Thorough comprehension of the molecular and biochemical mechanisms underlying cellular responses is coming from omics techniques and RNA sequence analysis of samples from simulated and real microgravity experiments (Ma et al., 2014; Mao et al., 2018; Cui et al., 2020; Kasiviswanathan et al., 2020). The expectations from these types of experiments are high.

Nevertheless, it is important to stress the concept that all the information obtained for space research can be exploited on the Earth for fragile (aged, diabetic) or bed-ridden patients, whose clinical characteristics are very similar to astronauts. A common feature that accompanies space travelers and aged/fragile patients is indeed endothelial dysfunction, which, among the others, is responsible for angiogenesis impairment and not efficient healing of wounds and ulcers.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, investigation, and writing (original draft preparation) were carried out by LM; writing review and editing were done by LM, SG, and FC; supervision and funding acquisition were provided by LM. All authors have read and agreed to the published version of the manuscript.

FUNDING

The review was written within the activities of the ESA-Topical Team on "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration" and of the ESA-MAP Project "WHISPER—Wound Healing In Space: problems and PErspectives for tissue Regeneration and engineering," SciSpacE Microgravity Application Promotion Programme, ESA Contract Number 4000130928/20/NL/PG/pt.

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frontiers in Bioengineering and Biotechnology

REVIEW
published: 25 October 2021
doi: 10.3389/fbioe.2021.716184

Platelets in Wound Healing: What Happens in Space?

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Beyond their fundamental role in hemostasis, platelets importantly contribute to other processes aimed at maintaining homeostasis. Indeed, platelets are a natural source of growth factors and also release many other substances—such as fibronectin, vitronectin, sphingosine 1-phosphate—that are important in maintaining healthy tissues, and ensuring regeneration and repair. Despite rare thrombotic events have been documented in astronauts, some *in vivo* and *in vitro* studies demonstrate that microgravity affects platelet's number and function, thus increasing the risk of hemorrhages and contributing to retard wound healing. Here we provide an overview about events linking platelets to the impairment of wound healing in space, also considering, besides weightlessness, exposure to radiation and psychological stress. In the end we discuss the possibility of utilizing platelet rich plasma as a tool to treat skin injuries eventually occurring during space missions.

Keywords: platelets, microgravity, platelet rich plasma, wound healing, regeneration

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *28 May 2021*
Accepted: *11 October 2021*
Published: *25 October 2021*

Citation:
Locatelli L, Colciago A, Castiglioni S and Maier JA (2021) Platelets in Wound Healing: What Happens in Space? Front. Bioeng. Biotechnol. 9:716184. doi: 10.3389/fbioe.2021.716184

INTRODUCTION

In the adult, approximately one trillion platelets circulate in the blood. These disc-shaped anucleate cells arise from the fragmentation of the cytoplasm of megakaryocytes, residing in the bone marrow. Upon release in the bloodstream, platelets circulate for approximately 10 days before being cleared by the splenic and hepatic reticuloendothelial system (Dowling et al., 2010). In specific conditions, they undergo apoptosis through the intrinsic pathway (McArthur et al., 2018) and activate autophagy, which importantly modulates their function (Banerjee et al., 2019). Platelets contain mitochondria, display a range of coding and non-coding RNAs, synthesize some proteins and store a considerable number of preformed bioactive molecules in uniformly distributed secretory granules derived from megakaryocytes (Gianazza et al., 2020). Beyond the lysosomes containing a panoply of hydrolases, the most abundant are the α granules (Blair and Flaumenhaft, 2009), which account for about 10% of the platelet volume and mostly bud from the trans-Golgi network. Alpha granules contain hundreds of proteins such as coagulation factors, growth factors (GFs), adhesive molecules, pro- and anti-angiogenic factors, cytokines and chemokines (Chen et al., 2018). After their release, these proteins potentiate platelet responses in an autocrine fashion or target other cell types through paracrine mechanisms. It is now clear that the content of α granules is heterogeneous and this might determine a differential exocytosis of granule's cargo in response to distinct stimuli. Dense granules, usually 3–8 per platelet, originate from exosomes and contain small molecules among which serotonin, histamine, calcium (Ca^{2+}), magnesium, ADP, ATP, pyrophosphates, and polyphosphates, all important to magnify platelet activation (Ambrosio and Di Pietro, 2017). T granules were the last to be identified as an electron-dense tubular system-related

compartment containing toll-like receptor (TLR) 9 and protein disulphide isomerase (Thon et al., 2012). Platelet granule release is essential for the full repertoire of platelet activities.

PLATELETS

Along with their traditional role in hemostasis, increasing experimental and clinical evidence points to platelets as relevant modulators of some physio-pathological processes such as inflammation, immunity, wound healing and tissue regeneration, because they release GFs, cytokines, and extracellular matrix modulators that sequentially promote angiogenesis, restoration of damaged connective tissue, proliferation and differentiation of mesenchymal stem cells into tissue-specific cell types (Doucet et al., 2005).

Platelets and Hemostasis

Platelets are sentinels of vascular integrity as they sense and respond to perturbations in the blood and disruption of the endothelial layer (Becker et al., 2018). Physiologically, platelets flow in close proximity to the endothelial cells (Becker et al., 2018), which continuously release anti-platelets molecules, such as nitric oxide and Prostaglandin I2 (PGI2), or prostacyclin. Moreover, the normal healthy endothelium is covered by the glycocalyx, a 0.5–5 μm-thick (Uchimido et al., 2019) proteoglycan rich structure that prevents endothelial-platelet interaction. After a damage to a blood vessel, platelets adhere to the vascular wall through the engagement of different receptors which bind to cellular and extracellular matrix constituents of the vessel wall. Initially, the platelet receptor glycoprotein (GP) Ib-IX-V complex tethers immobilized von Willebrand factor (vWF), a multimeric adhesive protein secreted from activated endothelial cells. This interaction allows the binding of platelet collagen receptor GpVI to its ligand in the uncovered subendothelial matrix and triggers intracellular signals that activate integrins, which are involved in cell-cell and cell-matrix interactions, thereby leading to platelet stable adhesion, activation and aggregation (Varga-Szabo et al., 2008). The adherence of platelets to the vascular wall activates them to undergo a dramatic shape change and to release the content of their granules and also extracellular vesicles (Estevez and Du, 2017; Lopez et al., 2019). These events result from cytoskeletal rearrangements through the rapid reorganization of microtubules and the polymerization of actin, which is prompted by increased intracellular Ca^{2+} initially due to its release from intracellular stores and then to its entry through the plasma membrane (Varga-Szabo et al., 2009). The cytoskeleton, then, directs the centralization of the granules, followed by their fusion mainly with the open canalicular system (OCS), a tunneling network that markedly increases platelet surface area, but also with the plasma membrane (Heijnen and van der Sluijs, 2015). This release reaction is very complex and tightly controlled by intracellular kinases, proteases and Ca^{2+}. To further complicate the scenario, it is reported that, in parallel with the degranulation, also microparticles and exosomes, both implicated in intercellular communication, are released and, again, Ca^{2+} plays a prominent role (Melki et al., 2017). Within minutes, platelets aggregate to form the primary hemostatic plug. The secreted microparticles, together with the transbilayer movement of negatively charged phospholipids, provide binding sites for components of the coagulation system with the efficient production of thrombin, which cleaves fibrinogen in fibrin. Fibrin recruits more platelets, and also erythrocytes and leukocytes, and consolidates the initial plug into the so called secondary hemostatic plug. Concomitantly, counter-regulatory pathways are activated to limit the extension and the dimension of the plug (Palta et al., 2014). Platelet adhesion, activation and aggregation are finely tuned by a plethora of pre-formed and neo-synthesized mediators (Nieswandt et al., 2011).

While all the aforementioned events are fundamental to plug holes in injured vessels thus protecting against blood loss, an uncontrolled and dysregulated activation of hemostasis leads to the formation of an intravascular clot which partially or completely blocks the lumen of the vessel, with relevant clinical implications (Satoh et al., 2019). This process is known as thrombosis and is triggered by the so-called Virchow's triad featured by endothelial damage, altered blood flow and hypercoagulability. On the contrary, impaired hemostasis leads to bleeding diathesis because primary hemostasis is impaired (Vinholt, 2019).

Beyond Hemostasis: Platelets, Vascular Integrity and Inflammation

Platelets function as gatekeepers of the integrity of the vascular wall through a complex bidirectional communication with the endothelium. In the bone marrow, megakaryocytes constitutively secrete Vascular Endothelial Growth Factor (VEGF) and other angiogenic factors, which promote the survival of bone resident microvascular endothelial cells by upregulating the antiapoptotic protein Bcl2. Conversely, bone marrow endothelial cells support the proliferation and differentiation of the megakaryoblasts as well as megakaryocytic fragmentation by releasing specific trophic cytokines (Nachman and Rafii, 2008). Under physiological conditions, a very low grade platelet activation is likely to occur as a response to rheologic events, thus accounting for a tonic, controlled release of low amounts of endothelial trophogens-VEGF, angiopoietin 1, Brain-Derived Neurotrophic Factor (BDNF), Sphingosine-1-Phosphate (S1P)- stored in the granules or for their exposure on the cell surface (Randriamboavonjy and Fleming, 2018). VEGF and BDNF function as survival factors for the endothelium of mature vessels (Donovan et al., 2000), whereas angiopoietin 1 and S1P contribute to the stabilization of the blood vessels (Gavard et al., 2008; Xiong and Hla, 2014). Consequently, the structural and functional integrity of the vascular-endothelium cadherin complex is preserved, thus maintaining the stability of the vessels. Accordingly, thrombocytopenia is associated with alterations of the intercellular adherens junctions because of the disassembly of cadherin complexes, which culminates with the extravasation of erythrocytes into the neighboring tissues (Nachman and Rafii, 2008).

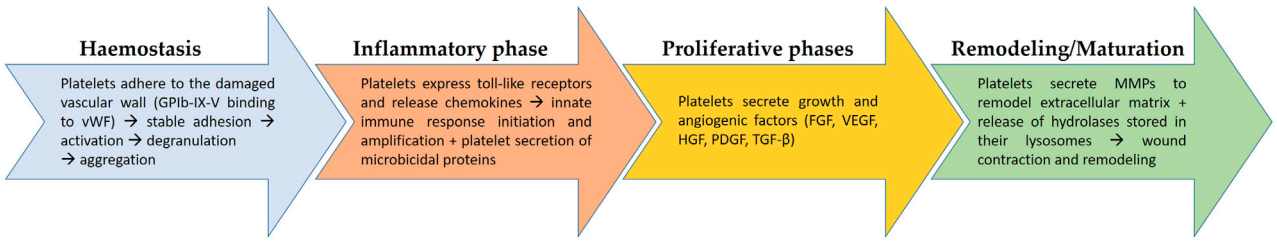

FIGURE 1 | The role of platelets in the different sequential steps of wound healing. In wound healing, four steps are recognized, i.e., hemostasis, inflammation, proliferation, and remodeling/maturation. From the beginning of the process platelets play a fundamental role, starting from the aggregation and formation of a clot to stop bleeding (hemostasis). They also contribute to the recruitment of immune cells (inflammatory phase), to the formation of granulation tissue (proliferative phase), to the remodeling and contraction of the wound to reconstitute structural continuity and, possibly, function. vWF, von Willebrand factor; FGF, Fibroblast Growth Factor; VEGF, Vascular Endothelial Growth Factor; HGF, Hepatocyte Growth Factor; PDGF, Platelet-Derived Growth Factor; TGF-β, Transforming Growth Factor β; MMPs, Matrix Metallo-Proteinases.

Platelets also participate in inflammation. They express toll-like receptors, which initiate the innate immune response, and bind components of the complement system, thus resulting in the formation of the membrane attack complex that lyses pathogens (Li et al., 2017; Eisinger et al., 2018). Moreover, platelets accumulate microbicidal proteins, i.e., thrombocidin 1 and 2, in their granules (Eisinger et al., 2018). Consequently, it is not surprising that thrombocytopenia is considered as an independent risk factor of mortality in sepsis (Assinger et al., 2019).

While in physiological conditions platelets dampen neutrophil degranulation and histotoxic functions, in an inflammatory environment they directly interact not only with the activated endothelial cells, but also with leukocytes. Furthermore, platelets release chemokines that recruit leukocytes and stimulates their adhesion to the endothelium by upregulating endothelial adhesion molecules which grant firm adhesion and extravasation (Assinger et al., 2019). Neutrophils are central in innate immunity and are rapidly engaged at the site of inflammation. Platelets enhance neutrophil's phagocytosis and production of free radicals, and induce the formation of Neutrophil Extracellular Traps (NETs) that protect against pathogens but can also occlude the vasculature or cause immune dysfunction (Lisman, 2018). In parallel, neutrophils secrete proteases that amplify platelet responses by activating protease-activator receptors (Lisman, 2018).

Activated platelets also release C-C Chemokine Ligand 5 (CCL5), which recruits monocyte and T-lymphocytes and preludes their transmigration towards the site of inflammation (Randriamboavonjy and Fleming, 2018). In particular, platelets regulate T-lymphocyte trafficking and activation (Randriamboavonjy and Fleming, 2018). On the other hand, T cells trigger platelet to release CCL5, which further engages T cells (Margraf and Zarbock, 2019).

It should be recalled that, while orchestrating host defense, platelets continue to serve as gatekeepers of the vascular wall to secure vascular integrity (Ho-Tin-Noé et al., 2018). Moreover, it is emerging that platelets also secrete pro-resolving mediators of the lipoxin family, which grant the transition from the inflammatory to the resolution phase (Rossaint et al., 2018).

Platelets and Wound Healing

Healing or replacing injured tissues is the result of millennia of evolution that has allowed the refinement of increasingly sophisticated processes fundamental to cope with the onslaught of all the biological, physical and chemical challenges that dot our everyday life. Different cell types and a panoply of GFs, cytokines and active metabolites are dynamically coordinated in wound healing. Different sequential steps have been defined to outline such a complex process i.e., hemostasis, inflammation, proliferation, and remodeling/maturation (**Figure 1**). Platelets are implicated in all these phases, from the early moments, where they are the most abundant cell type present, to the late steps (Wilkinson and Hardman, 2020). Upon tissue injury, platelets rapidly form a fibrin clot that stops bleeding, provides a provisional scaffold for inflammatory cells, and harbors a reservoir of cytokines, chemokines, and GFs that drive the early events of repair, among which the recruitment of neutrophils as the first line of defense against microorganisms. Indeed, within 12–24 h from injury, neutrophils represent ~50% of all the cells in the wound while after 3–5 days macrophages predominate (Rodrigues et al., 2019). Neutrophils and platelets cooperate to orchestrate the resolution of inflammation by releasing pro-resolving mediators and by polarizing macrophages towards a repair phenotype (Uchiyama et al., 2021). Moreover, platelets release a large array of growth and angiogenic factors, thus playing a role in the proliferative phase. Neovascularization is a pivotal process to meet the high metabolic demands of the healing tissue and is finely regulated by the balanced release of pro- and anti-angiogenic factors. In addition to sprouting angiogenesis induced by the release of VEGF, Hepatocyte Growth Factor (HGF) and Fibroblast Growth Factor (FGF), platelets also stimulate the recruitment of CD34+ bone marrow derived endothelial progenitors through the secretion of Stromal cell-Derived Factor (SDF)-1α (Ho-Tin-Noé et al., 2011). The arsenal of GFs stored in platelet's granules can aid the proliferation of many cellular protagonists in healing, i.e., keratinocytes in case of

TABLE 1 | Summary of the studies on platelets in microgravity using different experimental models.

Model	Method	Type of microgravity	Time of exposure	Effects
Humans	Spaceflight	Real, different g forces experienced	From days to months	Auñón-Chancellor et al. (2020); Limper et al. (2021); Garrett-Bakelman et al. (2019)
	Bed rest	Simulated	From days to months	Brzhozovskiy et al. (2019); Venemans-Jellema et al. (2014)
Animals	Parabolic flight	Real, different g forces experienced	Seconds	Fuse et al. (2002)
	Hindlimb	Simulated	From minutes to days	Dai et al. (2009)
	Spaceflight	Real, different g forces experienced	Days	Davidson et al. (1999)
	RWV	Simulated	From minutes to days	Cialdai et al. (2020)
Cells	Spaceflight	Real, different g forces experienced	Days	Davis et al. (1996); Plett et al.,2001; Plett et al.,2004; Akiyama et al. (1999)
	Parabolic flight	Real, different g forces experienced	Seconds	Schmitt et al. (1993)
	RWV	Simulated	From minutes to days	Li et al. (2010)

The type of microgravity, the duration of microgravity exposure and the results obtained are reported.

wounds or bone cells in case of fractures. Furthermore, platelet-released Transforming Growth Factor (TGF) ßs and Platelet-Derived Growth Factor (PDGF) act on fibroblasts so that the initial provisional fibrin scaffold is replaced with a granulation tissue rich in immature collagens, fibronectin and proteoglycans (Eisinger et al., 2018). Last, platelets aid the remodeling of the extracellular matrix secreting Matrix Metallo-Proteinases (MMPs) and releasing the hydrolases stored in their lysosomes. Our increasing understanding of the role of platelet in wound healing and tissue regeneration resulted in the development of autologous Platelet-Rich Plasma (PRP) gels, which are largely utilized in a number of clinical settings, from healing of skin wounds and diabetic ulcers to regeneration of tendons and ligaments, from eye lesions to bone loss (Arora and Arora, 2021).

PLATELETS IN SPACE

Life on this planet developed and evolved under a static gravity of 9.81 m/s^2 and surrounded by the atmosphere, a perfect protective shield from radiations. Moving away from Earth, gravity and atmosphere disappear. Therefore, in long-duration space missions, radiations and microgravity represent primary hazards to astronaut's health (Garrett-Bakelman et al., 2019). Moreover, confinement, isolation, sleep disturbances, alteration of circadian rhythms and possible conflicts among the members of the crew create a variety of potentially stressful demands, known to contribute to a number of diseases (Cohen et al., 2007). We here discussed available evidence on the role of microgravity, radiations and stress on platelets.

Platelets and Microgravity

Cellular, animal, and human studies performed in simulated or real microgravity demonstrated influences of weightlessness and gravity on haemostasis and, in particular, on platelets, even if available data depict a contrasting picture probably due to the differences in unloading conditions. Briefly, it should be recalled that experiments in real microgravity can be performed only in orbit, i.e., onboard space stations or rockets and capsules, while short duration of reduced gravity can be achieved by parabolic flights (Pletser, 2020). Ground-based analogues for human spaceflight, such as immersion and head down tilt bed rest, are available, but present some limits (Pandiarajan and Hargens, 2020). For rodents, hindlimb unloading is widely used to simulate microgravity (Morey-Holton and Globus, 2002). For studies at the cellular level, several devices, i.e., clinostats, among which the random positioning machine (RPM), and the rotating wall vessel (RWV), are commonly utilized and simulate some aspects of microgravity (for detailed description please see (Maier et al., 2015; Riwaldt et al., 2021) and **Table 1**.

Platelet, Microgravity, and Hemostasis

Turning to the effects of gravity on platelets, parabolic flight induces thrombocytopenia in mice (Fuse et al., 2002). Two *in vitro* experiment in real microgravity during the missions STS-63 (Discovery) and STS-69 (Endeavour) showed a reduction of the proliferation of CD34+ bone marrow progenitors compared to ground controls (Davis et al., 1996). Consistently, in simulated microgravity, Plett at al. showed inhibition of migration, delay in cell cycle progression with consequent growth retardation and impaired differentiation patterns of hematopoietic progenitors (Plett et al., 2001; Plett et al., 2004), which might account for alterations in platelet counts.

Also platelet functions were investigated in microgravity. Parabolic flight does not inhibit platelet activation. Indeed, Ca^{2+}-calmodulin-mediated events and Protein kinase C (PKC) -dependent pathways are maintained. Moreover, no significant modifications in shape changes, phosphorylation patterns or degranulation of platelets were detected after parabolic flight (Schmitt et al., 1993). The limitations of this study are the low level of microgravity reached with parabolic flight (10^{-2} g) and the short time of microgravity experienced (5 or 10 min of cumulative microgravity), which might explain the discrepancy between this and other studies highlighting platelets' dysfunction in microgravity.

Simulated microgravity decreases platelet adhesion to vWF by downregulating GPIbα on platelet surface (Dai et al., 2009). The

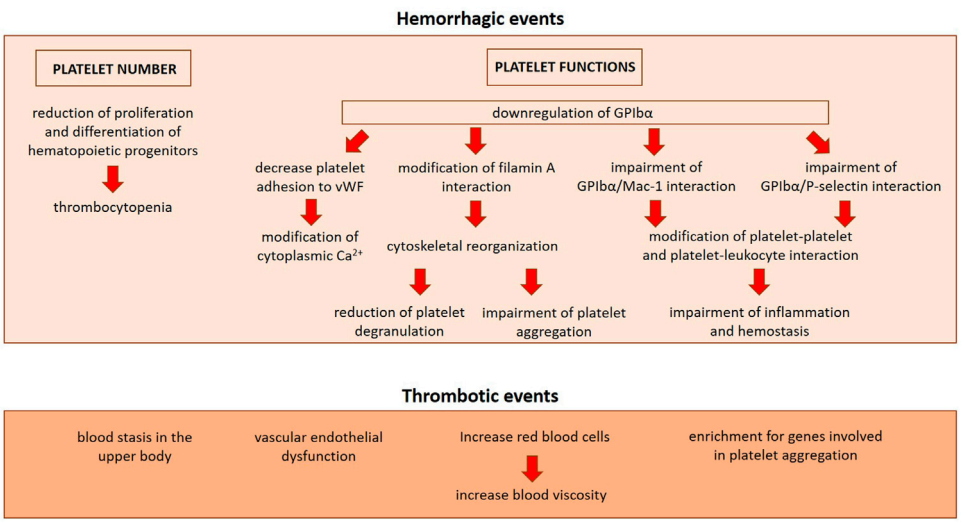

FIGURE 2 | The role of microgravity in inducing hemorrhagic or thrombotic events. Microgravity induces hemorrhagic events by decreasing platelet number and impairing platelet functions. On the other hand, microgravity induces blood stasis in the upper body, vascular endothelial dysfunction and alterations in blood volume and blood viscosity, all events which might contribute to the increase of thrombotic incidents.

interaction of GPIbα with vWF induces different intracellular changes, among which the increase of cytoplasmic Ca^{2+} level. Li et al. demonstrated that different gravity conditions induce a modification of intracellular Ca^{2+} concentration suggesting that platelet intracellular Ca^{2+} plays a key role in the alterations of platelet functions in different gravity conditions (Li et al., 2010). Since GPIbα associates with filamin A, which directly binds the actin cytoskeleton, its downregulation contributes to cytoskeletal disorganization in simulated microgravity. Importantly, cytoskeleton modifications impair platelet functions, reduce platelet release reaction and severely impair platelet aggregation after induction with rystocetin or collagen (Dai et al., 2009).

In addition to binding vWF, GPIbα also binds the integrin Mac-1 which is expressed on leukocytes. Notably, heterotypic cell—cell interactions between leukocytes and platelets enhance pro-inflammatory and pro-thrombotic events (Wang et al., 2017). GPIbα also binds P-selectin, thus promoting thrombus propagation independently of vVW (Prakash et al., 2017). Therefore, GPIbα downregulation in microgravity suggests an impairment of platelets mediated events in inflammation and hemostasis (**Figure 2**).

Interesting data were obtained by analysing astronaut's medical records. A recent study reports the proteomic analysis of blood plasma samples obtained from cosmonauts before and after missions on the international space station (ISS) as well as from volunteers before and after 21 days of bed rest and dry immersion. The study revealed nine common proteins significantly regulated by gravity. Most of these proteins among which SERPIN1, SERPIN3, SERPINC1, SERPING1, A2M, are involved in platelet degranulation being mainly released from α granules (Brzhozovskiy et al., 2019).

Despite the evidence demonstrating that microgravity reduces platelet number and activity, not only hemorrhagic but also thrombotic events have been shown after microgravity exposure. This is not completely surprising given the protective effects exerted by platelets on the endothelium (Nachman and Rafii, 2008). In 2019 the first known blood clot was treated in space. After 2 months onboard the ISS, during an ultrasound examination, an obstructive internal jugular venous thrombosis was revealed in an astronaut (Auñón-Chancellor et al., 2020). The increase of thrombotic incidents may be explained with several factors among which blood stasis in the upper body, vascular endothelial dysfunction and alterations in blood volume and blood viscosity that are documented in real microgravity (**Figure 2**). When entering weightlessness, the astronauts experience an initial increase in central blood volume, followed by intravascular volume contraction due to reduced thirst and increased urine output. Also the increased levels of fibrinogen *ß* chain and the elevated red blood cell counts, both documented in astronauts after long-term spaceflight, might play a role in the formation of thrombi (Limper et al., 2021).

In the NASA Twins Study, the Gene Ontology enrichment analysis revealed an enrichment for genes involved in platelet aggregation and for genes involved in the response to PDGF in the astronaut subjected to one year-long mission compared with his twin on Earth (Garrett-Bakelman et al., 2019). The head-down bed-rest experiments documented reduced deformability and increased aggregation of red blood cell and decreased platelet activation (Venemans-Jellema et al., 2014).

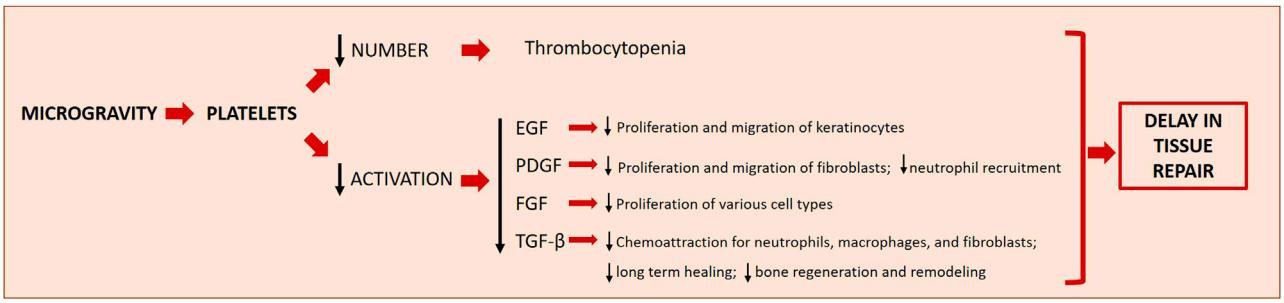

FIGURE 3 | Microgravity effects on platelets and the downstream effects on wound healing. Microgravity induces thrombocytopenia and decreases the activation of platelets, two phenomena which concur to delay tissue repair in space. The decreased production/release of different growth factors (EGF, PDGF, FGF and TGF-β) has downstream effects on other important players of the different steps of wound healing. EGF, Epidermal Growth Factor; PDGF, Platelet-Derived Growth Factor; FGF, Fibroblast Growth Factor; TGF-β, Transforming Growth Factor β.

Platelets, Microgravity, and Wound Healing

Wound healing was studied in microgravity using rats subcutaneously implanted with polyvinyl acetal sponge disks releasing PDGF-BB and basic FGF. The capacity to form granulation tissue on the sponge disks was studied during 10 days on the orbiting space shuttle Endeavour. The authors demonstrated a blunted response to the GFs, regarding the cellularity and collagen deposition, in the flight sponges compared to the ground controls. These data suggest that microgravity affects tissue responsiveness retarding the capacity of wound to heal (Davidson et al., 1999). Accordingly, during space flight the response of wounds to PDGF was lower than in control wounds on the ground (Davidson et al., 1999). Similar results have been obtained by an "*in vivo*" model of wound healing based on the use of leeches (Hirudo) exposed to modelled microgravity by RPM, showing a delay in healing capacity probably related to a decrease in collagen fibre density (Cialdai et al., 2020). As mentioned above, wound healing is a complex process orchestrated by the coordinated intervention of many different cell types and by a myriad of different molecules. The initial steps are driven by platelets. Therefore, microgravity-associated thrombocytopenia is itself a critical issue in wound healing. Moreover, microgravity impairs platelet adhesion to the injured tissue, thus reducing the efficiency of clot formation, and blunts the release of growth and angiogenic factors, thus retarding the activation of cell proliferation and migration (Farahani and DiPietro, 2008) (**Figure 3**). Indeed, platelet-released GFs, such as TGF-*ß*, PDGF, and Epidermal Growth Factor (EGF), modulate the healing process. PDGF is mitogenic and motogenic for fibroblasts and stimulates the recruitment of neutrophils (Werner and Grose, 2003). Large amounts of TGF-ß are released from platelets immediately after wounding (Werner and Grose, 2003) and this initial kick-start of active TGF-ß serves as a chemoattractant for neutrophils, macrophages, and fibroblasts. EGF stimulates keratinocyte migration, fibroblast function and the formation of granulation tissue (Hardwicke et al., 2008). The impaired release of GFs is aggravated by the evidence that microgravity interferes with receptor binding and signal transduction. It is reported that microgravity inhibits EGF-induced signal transduction independently from the redistribution of EGF receptor in the plasma membrane of epidermal cells (Rijken et al., 1993). Also the expression of PDGF receptors and of the various isoforms of TGF-ß is downregulated in simulated microgravity (Akiyama et al., 1999; Farahani and DiPietro, 2008). As for TGF-ß, since microgravity generates a low shear stress environment, it is likely that altered biomechanical properties decrease TGF-ß synthesis. Moreover, flight-induced psychological stress might play a role, because of the increase of glucocorticoids, which are known to inhibit TGF-ß transcription (Farahani and DiPietro, 2008).

Furthermore, because fibrin structure determines healing outcomes, it is noteworthy that microgravity affects the branches and porosity of fibrin matrices resulting in the formation of more homogeneous fibrin gels than on ground (Nunes et al., 1995; Roedersheimer et al., 1997). It is feasible that the reduced platelet content in the fibrin plug due to microgravity diminishes the contractile force and facilitates the lysis of the fibrin clot. Platelets are just one of the players in wound healing, but it is clear that their reduced number together with their functional impairment contribute to delay different steps of the process.

Platelet and Radiations

During space travels, beyond the Earth's protective magnetosphere, astronauts experience acute and chronic exposure to ionizing radiations, in particular those generated by solar particle event (SPE) (Hu et al., 2009). A SPE is an intense release of ionizing radiation, specifically low energy protons, which occurs in specific regions of the Sun. These radiations represent a serious risk for astronauts mostly during extravehicular activities (Kerr, 2013; Zeitlin et al., 2013). Astronauts experience SPE doses ranging from 0 to 50 cGy/h, and the largest expected tissue dose is ~2 Gy (Townsend, 2005; Hu et al., 2009). Both *in vivo* and *in vitro* experiments highlight that blood cells are particularly sensitive to ionizing radiation (Dainiak, 2002) and platelets show a gradual decline in number over time (Maks et al., 2011; Romero-Weaver et al., 2013) with a marked drop around day 10 of spaceflight. Platelets number declines more gradually than other circulating cells after irradiation (Maks et al., 2011).

Several studies have reported that the hematopoietic system is particularly sensitive to radiation damage, resulting also in thrombocytopenia (Dainiak, 2002; Stasi, 2012). This seems to be mediated directly, by the direct damage to hematopoietic cells, and indirectly, through the radiation-mediated alteration of endothelial cell function (Wen et al., 2016; Chen et al., 2017). Indeed, the vascular niche is the site of terminal maturation of megakaryocytes and thrombopoiesis (Niswander et al., 2014), where endothelial cells promote the differentiation, maturation and localization of megakaryocytes through the expression of numerous autocrine and paracrine factors (Gaugler et al., 1998; Mazo et al., 2002; Himburg et al., 2016) among which VEGF, whose production was demonstrated to be sensitive to radiation (Chen et al., 2017).

For what regards animal models, Romero-Weaver and colleagues exposed mice to SPE-like proton radiation and/or microgravity simulated by hindlimb unloading and analyzed the number of different blood cells among which platelets (Romero-Weaver et al., 2014). They found that platelet counts were decreased significantly in a dose dependent manner by proton radiation, but not significantly affected by hindlimb unloading treatment alone. Exposure to radiation alone caused a significant decrease in platelet number with a drop on day 10 post-irradiation. This reduction in platelet numbers might increase the risk of hemorrhagic events and, eventually, delay healing (Dai et al., 2009).

Of note, differences determined by gender were found in platelets response to proton radiation by Billings and colleagues. They show that non-irradiated female mice have 13% less platelets than their male counterparts and recover slower than males post irradiation. Both males and females display platelet decrease at day 4 post irradiation with a drop at days 11–12, followed by a consistent rebound in males and a slower in females (Billings et al., 2014). The opposite gender trend was reported in humans (Liu et al., 2021) analyzing the effects of low cumulative doses of radiations on medical workers. Males have lower platelet counts than females, but they both display a first increase post irradiation and then a decrease in the number of platelets, in a dose-dependent manner. It is important to note that platelet aggregation and release reaction were not altered by the irradiation (Rock et al., 1988).

Platelet and Psychological Stress

Psychological and social issues affect astronauts proportionally to the duration of the space mission (Oluwafemi et al., 2021). Not only isolation, confinement, separation from families and interpersonal tensions but also sleep disorders generate stress. Numerous studies have shown a relation between psychological stress and somatic disorders (Kendler et al., 1999; Leonard, 2006; Anisman, 2009; Haroon et al., 2012). First of all, stress activates the hypothalamic-pituitary–adrenal (HPA) axis, resulting in increased levels of glucocorticoids which impair wound healing (Slominski and Zmijewski, 2017). This is due to the fact that glucocorticoids inhibit inflammation, required in the early phases of repair, and retard keratinocyte migration and wound closure (Slominski and Zmijewski, 2017). However, glucocorticoids exert no effects on platelet number and functions (Liverani et al., 2012).

Platelets contain the largest amount of serotonin (5-HT) outside the central nervous system and express serotonin receptors 2A and 3A (5-HT-2A receptor, 5-HT-3A receptor), α-2, β-2 adrenoreceptors, benzodiazepine and the serotonin transporter (SERT) (Camacho and Dimsdale, 2000). Their activation might occur, in addition to the canonical pathways, by various lifestyle factors such as physical and mental stress (Camacho and Dimsdale, 2000; El-Sayed, 2002; Jurk and Kehrel, 2005). The HPA axis also activates the sympathetic nervous system (SNS) and the serotonergic system, thus activating platelets. Increased platelet activity is reported in emotional stress through increased serotonin binding to 5-HT-2 receptors on platelets (Markovitz and Matthews, 1991; Garvey et al., 1995) or to increased platelet 5-HT reuptake, as described in patients with anxiety or depression (Pecknold et al., 1988). In general, it seems that stress does not importantly affect platelet function and, therefore, stress associated delay of wound healing is mainly due to effects on all the other cell types involved in the process (Gouin and Kiecolt-Glaser, 2011).

PLATELET RICH PLASMA: THE FUTURE OF WOUND HEALING IN SPACE?

With the increase of manned missions in space, the chances of injury due to traumatic events or unexpected emergency surgery will increase. Wound healing might represent one of the major problems onboard, and this raises the need to promote studies to define adequate countermeasures. As mentioned above, a great amount of data demonstrate the improvement of tissue repair and wound healing upon PRP application (Arora and Arora, 2021). These outcomes together with the feasibility to obtain PRP (once prepared from a few milliliters of autologous blood, it can be stored frozen for many months onboard) indicate that PRP might become a new challenge in the field of tissue healing also in space.

The rationale of using PRP relies on the evidence that, upon degranulation, platelets release GFs, thereby accelerating the recruitment of the cells implicated in the healing process. Indeed, PRP application yielded excellent results in oral and maxillofacial surgical procedures for its efficacy on soft tissue repair (Mijiritsky et al., 2021). Moreover, autologous PRP represents a promising adjuvant therapy for the treatment of diabetic foot ulcers, since it ameliorates the healing process and reduces the risk of infections (Shao et al., 2020). In addition, PRP has been proposed as a local supply of cytokines and GFs in different hyaluronan-controlled and placebo-controlled clinical trials (Andia and Maffulli, 2013) in patients with symptomatic osteoarthritis, even if the cellular and molecular mechanisms of PRP effects remain poorly elucidated. Furthermore, PRP shows anti-inflammatory effects likely by interfering with Nuclear Factor kappa B (NFkB) signaling (Andia and Maffulli, 2013), increases cartilage height and reduces the loss of cartilage matrix by diminishing chondrocyte apoptosis (Testa et al., 2021). Moreover, PRP ability to polarize macrophages towards a M2 repairing phenotype is of utmost importance to reduce the proinflammatory chronic effects of joint M1 macrophages (Uchiyama et al., 2021) (**Figure 4**).

Very few studies are published concerning the application of platelet derivatives in different gravity conditions. PRP treatment can partially counteract microgravity-induced alterations in cultured fibroblasts (Cialdai et al., 2020). When applied to a fibroblast cell culture exposed to simulated microgravity in RPM, PRP prevents the

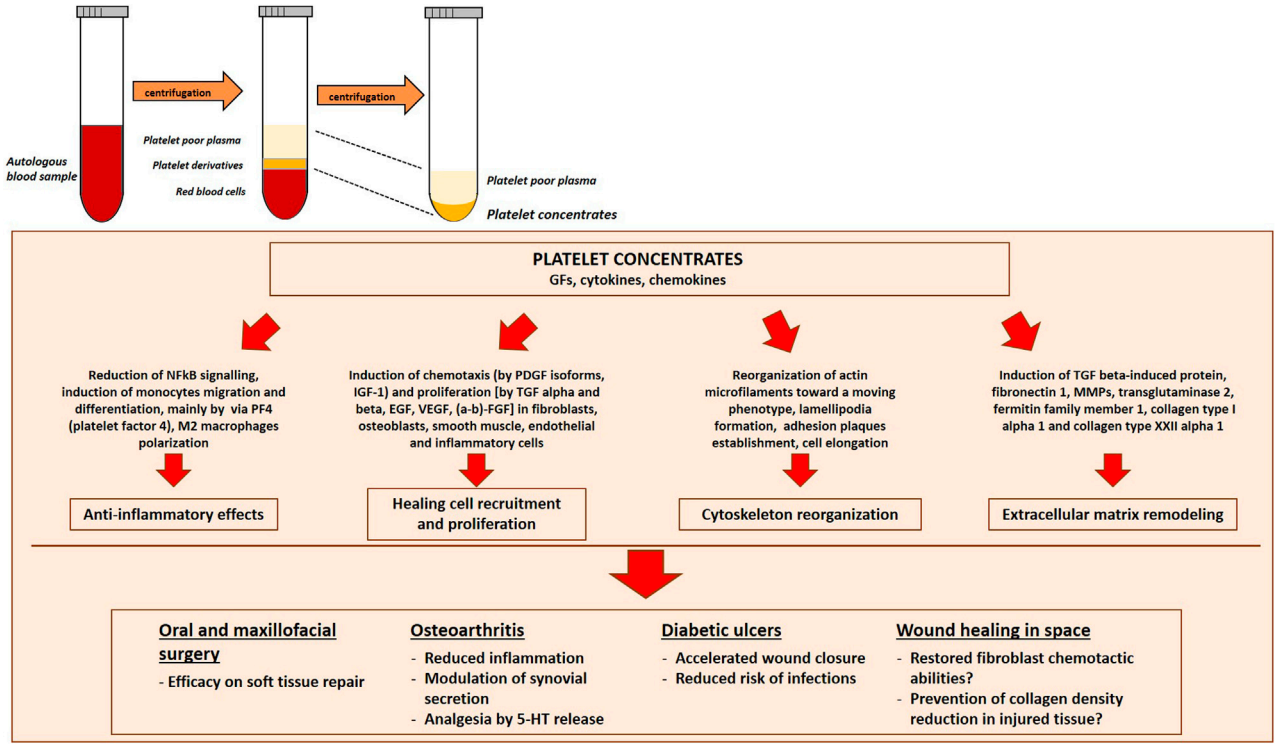

FIGURE 4 | Platelet Rich Plasma (PRP) preparation and efficacy in different clinical conditions. PRP is prepared by centrifugation of peripheral blood to concentrate platelets. Platelet derivatives are promising tools to promote regeneration and healing for future space travel. Indeed, they have yielded excellent results in different medical fields, from oral and maxillofacial surgical procedures, to the treatment of symptomatic osteoarthritis patients and diabetic ulcers. NFκB, Nuclear Factor kappa B; TGF-β, Transforming Growth Factor β MMPs, Matrix Metallo-Proteinases, 5-HT, serotonin.

formation of 3D aggregates, probably by remodeling the cytoskeleton (Casati et al., 2014) and restores, at least in part, the chemokinetic abilities of fibroblasts largely compromised by gravitational unloading (Cialdai et al., 2020). The regenerative effects of PRP were recently proved also in an "*in vivo*" model of tissue repair in microgravity, based on the use of leeches (Cialdai et al., 2020), considered a good model for the study of tissue repair (Tettamanti et al., 2004) as the wound healing process occurs similarly to vertebrates. To mimic a wound, a surgical lesion (length 10 mm, depth ~2 mm) was performed on the dorsal skin of each leech before exposing them to simulated microgravity in the RPM. The supply of PRP to the medium prevented both healing delay and alterations in tissue structure, narrowing the surgical wound, enhancing re-epithelization and preventing the decrease in collagen meshwork density of collagen fibers of the peri-lesional connective tissue (Cialdai et al., 2020). The first results are promising, but more studies are necessary to assess PRP usefulness in microgravity.

CONCLUSION

Since 1971, astronauts have spent months in space stations orbiting Earth. Future human missions to Mars will require astronauts to live in space more than 2 years. A human outpost is foreseen on the moon to be used as a long-term foothold for lunar exploration and as a gateway for deep space missions. Moreover, if and when Earth is incapable of supporting life, space might be an alternative for human survival. Last, 20 years after the first space tourist, more people are experiencing spaceflight. Therefore, long term permanence and more people in space require to optimize how to face the most likely injury that might occur, i.e., a wound. PRP might represent a safe, relatively easy and efficient tool to accelerate tissue repair in an extreme environment. Only few data are available at the moment to prove its effectiveness also during weightless condition and further studies are needed to test whether long term storage of PRP in space affects the stability and activity of the GFs and cytokines and, thus, PRP regenerative properties.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

This review was written in the frame of the European Space Agency (ESA) Topical Team on "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration", partially

supported by MAP Project "WHISPER—Wound Healing in Space: problems and Perspectives for tissue Regeneration and engineering", SciSpacE Microgravity Application Promotion Program, ESA; Contract Number 4000130928/20/NL/PG/pt. The authors acknowledge support from the University of Milan through the APC initiative.

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frontiers in Bioengineering and Biotechnology

PERSPECTIVE
published: 25 October 2021
doi: 10.3389/fbioe.2021.720217

Wound and Skin Healing in Space: The 3D Bioprinting Perspective

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Skin wound healing is known to be impaired in space. As skin is the tissue mostly at risk to become injured during manned space missions, there is the need for a better understanding of the biological mechanisms behind the reduced wound healing capacity in space. In addition, for far-distant and long-term manned space missions like the exploration of Mars or other extraterrestrial human settlements, e.g., on the Moon, new effective treatment options for severe skin injuries have to be developed. However, these need to be compatible with the limitations concerning the availability of devices and materials present in space missions. Three-dimensional (3D) bioprinting (BP) might become a solution for both demands, as it allows the manufacturing of multicellular, complex and 3D tissue constructs, which can serve as models in basic research as well as transplantable skin grafts. The perspective article provides an overview of the state of the art of skin BP and approach to establish this additive manufacturing technology in space. In addition, the several advantages of BP for utilization in future manned space missions are highlighted.

Keywords: wound healing, skin, bioprinting, space, microgravity, bioinks, biofabrication

OPEN ACCESS

Edited by:
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Università degli Studi di Firenze, Italy

Reviewed by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *03 June 2021*
Accepted: *13 September 2021*
Published: *25 October 2021*

Citation:
Cubo-Mateo N and Gelinsky M (2021) Wound and Skin Healing in Space: The 3D Bioprinting Perspective. Front. Bioeng. Biotechnol. 9:720217. doi: 10.3389/fbioe.2021.720217

INTRODUCTION

The human body, in general, has a huge capacity for wound healing; although, it depends on the general health situation of the individual, the extent of damage suffered by tissues, and the capacity of those cells to multiply. Based on the proliferation capacity of the cells, we can find labile (always in renewal, as skin or bone), stable (able to regenerate when damaged, as liver or kidney), and permanent (almost no regeneration, as cardiac and skeletal muscle) tissues (Paul and Sharma, 2021). Skin, which is the outer covering of the body, is a labile tissue, so cells are under constant active division, replacing the damaged or aged layers continuously. Withal, when the injury is too big as in extensive or severe burns [e.g., in case of partial thickness burns >20% of the total body surface area in an adult, or 10% in children and seniors (European Burns Association., 2017)], the remaining cells are not capable of closing the wound. In these cases, it becomes necessary to transplant healthy skin from other areas of the body (autograft) and utilize artificial skin substitutes or even skin from a human donor (allograft). The optimal option is always the use of autologous cells or tissues, as immunological rejection is avoided. However, harvesting skin for autologous transplantation or isolation of cells leads to the formation of additional lesions.

In the last decades, new materials and methodologies have been developed for the fabrication of improved skin substitutes, including the utilization of emerging technologies (Tottoli et al., 2020; Dai et al., 2020; Tavakoli and Klar 2021). In this regard, applicable also for other tissues as bone or cartilage, three-dimensional (3D) bioprinting (BP) allows the production of complex, cellularized constructs that may overcome the limitations present in the classical methods of tissue engineering, where commonly a prefabricated scaffold is seeded with cells. BP is an additive manufacturing

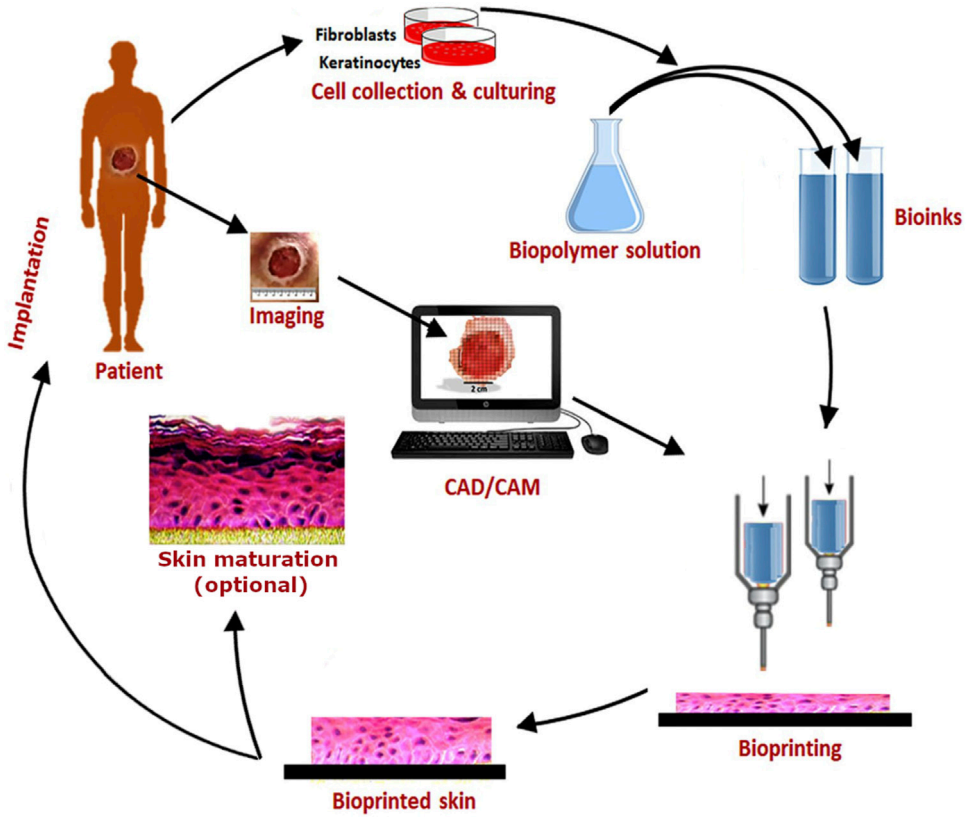

FIGURE 1 | Schematic showing the workflow of BP using the example of extrusion BP of skin utilizing two different cell types (fibroblasts and keratinocytes) and bioinks. Original image taken with permission from Augustine (2018) was adapted.

technology and can be seen as a specific type of tissue engineering method. In contrast to the conventional approach, in BP, the live cells are printed together with suitable biomaterial(s) so that a tissue-like construct can be fabricated in one process step.

All the above mentioned is effective under Earth conditions. But, when considering a long-term space exploration mission, as travelling to Mars or settlements on the Moon, it becomes necessary to consider pathologic wound healing due to altered gravity and radiation effects (Grimm et al., 2020). This, in addition to the deconditioning caused by such environmental factors over a prolonged period, makes it necessary to find new strategies for wound healing in space, both concerning advanced research models and for medical treatments.

In this article, a perspective from the BP point of view for healing of skin wounds in space is described, along with a brief review of selected studies about BP of the skin. As the international space agencies have started to implement BP devices at the International Space Station (ISS) (Cubo-Mateo et al., 2020), such approaches are no longer science fiction but will become feasible soon.

THREE-DIMENSIONAL BIOPRINTING

BP is a type of additive manufacturing in which live cells are included directly in the printing process, mostly in combination with biomaterials. Two main technologies have to be distinguished, extrusion and inkjet (drop-on-demand) BP. In extrusion BP, continuous strands consisting of cells that are suspended in a gellable, viscous liquid called bioink are deposited in a layer-by-layer fashion so that easily 3D constructs can be generated (Askari et al., 2021). In contrast, in inkjet BP, discrete droplets containing single or few cells, small cell aggregates, or even organoids without or with the addition of biomaterial components are deposited (Gudapati et al., 2016). In case of utilization of cell aggregates or spheroids, the term bioassembly is also common for this method (Mironov et al., 2009). The main advantage of BP compared to conventional tissue engineering is the opportunity to deposit different cell types along with specific biomaterials with high spatial resolution. For multilayered tissues like the skin, consisting of different cell types, this facilitates the fabrication process significantly. The whole field of BP has been tremendously developing since a couple of years, and the current state of the art is being described and reviewed continuously (Shapira and Dvir, 2021; Zhang et al., 2021). BP technologies also offer new opportunities for process automation and standardization which is beneficial for translation into clinical applications, as well as for utilization in isolated environments with strictly limited facilities, like in space flight. The principle of BP is illustrated in **Figure 1** using the example of extrusion skin BP.

TABLE 1 | Selected skin BP approaches performed under standard gravity conditions. No. 1–5 describe conventional studies (*in vitro* BP), No. 6–8 examples for *in situ* skin BP.

No	References	Dermal bioink	Fibroblasts (FB) density	Epidermal bioink	Keratinocytes density (KC)	Comments
1	Lee et al. (2009)	Rat tail type I collagen, diluted in DPBS	1×10^6 primary hFB/mL of bioink	Collagen type I diluted in DPBS	1×10^6 hKC/mL of hydrogel	Drop-on-demand BP. Separate printing of biomaterial components and suspended cells
2	Koch et al. (2012)	MatriDerm™, rat tail type I collagen	1.5×10^6 NIH-3T3 FB, resuspended in 1 × DMEM/Ham's F12 medium	Rat tail type I collagen	Same density than for FB. HaCaT KC	LIFT BP. Almost no support material between cells. High accuracy, important for future vascular network forming
3	Cubo et al. (2017)	Human plasma from blood (+tranexamic acid, $CaCl_2$, and NaCl 0.9%) with embedded primary hFB	1×10^3 hFBs/cm^2 (for a 3-mm thick gel: 3×10^3 FB/mL of bioink)	None, human primary KC suspended in KC medium	>1.5×10^4 hKCs/cm^2	Extrusion BP. Includes *in vivo* study (nude mice). Layers cannot be piled up, too liquid. Quick patient treatment possible: final cell expansion *in situ* inside the plasma hydrogel and *in vivo* maturation. Natural wound healing process
4	Baltazar et al. (2020)	Rat tail type I collagen, FBS and reconstitution buffer (embedded FB, and in some cases, 7×10^5/ml human EC with or without 3.5×10^5/ml human PC)	7.0×10^5 hFB/mL of bioink	KC growth medium and skin differentiation supplemented medium	2×10^6 hKC/mL of media	Extrusion BP. Includes *in vivo* study (nude mice). The PC in the dermal bioink associate with EC-lined vascular structures and appear to improve KC maturation
5	Admane et al. (2019)	5% silk fibroin and 5% gelatin	2×10^6 hFB/mL of bioink	5% silk fibroin and 5% gelatin	5×10^6 hKC/mL of bioink	Extrusion BP. Good reproduction of the dermal–epidermal interface and in-depth gene expression analysis
			***In situ* skin BP approaches**			
6	Skardal et al. (2012)	Fibrinogen and type I rat tail collagen	No FB; 1.66×10^7 human AFS and MSC/mL	No epidermal layer	No KC	Drop-on-demand BP, directly onto a wound generated in nude mice. Use of MSC and AFS beneficial to wound healing and immune privileged even though they originated from an allogenic source
7	Hakimi et al. (2018)	Bovine fibrinogen, sodium hyaluronate, type I rat tail collagen dissolved in PBS	0.5×10^6 hFBs/mL	Bovine fibrinogen, sodium hyaluronate	1.25×10^6 hKC/mL of hydrogel	Handheld BP, directly onto wounds generated in nude mice and Yorkshire pigs. Quick wound coverage: 0.3–1.6 cm^2/s. Normal reepithelization and wound contraction
8	Albanna et al. (2019)	25 mg/ml bovine fibrinogen and 1.1 mg/ml rat tail type I collagen	3.75×10^7 FB/mL of hydrogel (allogenic: human, autologous: murine and porcine FB)	25 mg/ml fibrinogen and 1.1 mg/ml collagen type I	7.5×10^7 kC/ml of hydrogel (allogenic: human, autologous: murine and porcine KC)	Ink-jet BP, directly onto wounds generated in nude mice and specific pathogen-free Yorkshire pigs. Wounds treated using *in situ* skin BP demonstrated faster wound closure compared to untreated and matrix-treated group

Abbreviations: h = human, FB = fibroblasts, KC = keratinocytes, EC = endothelial cells, PC = placental pericytes, hDPC = human dental pulp cells, AFS = amniotic fluid–derived stem cells, MSC = bone marrow–derived stem cells, LIFT = laser-induced forward transfer, DPBS = Dulbecco's phosphate-buffered saline.

CURRENT STATUS OF SKIN BIOPRINTING

Human skin is consisting of two main layers, the epidermis as the outermost part and the dermis. While the epidermis contains only one cell type, the keratinocytes, the dermis is dominated by fibroblasts. However, the dermis also contains blood vessels and nerves and several skin appendages like sebaceous and sweat glands – and therefore numerous other cell types. If skin patches are produced for wound healing applications in most cases only fibroblasts and keratinocytes are included to keep the fabrication simple. The skin is one of the human tissues for which replication utilizing BP has been intensively explored, and several studies have been published that describe approaches for the fabrication of artificial skin of variable complexity (Perez-Valle et al., 2020; Tan et al., 2021; Weng et al., 2021). For skin BP, several methods including extrusion and inkjet printing are applied, and also additional BP technologies like laser-induced forward transfer (LIFT) (Koch et al., 2012). **Table 1** summarizes selected studies

which describe BP of multilayer skin constructs, consisting of (at least) a dermal and epidermal compartment. This list shall provide a concise overview about the variety of approaches for using BP to fabricate skin models. One has to distinguish two fundamentally different approaches in skin BP: one is the fabrication of skin-like grafts in the lab, which commonly are further cultivated *in vitro* before implantation onto a wound, whereas the other describes the direct deposition of cells and materials onto the lesion of the patient (or experimental animal). The latter has been defined as "*in situ*" or "*in vivo* BP" and can be performed with a robotic arm device or just a handheld deposition system (Singh et al., 2020).

In the studies included in **Table 1**, the skin-like constructs that are bioprinted are simplified compared to the native tissue. For printing the dermal layer commonly only fibroblasts are utilized and for the epidermal layer keratinocytes. However, few studies have already described the inclusion of additional cell types like preadipocytes (for providing a hypoderm-like third layer for the treatment of full-thickness skin defects or burns) or endothelial cells (to support fast vascularization of the constructs). Ng and co-workers have demonstrated bioprinting of pigmented skin by inclusion of melanocytes (Ng et al., 2018). Due to the fact that the extracellular matrix (ECM) is the largest component of the dermis, constituting over 70% of this tissue (Widgerow et al., 2016), the fibroblasts are in most cases applied as part of a bioink, consisting of hydrogel-forming (bio)polymer solutions, whereas the keratinocytes are bioprinted both with the matrix components or just as a cell suspension on top of the dermal layer.

A variety of bioinks have been investigated and applied so far for BP of the dermal layer, and most of them are based on single biopolymers or blends (Perez-Valle et al., 2020; Masri and Fauzi, 2021). As native ECM of the dermis mainly consists of collagen type I, this biopolymer is an obvious suitable choice and also many other types of biopolymers, including gelatin, fibrin, chitosan, and alginate have been applied successfully. As the scaffolds in conventional tissue engineering, the bioinks in BP shall provide only temporary support, being replaced by the natural ECM synthesized by the embedded cells (i.e. fibroblasts in case of the dermal layer) or cells invading the implanted graft from the surrounding tissue over time.

WOUND AND SKIN HEALING IN SPACE

The skin provides the outer covering of the body and therefore has a protective function: it avoids excessive water loss and prevents pathogens from entering the organism. It also regulates body temperature and contains several types of glands and sensors (nerve endings) that allow us to feel objects and to secret metabolites. With increasing age, the human skin becomes more fragile and thin and requires longer periods to heal from injuries (Dyer and Miller, 2018). In space, environmental factors such as microgravity and radiation can have a severe impact on different tissues, and the effect can be quickly seen on the skin, bones and cartilages, muscles, and some internal organs like the heart (Afshinnekoo et al., 2020).

Astronauts lose more skin cells (keratinocytes) in space than on the Earth, and their skin ages faster during space flight; a common complaint of astronauts is cracking skin and rashes or itchiness. Apart from that, a thinning of the skin and increased sensitivity combined with delayed healing of wounds and an increased tendency to skin infections have been reported during and after long-term stays in space. These data have been obtained from three experiments carried out at the ISS regarding tissue development of humans and mice in space (Lademann and Fluhr, 2008; Tronnier et al., 2008; König et al., 2015; Neutelings et al., 2015; Braun et al., 2019). More details and the newest findings regarding wound and skin healing in space are described elsewhere in the present special issue of which this article is part of.

Bioprinting of Skin for Wound Healing in Space

While it is already widely accepted that additive manufacturing using nonbiological materials will play a crucial role in the further development of space flight (Ghidini 2018), the international space agencies have started to become interested in BP too. The authors recently have described the relevance of BP for future long-term and far-distant manned space missions, and the current status of the establishment of bioprinters at the ISS in a separate study (Cubo-Mateo et al., 2020). To summarize, the two main objectives for using BP in space are: on the one hand, the opportunity to fabricate complex, multicellular, and 3D tissue models to investigate the effects of space conditions on cells and tissues on-site; on the other hand, there is the hope that BP once could provide tissue constructs for the medical treatment of injured or diseased astronauts. As skin is the tissue being mostly exposed to the environment, it also has the highest risk of being injured. In a study about traumatic injuries during long-duration spaceflight, those of the skin were reported to be among the most frequent and likely ones (Kirkpatrick et al., 2009). Together with the fact of impaired wound healing in space, the ability to be able to bioprint skin tissue might be of crucial importance for future manned space missions like lunar settlements or Mars exploration. BP therefore can help to increase the autonomy of the crew on long-term missions, and as the bioprinted constructs are already cellularized and ready-to-use, the wound healing (even when impaired because of the environmental factors) can be improved and accelerated.

It has been already demonstrated that human cells can be grown under altered gravity conditions thanks to rotary devices that allow the generation of 1G conditions in space (as rotatory vessels or centrifuge chambers) (Grimm et al., 2020). Therefore, if the BP technology can be adapted to work under space conditions (which is quite likely), the previous cell expansion and the posterior maturation of the printed tissues could be managed inside such devices. As maturation of the epidermal layer of bioprinted skin requires contact to air, cultivation under simulated gravity would help in providing the necessary air–liquid interface.

For applications in space flight, accessibility of all components is of utmost importance because of the very limited payload capacities. Therefore, it would be obvious to utilize autologous cells, isolated from an injured astronaut in case of the need for BP of a tissue for medical application. In addition, this would lead to an autologous skin graft, which is being preferred because of immunological compatibility. An interesting possibility would be to create cell

banks for (and from) the crew with healthy cells of the most interesting tissues, including mesenchymal stem cells, taken even before launch from the Earth. Also, 3D models of their organs and bone structures could be archived from CT/MRI (computed tomography/magnetic resonance imaging) data sets. Regarding materials, also the other components for bioink preparation could be provided on-site: this can be biopolymers like alginate, isolated from algae which could be cultivated in space—or constituents of human blood like whole plasma or fibrinogen which again could be derived from the astronauts themselves. It already could be demonstrated that human plasma and fibrinogen are suitable bioink components for skin BP (Cubo et al., 2017; Albanna et al., 2019).

Also the *in situ* BP approach, briefly explained above, is of interest for applications in space flight as it would provide a fast and easy opportunity to support wound healing without the necessity to further cultivate and mature the bioprinted constructs prior to deposition. Currently, the space company OHB is developing for the German Space Agency at DLR a handheld skin BP device, based on the extrusion printing principle, which shall be sent to the ISS at the end of the year 2021 for evaluation (DLR, 2020).

DISCUSSION AND CONCLUSIONS

BP is a tremendously developing field of research. Several BP technologies and a huge variety of suitable biomaterials for the printing of live cells have been developed and are available (Chimene et al., 2016; Gungor-Ozkerim et al., 2018; Li et al., 2020). Skin BP is one of the applications being already investigated in depth, and numerous studies have proven the applicability in principle, utilizing various BP technologies, cell types, and biomaterials. Within the available technologies of the additive manufacturing family, the most developed methods for BP are based on extrusion or drop-on-demand printing. Between these two, probably extrusion BP is more mature and easier to translate first into space because it presents a more basic mechanism and requires a smaller number of cells, as it works with cell-material suspensions. Therefore, also less time is required for cell expansion prior to the BP process when the cells are "diluted" in the respective bioinks. In addition, in extrusion BP more viscous bioinks can be utilized compared to inkjet BP that would present higher stability while printing in microgravity and for the post-printing process steps. Finally, such pasty cell-laden bioinks can be designed to be quite sticky which would facilitate the layer-by-layer assembly process under microgravity conditions and also allows *in situ* BP applications, i.e., direct deposition of such bioinks onto skin wounds (Cubo-Mateo et al., 2020).

The main advantages of utilizing BP technologies for fabrication of transplantable skin grafts can be summarized as follows:

- Compared to conventional tissue engineering approaches, BP can be done in a semi-automated manner which is beneficial when applied during space flight,
- Again compared to TE, the fabrication process in BP is faster and simpler as biomaterials and cells are deposited together whereas in TE first a scaffold has to be made which then is seeded with the different cell types, one after the other,
- BP provides better control concerning the dimensions and internal structure (e.g., thickness of dermal and epidermal layers) of the skin grafts,
- For every cell type included in the BP process, optimal biomaterials can be selected, e.g., mimicking the respective local ECM composition,
- In principal, BP allows manufacturing of complex tissue equivalents by integration of hair follicles, melanocytes, eccrine sweat and sebaceous glands, etc., or cell types supporting fast vascularization.

Initially, BP will be applied in space for the fabrication of more complex, multicellular, and 3D tissue models for research purposes, which mimic the native human tissues better than conventional cell cultures used so far. For this purpose, the international space agencies already have started to install such devices at the ISS. However, there is hope that the technologies will develop further so that once BP might be able to support medical treatments and help astronauts on far-distant space missions or in extraterrestrial human settlements, e.g., on the Moon, to survive. As the skin is the tissue mostly at risk to become injured and wound healing is known to be impaired in space, further developments of BP of the skin is believed to be an important topic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was partly funded by the German Space Agency at DLR (project #50WB 2032). We thank the Saxon State and University Library (SLUB) for covering the open access publication fees.

ACKNOWLEDGMENTS

This study has been performed in the framework of the Topical Team "3D Bioprinting of living tissue for utilization in space exploration and extraterrestrial human settlements" of the European Space Agency (ESA), coordinated by MG. The authors thank the ESA PLT-Space team (Tommaso Ghidini, Sandra Podhajsky, Klaus Slenzka, and Daniela Knickmann) and the team of the DLR-funded project "Handheld Bioprinter" (Michael Becker, Markus Braun, Nathanael Warth, and coworkers) for stimulating discussions.

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frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 07 February 2022
doi: 10.3389/fbioe.2021.724101

Gravity-Vector Induces Mechanical Remodeling of rMSCs *via* Combined Substrate Stiffness and Orientation

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *12 June 2021*
Accepted: *30 December 2021*
Published: *07 February 2022*

Citation:
Zhang C, Lü D, Zhang F, Wu Y, Zheng L, Zhang X, Li Z, Sun S and Long M (2022) Gravity-Vector Induces Mechanical Remodeling of rMSCs via Combined Substrate Stiffness and Orientation. Front. Bioeng. Biotechnol. 9:724101. doi: 10.3389/fbioe.2021.724101

Distinct physical factors originating from the cellular microenvironment are crucial to the biological homeostasis of stem cells. While substrate stiffness and orientation are known to regulate the mechanical remodeling and fate decision of mesenchymal stem cells (MSCs) separately, it remains unclear how the two factors are combined to manipulate their mechanical stability under gravity vector. Here we quantified these combined effects by placing rat MSCs onto stiffness-varied poly-dimethylsiloxane (PDMS) substrates in upward (180°), downward (0°), or edge-on (90°) orientation. Compared with those values onto glass coverslip, the nuclear longitudinal translocation, due to the density difference between the nucleus and the cytosol, was found to be lower at 0° for 24 h and higher at 90° for 24 and 72 h onto 2.5 MPa PDMS substrate. At 0°, the cell was mechanically supported by remarkably reduced actin and dramatically enhanced vimentin expression. At 90°, both enhanced actin and vimentin expression worked cooperatively to maintain cell stability. Specifically, perinuclear actin stress fibers with a large number, low anisotropy, and visible perinuclear vimentin cords were formed onto 2.5 MPa PDMS at 90° for 72 h, supporting the orientation difference in nuclear translocation and global cytoskeleton expression. This orientation dependence tended to disappear onto softer PDMS, presenting distinctive features in nuclear translocation and cytoskeletal structures. Moreover, cellular morphology and focal adhesion were mainly affected by substrate stiffness, yielding a time course of increased spreading area at 24 h but decreased area at 72 h with a decrease of stiffness. Mechanistically, the cell tended to be stabilized onto these PDMS substrates *via* β1 integrin–focal adhesion complexes–actin mechanosensitive axis. These results provided an insight in understanding the combination of substrate stiffness and orientation in defining the mechanical stability of rMSCs.

Keywords: substrate stiffness, orientation, mechanosensing, nucleus translocation, cytoskeletal remodeling, focal adhesion complex reorganization, cellular morphology

Abbreviations: 3D, three-dimensional; CD, cytochalasin D; CSK, cytoskeletal; ECM, extracellular matrix; FAC, focal adhesion complex; MSC, mesenchymal stem cell; PDMS, poly-dimethylsiloxane; SCs, stem cells.

INTRODUCTION

The stem cell niche, defined as the surrounding microenvironment of both the neighboring cells and the extracellular matrix (ECM), provides biochemical and biomechanical signals to regulate stem cell self-renewal and fate commitment (Lemischka and Moore, 2003; Moore Lemischka, 2006; Mitsiadis et al., 2007; Aichinger et al., 2012; Kordes and Haeussinger, 2013; Turksen, 2015). Physical or mechanical factors (ECM stiffness, mechanical force, topography, cell shape or colony sizes, and others) play a considerably important role in these processes (Discher et al., 2009; Li et al., 2011). It is well known that matrix stiffness directs stem cell fate specification, as it was seen that mesenchymal stem cells (MSCs) can differentiate into osteoblasts, myoblasts, and neurons on a substrate that mimics bone, muscle, and neural stiffness, respectively (Engler et al., 2006; Rowlands et al., 2008; Tse and Engler, 2011). Hereinto mechanosensing is initiated from the varied cell–ECM traction force induced by different substrate stiffness levels, which then alters the intracellular prestress and stem cell or nucleus stiffness and results in the mechanical remodeling of stem cells on their niches (Chowdhury et al., 2010; Swift et al., 2013; Buxboim et al., 2014; Harada et al., 2014). Evidently, these *in vitro* studies, by mimicking or replicating *in vivo* ECM stiffness on a planar substrate, open a window from a biomechanical or biophysical viewpoint for the mechanical remodeling of various types of stem cells.

Substrate orientation also regulates the mechanical remodeling of the cells. These studies are usually designed to elucidate how the gravity vector manipulates the gravisensing mechanisms for a plant or mammalian cell (Vassy et al., 2001; Morita, 2010)—for example, the spreading and mitosis of Chinese hamster ovary cells are sensitive to the change in gravity vector, and randomizing the direction of the gravity has no effects on the division orientation of the point-attached cell in a vertical plane (Helmstetter, 1997). Directed nucleolus sedimentation inside a *Xenopus* oocyte is dominant over thermal fluctuation, implying that the sedimentation of a relatively dense nucleus could initiate cell gravisensing (Feric and Brangwynne et al., 2013). While these static models of orientation change are helpful in elucidating cell mechanosensing, it is still unclear in this process what a role the extracellular microenvironment, such as ECM stiffness, plays and how it is correlated with substrate orientation alteration.

Mechanotransduction is crucial to understand the above-mentioned mechanosensing process. On one hand, substrate stiffness is well sensed by membrane-anchored integrin molecules. F-actin binds to matrix proteins at the focal plane *via* integrin-anchored focal adhesion complexes (FACs) as well as to myosin II elements inside the cell, which initiates extra-intracellular mechanotransduction (Giannone et al., 2007; Irianto et al., 2016). Meanwhile, there are substantial physical links between the nucleoskeleton and cytosolic actin, intermediate filament, or microtubule components (Dahl et al., 2004; Lammerding et al., 2004; Lammerding et al., 2006; Dahl et al., 2008) *via* the linker of nucleus and cytoskeleton (LINC) complex (Crisp et al., 2006; Dahl et al., 2008). These signaling pathways result in a major mechanotransduction axis from the ECM to the nucleus through cytoskeletal (CSK) remodeling. On the other hand, the gravitational force acting on or lost from the organelles is sensed by the cytoskeletons. Loss of gravity alters prestress in the cytoskeleton and is transmitted to the mechanosensitive structures of actin, intermediate filament, and microtubule (Vassy et al., 2001; Bershadsky et al., 2006; Meloni et al., 2011), suggesting that the CSK network could serve as the preferential candidate for intracellular gravisensing. This mechanical signaling can be transmitted though the interactions between F-actin and FACs and induces FAC remodeling to lead the cell adhered stably on substrate (Humphries et al., 2007), which is finally transmitted to the nucleoskeleton *via* LINC. Thus, both the substrate stiffness and orientation likely share the common mechanotransductive pathways, which is required to be identified in a combined approach.

Previously, we quantified how substrate stiffness and microtopography cooperatively direct the differentiation of rMSCs (Li et al., 2013) and maintain the stemness of mouse embryonic stem cells (Lü et al., 2014), suggesting that the CSK remodeling is one of key factors in these processes. Recently, we also elucidated how the substrate orientation affects the mechanical stability of an osteoblast-like cell, where the nucleus translocation due to density difference is mechanically supported by CSK remodeling and FAC reorganization (Zhang et al., 2017). Here we combined the substrate stiffness with substrate orientation mainly because the former is biologically relevant and the latter is a well-defined *in vitro* model. rMSC remodeling was systematically tested for stiffness-varied substrates in three orientations, and the related intracellular events were analyzed for biological homeostasis of the cells.

MATERIALS AND METHODS

Ethics Statement

All experiments involving the use of live animals were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all the protocols were approved by the CULA at the Institute of Mechanics, Chinese Academy of Sciences.

Cells and Reagents

Rat bone marrow-derived stem cells (rMSCs) were isolated from 3- to 4-week-old male Sprague–Dawley rats as described previously (Li et al., 2013). Briefly, the rats were sacrificed by cervical dislocation, and the femurs and tibias were then collected. The marrow was flushed out and blown into single cells by an injector, and the collected cells were added into L-DMEM medium (GE Healthcare Life Sciences, Logan, UT, United States) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, United States) and 1 ng/ml bFGF (R&D systems, Minneapolis, MN, United States). The cells were then maintained in a humidified incubator with 95% air/5% CO_2, 37°C, by refreshing the medium every 3 days. When grown up to 85–90% confluence, the cells were detached using 0.25% trypsin-EDTA for 1 min and passaged into T-25

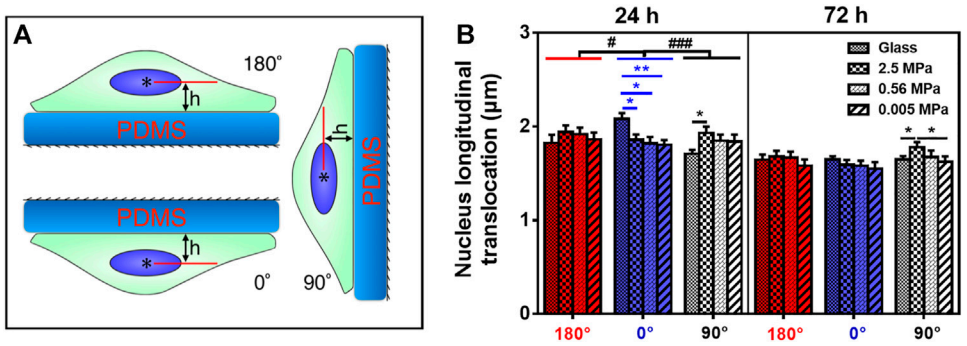

FIGURE 1 | Nucleus translocation of rat bone marrow-derived stem cells (rMSCs) on different stiffness in three oriented substrates. **(A)** Schematic of rMSCs placed onto poly-dimethylsiloxane (PDMS) substrate at 180°, 0°, or 90°. The angle is defined as the one between the outer normal vector of the substrate and the gravity vector in respective orientation, and *h* is the distance between the nucleus centroid (*) to the surface of the substrate. **(B)** Nucleus longitudinal translocation of rMSCs onto oriented glass or PDMS substrate with three stiffness substrates. Data were presented as mean ± SE of ~45 cells from three repeated experiments at 24 or 72 h. * or **, *t*-test, $p < 0.05$ or 0.01; # or ###, two-way ANOVA test for different groups with different stiffness substrates with cell orientation, $p < 0.05$ or 0.001.

flask by 1:3 ratio. This procedure was repeated three or four times to collect rMSCs with ~95% purity.

For CSK staining, FITC-conjugated phalloidin was from Enzo Life Science (Enzo Biochem, Farmingdale, NY, United States), anti-vimentin (Alexa Flour 647-conjugated) and anti-α-tubulin (Alexa Flour 555-conjugated) rabbit monoclonal antibodies (mAbs) were from Cell Signaling Technology (Danvers, MA, United States). For FAC staining, anti-vinculin rabbit mAbs and donkey-anti-rabbit anti-IgG secondary polyclonal antibodies (DyLight 594-conjugated) were from Abcam (Cambridge, Cambridgeshire, UK). For β1 integrin staining, anti-β1 integrin mouse mAbs was from Santa Cruz (Dallas, TX, United States), and the goat-anti-mouse anti-IgG secondary polyclonal antibodies (Alexa Fluor 594-conjugated) were from Abcam. Hochest 33342 for nucleus stain was from Invitrogen (Thermo Fisher Scientific, Waltham, MA, United States). Goat-anti-rat anti-CD11b, CD34, CD45, or CD90 mAbs were purchased from Santa Cruz (United States) and used as biomarkers for identifying the rMSCs as described previously (Li et al., 2013).

Fabricating PDMS Substrate

Poly-dimethylsiloxane (PDMS) was used to construct the soft gel onto the glass surface *via* soft contact lithography technique (Whitesides et al., 2001; Cheng and Guo, 2004). Briefly, Sylgard 184 (Dow Corning, United States) was used, and two components of a "base" and a "curing" agent were mixed in 10:1, 33:1, or 70:1 ratio (v/v). The mixture was then poured uniformly on the top of glass coverslip. After additional degassing for 12 h, the PDMS was cured at 65°C for 3 h and at room temperature (~25°C) for 12 h. The cured PDMS substrate so obtained was then adhered onto the pre-processed, dustless coverslip (Corning, Corning, NY, United States) and treated with O_2 plasma for 1 min to make the surface of the substrate hydrophilic. In the current work, a planar PDMS substrate was used, with a stiffness of 2.5, 0.56, or 0.005 MPa (Wang, 2011; Yang et al., 2011; Sun et al., 2014), while the glass coverslip for tissue culture was used as control, with a stiffness of ~70 GPa, only for normalizing those data obtained from PDMS substrates with different stiffness levels.

Substrate-Oriented Cell Culture

Like those methods described previously (Li et al., 2010; Zhang et al., 2017), rMSCs were placed on three oriented glass coverslips in the presence or absence of PDMS cushion with different stiffness levels (~2 mm thickness) on the top. Briefly, the cells were seeded on either sterile glass or stiffness-varied PDMS substrate pre-coated with collagen I at 4 μg/cm^2 overnight. After 24-h pre-growth for steady cell adherence and spread, the substrate was transferred to a custom-made holder and orientated at 180°, 0°, or 90°, respectively (the angle between the substrate outer normal vector and the gravity vector; **Figure 1A**) for additional 24- or 72-h culture. To reach a similar confluence with the majority of isolated cells at given durations, the cells were seeded at a density of 1×10^2 or 3×10^2 cells/cm^2 for an additional 72 or 24 h. The effect of hydrostatic pressure among the three orientations was minimized and negligible due to the delicate protocol (Li et al., 2010; Zhang et al., 2017). Triplicate repeats were conducted in each orientation.

Nuclear and CSK Staining and Parameter Estimation

Morphological change and cytoskeletal expression were determined by immuno-cytochemistry. At given durations, cells grown on oriented glass or PDMS were rinsed with phosphate-buffered saline (PBS), fixed into 4% paraformaldehyde within 1 min for 15 min, and washed and permeated with 0.1% Triton 100-X for 10 min at room temperature. The collected cells were incubated in 1% bovine serum albumin/PBS for 60 min at 37°C to block non-specific staining. Filamentous actin, vimentin, and tubulin were stained with a mixture of phalloidin at 5 μg/ml, anti-vimentin mAbs at 1: 800, and anti-tubulin mAbs at 1:50 for 60 min at 37°C. The cells were then washed and incubated with Hoechst 33342 for 10 min

to stain the nuclei. Images of the stained cells were examined using a laser confocal microscope (LSM 710, Carl Zeiss AG, Oberkochen, Germany) with a 63× oil-immersion objective at a slicing height of 0.65 μm in a stepwise interval of 0.322 μm for three-dimensional (3D) imaging. Triplicate repeats were done in each case.

Several parameters were obtained from these images: 1) Nucleus translocation, defined as the longitudinal distance between the nucleus centroid and the substrate, was determined by Imaris software (Bitplane, Zurich, Switzerland) through the 3D reconstructed images of stained actin and nucleus; 2) CSK expression was quantified as the mean relative fluorescence intensity (arbitrary unit or AU) of stained actin, vimentin, or tubulin using ImageJ software (National Institutes of Health, Bethesda, MD, United States). To compare these values in distinct orientations in repeated experiments, a calibration curve was built at systematically varied laser power and PMT gain for the same fluorescent probes. To further test the expression difference between the two types of substrates, a ratio of each value onto PDMS to that onto glass was calculated in all the cases. Perinuclear actin stress fibers and vimentin cords, defined previously (Zhang et al., 2017), were used for quantitative analysis; 3) Anisotropy of perinuclear actin, defined in a recent protocol (Boudaoud et al., 2014), was also adopted to quantify the behavior of this type of fibrillar structures. Here a residual eigenvalue, q, calculated from the pixel intensity array of a given region of interest, denotes a completely isotropic fibril structure when $q = 0$ or a completely anisotropic fibril structure when $q = 1$. Noticing that the fibrillar isotropy and anisotropy respectively represent the randomized and aligned actin network, this eigenvalue was estimated in the region of the nucleus contour; 4) Cell morphological analysis was simply conducted on the cell contour identified by stained actin. Cell projected area, circularity (= $4\pi A$/perimeter^2), and aspect ratio (= long-axis length/short-axis length) was determined using ImageJ.

Staining and Functional Blocking of Mechanosensitive Molecules

FAC immunostaining was similar to the procedure detailed above for cytoskeleton staining. Briefly, after washing and fixing, the cells were co-incubated with phalloidin at 5 μg/ml and anti-vinculin mAbs at 1:200 for 60 min at 37°C, rinsed and incubated with secondary antibodies at 1:200 for 60 min at 37°C, and finally incubated with Hoechst 33342 for 10 min. FACs were visualized using confocal microscopy by collecting 0.65-μm-thickness information at the focal plane. The number of total FACs was counted for ~45 cells in each case, and the area of total FACs was calculated, respectively, using Matlab software. To exclude the potential impact of a different cell size, the resulting FAC number and area for cells were normalized per 1,000 μm^2 cell area. Similar protocol was applied for β1 integrin immunostaining.

To elucidate the potential mechanotransductive pathways, rMSCs were incubated with anti-β1 integrin, blocking mAbs at 1.5 μg/ml for 1 h per 24 h (Anguiano et al., 2017) in a total of 72 h of culture (Abcam, Cambridge, United Kingdom) or with 50 ng/ml F-actin depolymizer cytochalasin D (Sigma-Aldrich) for 24 h.

RNA Extraction and qPCR Test

The cultured rMSCs at 72 h were collected on various stiffness levels and orientations. Their total RNA was harvested using RNA extraction kit (Tiangen, Beijing, China) with an in-column DNase digestion step, according to the instructions of the manufacturer. The corresponding cDNA was generated using ReverTra Ace-a (Toyobo, Osaka, Japan) with 1 μg of RNA per reaction in a total volume of 20 μl. A reverse transcriptase-polymerase chain reaction was carried out using GoTaq® qPCR Master Mix with a two-step method as per the user manual (Promega, Madison, WI, United States) and then measured by a quantitative real-time amplification system (QuantStudio 7, Thermo Fisher). The optimized primers for PCR tests are summarized in **Supplementary Table S1**.

Statistical Analysis

Three-way ANOVA was performed to test the statistical significance of differences among the three factors of stiffness, orientation, and duration. Two-way ANOVA test, followed by Holm–Sidak test, was used to test the statistical significance of differences in the measured parameters between the two factors of orientation and duration or of orientation and stiffness. For comparisons between any two groups, Student's *t*-test or Mann–Whitney test was also performed upon data passing the normality test or not.

RESULTS

Differential Regulation of Substrate Orientation Onto PDMS Substrate

Different cell types present diverse phenotypes together with varied ECM stiffness in a pericellular microenvironment. Here we first tested nucleus longitudinal translocation in rMSCs onto typical PDMS substrates when glass coverslip served as the control (**Figure 1A**). The orientation dependence of nucleus translocation for rMSCs onto glass was presented with a higher value at 0° for 24 h (**Figure 1B**), consistent with that previously described for MC3T3-E1 cells (Zhang et al., 2017). When rMSCs were placed onto 2.5 MPa PDMS, nucleus translocation was relatively higher at 90° for 24 or 72 h (**Figure 1B**). These data indicated that the orientation dependence of nuclear translocation likely appeared for rMSCs on 2.5 MPa PDMS in a time-dependent manner. We further tested the combined impacts of substrate stiffness and orientation and found that both factors presented a significant difference in regulating nucleus longitudinal translocation at 2.5 MPa PDMS (symbol # in **Figure 1B**). Specifically, the translocation onto 2.5 MPa PDMS was lower at 0° for 24 h but higher at 90° for 24 and 72 h compared with those onto glass, potentiating the different mechanisms of substrate stiffness in regulating rMSC stability onto an oriented substrate.

It is well known that PDMS and glass substrates have distinctive properties in both elasticity and chemistry. Thus,

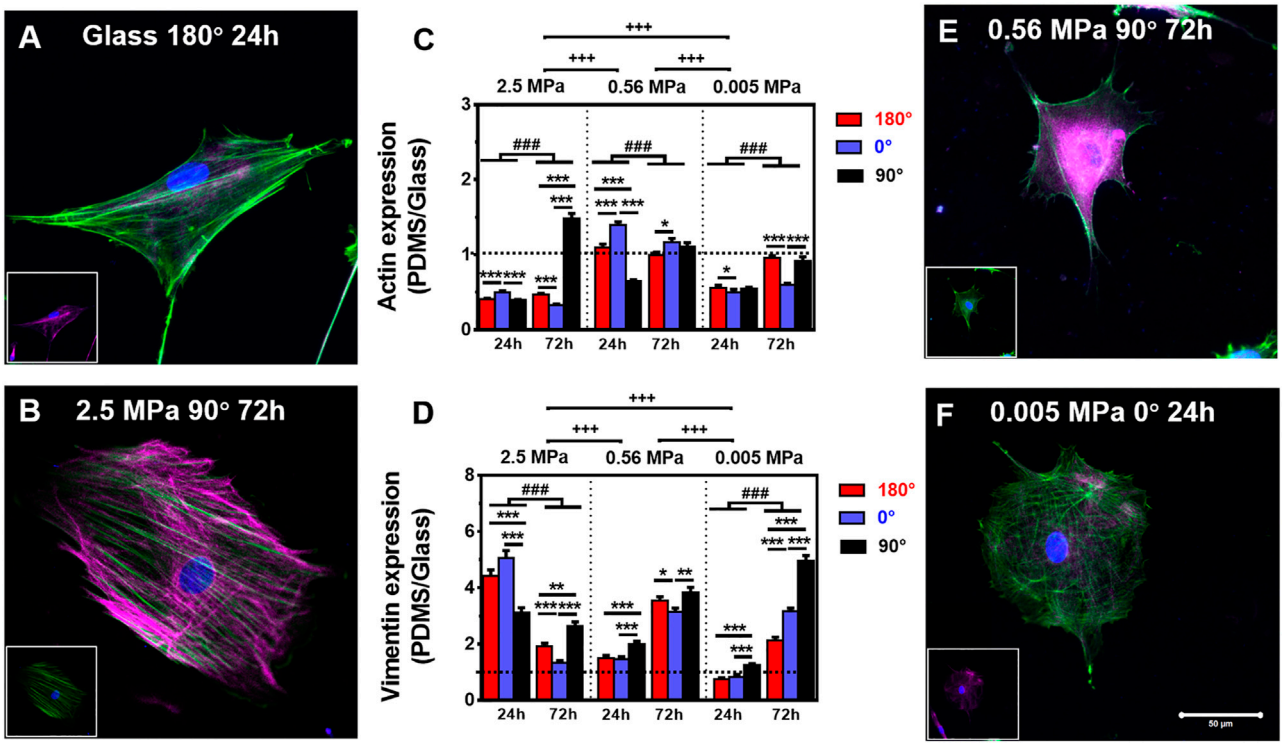

FIGURE 2 | Comparison of actin (green) and vimentin (magenta) expressions. **(A,B,E,F)** Typical merged cytoskeletal images for cells placed onto glass: **(A)** 180°, 24 h, 2.5 MPa, **(B)** 90°, 72 h, 0.56 MPa, **(E)** 90°, 72 h, or 0.005 MPa, **(F)** 0°, 24 h, poly-dimethylsiloxane (PDMS). The insert in each panel denotes the isolated cytoskeletal image for clarity. Bar = 50 μm. **(C,D)** Relative fluorescent intensity onto PDMS normalized to that onto glass was plotted for actin **(E)** or vimentin **(F)** in three orientations and presented as mean ± SE for ~45 cells from three repeated experiments at 24 or 72 h. *, **, or ***, *t*-test, $p < 0.05$, 0.01, or 0.001; $^{\#\#\#}$, two-way ANOVA test for different groups with different time with cell orientation, $p < 0.001$; $^{+++}$, three-way ANOVA test for differences among stiffness, orientation, and duration, $p < 0.001$.

we added two more PDMS stiffness levels to further test their mechanical remodeling under gravity vector. On either 0.56 or 0.005 MPa PDMS (**Figure 1B**), the rMSCs presented no orientation dependence of their nucleus translocation for 24 or 72 h, which is different from the above-mentioned observations onto 2.5 MPa PDMS. This was further verified by testing the statistical differences for combined effects of stiffness and orientation. No significant distinctive translocation in three orientations was observed on these softer PDMS ($^{\#}p \geq 0.408$) even with slightly lower values at 0° for 24 or 72 h. Taken together, these results indicated that the orientation dependence of nuclear translocation is also stiffness-dependent and only presented onto stiffer PDMS substrate.

Global CSK Expressions on PDMS Substrates

It was indicated previously that the orientation dependence of nuclear translocation is mainly relying on mechanical pathways *via* cytoskeleton remodeling and focal adhesion reorganization (Zhang et al., 2017). Next, we tested how CSK remodeling is associated with differential nucleus longitudinal translocation onto 2.5 MPa PDMS. Compared with high actin expression (**Figure 2A**) but low vimentin expression (insert in **Figure 2A**) onto glass, these CSK protein expressions seemed reversed onto 2.5 MPa PDMS, that is, with low actin expression but high vimentin expression at 24 h. Quantitative analyses further supported these observations. Indeed actin expression normalized to the one onto glass was ~50% reduced onto 2.5 MPa PDMS in all cases, except of the one at 90° for 72 h (**Figure 2B** and insert for vimentin), where it yielded ~1.5-fold higher onto 2.5 MPa PDMS (**Figure 2C**). Intriguingly, the exceptional difference in extremely high actin expression was positively related to the higher nucleus translocation at 90° (dotted bars in **Figure 1B**). By contrast, vimentin expression is ultimately reversed onto PDMS, that is, 3.0–5.0-fold higher for 24 h or 1.2–2.5-fold higher for 72 h in all cases (**Figure 2D**). These data indicated that placing rMSCs onto 2.5 MPa PDMS reduced the actin expression but fostered the vimentin expression as compared with those onto glass, implying that the mechanical stability of rMSCs could be achieved relying more on vimentin and less on actin onto 2.5 MPa PDMS or more on actin and less on vimentin onto stiff glass. More importantly, the differential distributions of actin and vimentin onto 2.5 MPa PDMS and glass were consistent with those above-mentioned orientation dependences of nucleus longitudinal translocation. Compared with those onto glass, the low nucleus translocation onto 2.5 MPa PDMS at 0° for 24 h

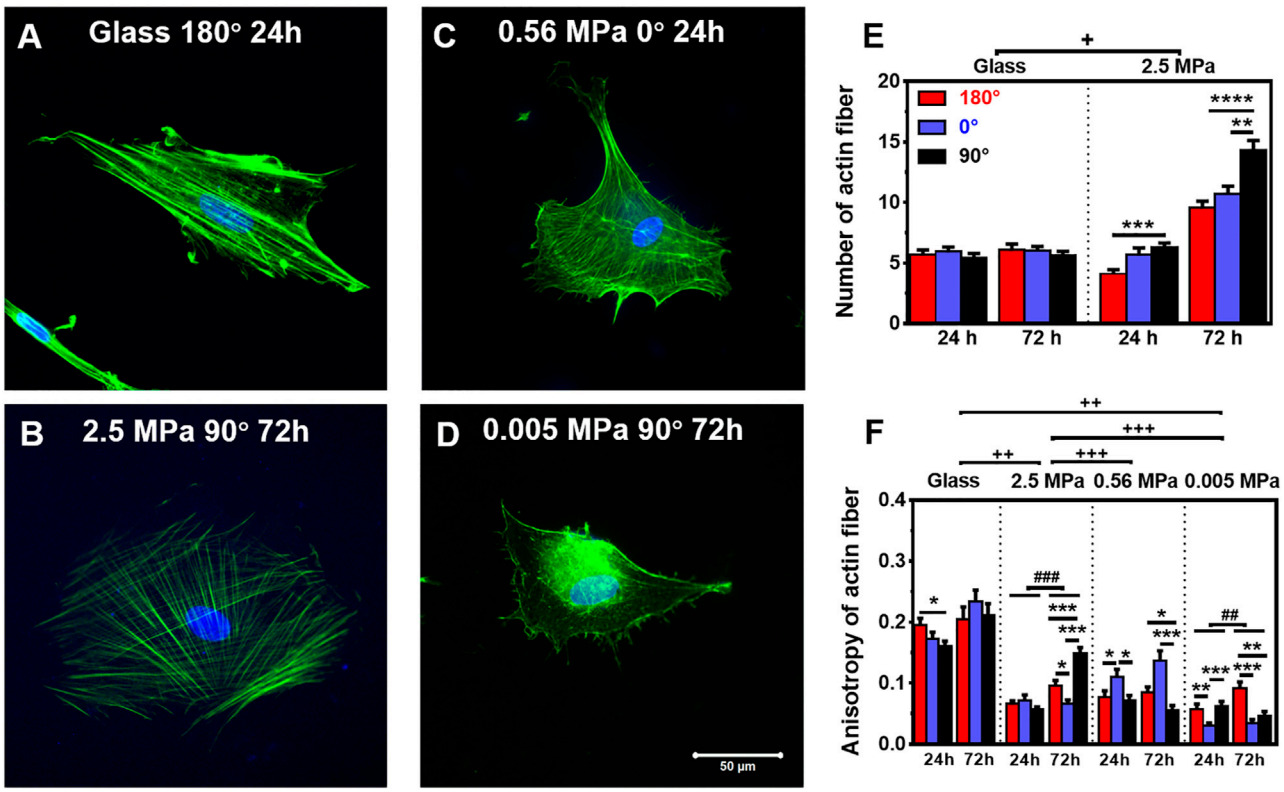

FIGURE 3 | Structure and number of actin stress fibers. **(A–D)** Typical images of perinuclear actin fibers onto glass **(A)** 180°, 24 h, 2.5 MPa, **(B)** 90°, 72 h, 0.56 MPa, **(C)** 0°, 24 h, or 0.005 MPa, **(D)** 90°, 72 h poly-dimethylsiloxane. Bar = 50 μm. **(E,F)** Number **(E)** and anisotropy **(F)** of stress fibers onto the two types of substrates at 24 or 72 h. Here the number was defined previously (Zhang et al., 2017), and the anisotropy term was adopted from Boudaoud *et al.* (2014) (as referred in "Materials and Methods"). Data were presented as mean ± SE for ~45 cells from three repeated experiments in three orientations. *, **, or ***, *t*-test, $p < 0.05$, 0.01, or 0.001; ## or ###, two-way ANOVA test for different groups with different time with cell orientation, $p < 0.01$ or 0.001; +, ++, or +++, three-way ANOVA test for differences among stiffness, orientation, and duration, $p < 0.05$ 0.01 or 0.001.

was associated with a relatively low actin and high vimentin expression, suggesting that high vimentin expression at 0° stabilizes the location of the nucleus when actin is reduced significantly by a short duration. By contrast, high nucleus translocation at 90° for 72 h was associated with high actin and high vimentin expressions, indicating that long-duration maintenance at 90° needs more cytoskeletons to resist the nucleus translocation or hold the nucleus steadily (also seen in those perinuclear vimentin distributions below). Additionally, the normalized tubulin expression fluctuated around unity (0.7–1.3) in all cases (**Supplementary Figure S1**), implying that tubulin contributes much less to this differential mechanism between the two types of substrates. Collectively, the orientation difference of nucleus translocation and global CSK presentation exists onto 2.5 MPa PDMS, with high vimentin expression at 0° for 24 h or high actin and vimentin expressions at 90° for 72 h. Meanwhile, those absolute fluorescence intensities of cytoskeletal protein expression also supported the above-mentioned observations using normalized ones. Onto 2.5 MPa PDMS, low actin, high vimentin, and low tubulin expressions were observed in three orientations for 24 or 72 h, even with a few exceptional cases of high actin expression at 90°, indifferent vimentin expression at 0°, and reversely high tubulin expression at 180° or 0° (**Supplementary Figure S2**).

We further tested global CSK expressions on softer PDMS substrates. On 0.56 and 0.005 MPa PDMS, CSK protein expressions presented different orientation-dependent patterns from the one on 2.5 MPa (**Figures 2E,F** and inserts). On 0.56 MPa PDMS, the cells yielded a high actin expression at 0° for 24 or 72 h, all of which were comparable with those on glass except of one case at 180° for 24 h (**Figure 2C**). By contrast, vimentin expression was relatively high at 90° for 24 or 72 h (**Figure 2D**) with 3.1–3.8-fold enhancement than those onto glass, consistent with the high nuclear translocation at 90° at long duration (*cf.* **Figure 1B**) and also implying a compensatory role of vimentin to support nucleus stability. On 0.005 MPa PDMS, the cells presented a relatively low actin expression, especially at 0° for 24 or 72 h, compared to those onto glass (**Figure 2C**). Vimentin expression was still lowered for 24 h but enhanced for 72 h especially at 90° (**Figure 2D**).

Finally, we tested typical mechanosensitive gene expressions at 72 h. Data indicated that the expression of actin, vimentin, or α-tubulin was indifferent on varied substrate stiffness and

TABLE 1 | Fractioned number of rMSCs with branched perinuclear actin fibers.

	Glass			2.5 MPa PDMS		
24	180°	0°	90°	180°	0°	90°
	3/45	2/45	2/45	6/46	10/45	13/45
72	3/45	5/45	3/45	24/45	25/45	36/45

orientation, implying that the cytoskeleton is favored to maintain their gene level in a conservative way on the current settings (**Supplementary Figure S3**).

Distinct Distributions of Perinuclear Cytoskeletons Onto PDMS Substrate

Not only the global presentation of actin within entire cell but also its localized distribution at the vicinity of the nucleus is crucial in manifesting CSK remodeling and maintaining nucleus stability. We further compared the distribution of perinuclear actin stress fibers onto glass or PDMS substrate. The fibers are likely uniformly aligned with high intensity onto glass (**Figure 3A**) but randomly oriented with low intensity onto 2.5 MPa (**Figure 3B**) or even unmeasurable onto 0.56 (**Figure 3C**) and 0.005 MPa (**Figure 3D**) PDMS, presenting a significant difference in perinuclear fibers between the two types of substrates. This observation was confirmed by the quantitative analyses that the number of perinuclear fibers was higher onto PDMS than those onto glass for 72 h (**Figure 3E**). At this duration, the fiber number onto 2.5 MPa PDMS was extremely higher at 90°, further supporting the consistency between global actin expression and nucleus translocation onto PDMS. We also compared the anisotropy of perinuclear fibers on all three PDMS substrates using the residual eigenvalue described previously (Boudaoud et al., 2014). It yielded lower values for cells onto PDMS (~0.05–0.15) than those onto glass (~0.15–0.25) in three orientations at two durations (**Figure 3F**), consistent with the above-mentioned observations of randomized fibers onto PDMS and aligned fibers onto glass from confocal images (**Figures 3A,B**). We also counted the number of cells with branched perinuclear actin network from the total cells observed. The fractioned number was again increased with time onto either substrate and yielded higher values onto 2.5 MPa PDMS than those onto glass (**Table 1**), further confirming the occurrence of high randomization or low anisotropy of the actin network for the cells onto PDMS. Similar to those orientation dependences of global actin expression (**Figure 2C**) and perinuclear fiber presentation (**Figure 3E**), the number of actin fibers was higher at 90° than that at 180° or 0° onto the same PDMS substrate for 72 h. These results implied that the relatively branched and stronger fibers are favorable in this orientation to maintain the nucleus stability. Taken together, mechanical support for rMSC stability is mostly attributed to the perinuclear stress fibers with a large number, low anisotropy, and low intensity onto PDMS as compared to those with a small number, high anisotropy, and high intensity onto glass. Onto a softer PDMS substrate at 0.56 MPa, a large number of tiny actin filaments (**Figure 3C**) tended to take over those stress fibers across the nucleus that appeared onto glass, with a relatively high expression and anisotropy but low mechanical support to nucleus translocation (**Figure 3F**). This substrate seemingly served as a transition one between the 2.5 and 0.005 MPa PDMS substrates since there was no visible filament with quite low anisotropy values onto 0.005 MPa PDMS (**Figure 3F**).

We also compared the perinuclear vimentin cords onto the two types of substrates. Onto 2.5 MPa PDMS (those images at 0.56 and 0.005 MPa were not able to be reconstructed for quantification), the cords were formed surrounding the nucleus (**Figure 4A**). The number of vimentin cords yielded higher values at 90° for 24 or 72 h (**Figure 4B**), partially supporting the orientation dependence of nucleus translocation and global vimentin expression at 90° for 72 h. By contrast, few vimentin cords were visible onto glass (**Figure 4B**). As indicated, the distribution of vimentin cord number was narrowed down to 0 or 1 onto glass but centered around 2 or 3 onto PDMS (**Supplementary Figure S4**). Moreover, vimentin onto PDMS tended to be dispersedly distributed from the nucleus to the cell edge, specifically extending into those lamellipodia (**Figure 4A**) where the actin presentation is low (*cf.*, **Figure 2B**). These results implied that the vimentin network also provided structural bases for supporting the cell as actin does.

FAC Reorganization on PDMS Substrate

Focal adhesion complex is required to anchor the cell onto the substrate mechanically. Thus, we compared the FAC reorganization onto glass (**Figure 5A**) or 2.5 (**Figure 5B**), 0.56 (**Figure 5C**), or 0.005 (**Figure 5D**) MPa PDMS in three orientations at two durations. Global differences were again found in normalized number (**Figure 5E**) and area (**Figure 5F**) of total FACs between the two types of substrates, presenting lower values onto PDMS (especially on softer substrates with 0.56 and 0.005 MPa) than those onto glass. Meanwhile, both the values of FAC number and area were higher at 0° onto glass for 24 h, consistent with the orientation dependence found for MC3T3-E1 cells (Zhang et al., 2017). By contrast, the value was indifferent onto three PDMS substrates in three orientations at two durations, implying that FAC formation happens in a stiffness-dependent but orientation- and time-insensitive manner onto these relatively soft substrates. This should not be surprising since the mechanical strength of the existing FACs is sufficiently enough to stabilize the cells (Zhang et al., 2017).

Stable Cell Morphology on the Substrates

Lastly, we compared the morphological alterations of rMSCs since the cell is stabilized onto the substrate *via* reorganized FACs. Here the cell contour was identified by actin staining images (**Supplementary Figure S5**), and the projected area, circularity, and aspect ratio were then determined on three PDMS and glass substrates. Global differences of the three parameters were observed between the two types of substrates or the two durations (**Figure 6**). At 24 h, there was a striking unanimity in all three orientations, that is, the area gradually increased with decreased stiffness, the circularity onto PDMS was

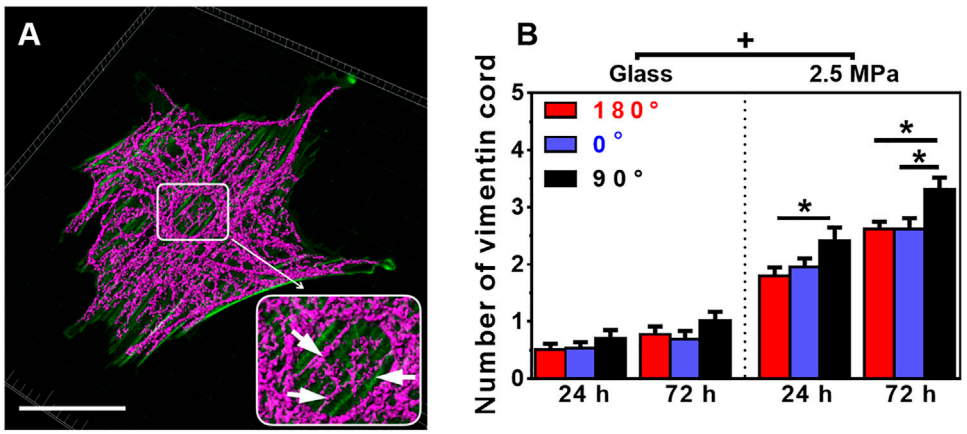

FIGURE 4 | Structure and number of vimentin cords. **(A)** Typical images of perinuclear vimentin cords (arrows in the insert) onto poly-dimethylsiloxane (PDMS) substrate (0°, 72 h). Bar = 50 μm. **(B)** The number of vimentin cords onto 2.5 MPa PDMS and glass, defined previously (Zhang et al., 2017), was compared in three orientations. Data were presented as mean ± SE for 45 cells from three repeated experiments at 24 or 72 h. *, *t*-test, $p < 0.05$; $^{+}$, three-way ANOVA test, $p < 0.05$.

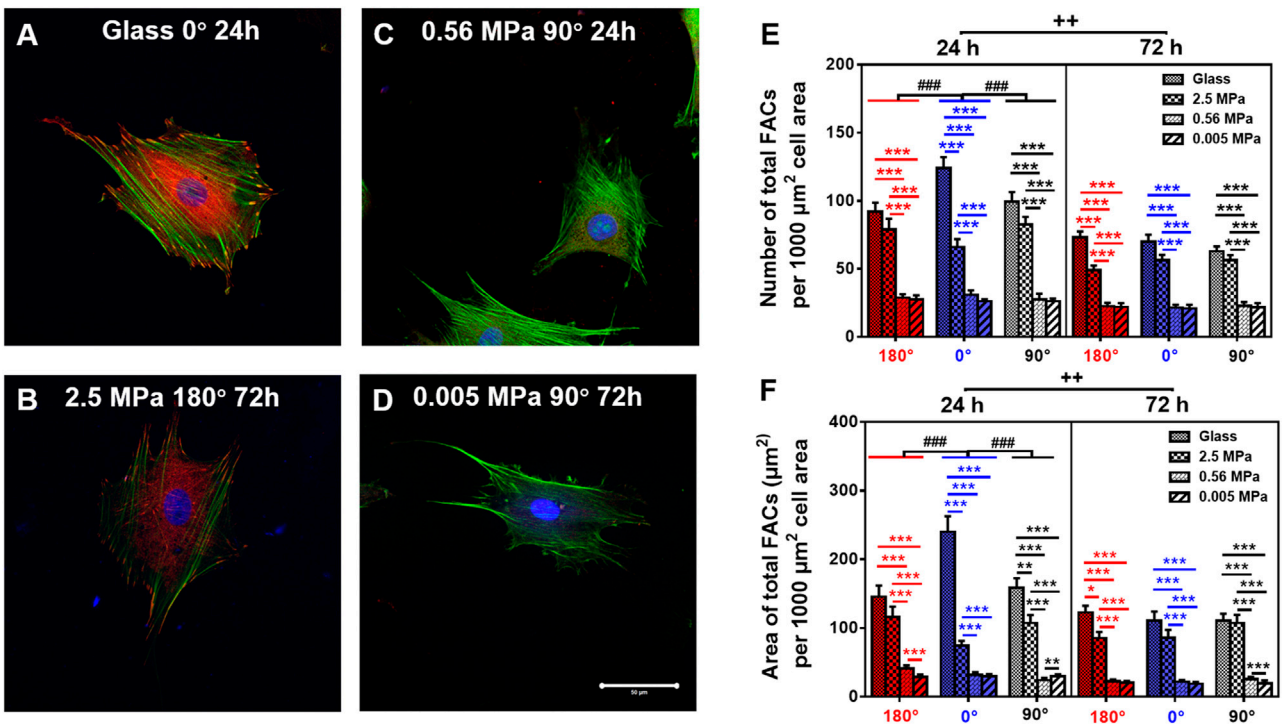

FIGURE 5 | Comparison of focal adhesion complex (FAC) (green, actin; red, vinculin) formation. **(A–D)** Typical images for cells placed onto glass **(A)** 0°, 24 h, 2.5 MPa, **(B)** 180°, 72 h, 0.56 MPa, **(C)**, 90°, 24 h, or 0.005 MPa, **(D)**, 90°, 72 h poly-dimethylsiloxane. Bar = 50 μm. **(E,F)** The number **(E)** and area **(F)** of total FACs (normalized per 1,000 μm^2 cell area) for cells onto the four different stiffness of substrate in three orientations were presented as mean ± SE of ~45 cells from three repeated experiments at 24 or 72 h. *, **, or ***, *t*-test, $p < 0.05$, 0.01, or 0.001; $^{\#\#\#}$, two-way ANOVA test for different group with different time with cell orientation, $p < 0.001$; $^{++}$, three-way ANOVA test for differences among stiffness, orientation, and duration, $p < 0.01$.

slightly higher at 0° but comparable at 180° or 90°, and the aspect ratio on PDMS was significantly lower in all three orientations. These data indicated that the cells tended to become less long and narrow onto PDMS even of a similar size with those onto glass. At 72 h, both the area and circularity were relatively higher, but the aspect ratio was lower, onto 2.5 MPa PDMS; while the area gradually decreased, the circularity was higher, but the aspect ratio was maintained onto 0.56 and 0.005 MPa PDMS, suggesting

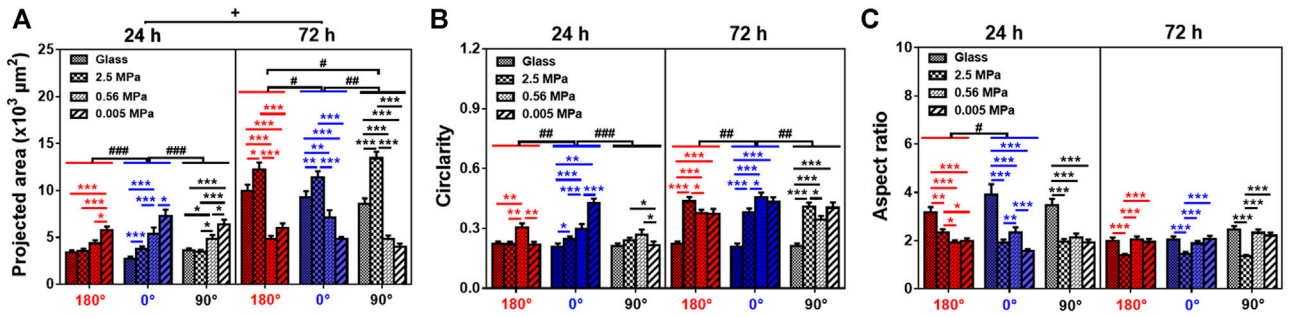

FIGURE 6 | Morphological change of rat bone marrow-derived stem cells (rMSCs). Area **(A)**, circularity **(B)**, and aspect ratio **(C)** of rMSCs onto glass or 2.5, 0.56, or 0.005 MPa poly-dimethylsiloxane in three orientations. Data were collected from those images for cell contour (cf. **Supplementary Figure S5**) and presented as mean ± SE of ~45 cells from three repeated experiments at 24 or 72 h. *, **, or ***, *t*-test, $p < 0.05$, 0.01, or 0.001; $^{\#}$, $^{\#\#}$, or $^{\#\#\#}$, two-way ANOVA test for different groups with different time with cell orientation, $p < 0.05$, 0.01, or 0.001; $^{+}$, three-way ANOVA test for differences among stiffness, orientation, and duration, $p < 0.05$.

that the cells became large in size with a spherical shape at 2.5 MPa but small in size with a spherical shape at 0.56 and 0.005 MPa. It was also noted that the decrease of the area at 72 h onto 0.56 and 0.005 MPa PDMS was correlated to the high vimentin increase (*cf.* **Figures 2D**, **4B**). Additionally, the area and circularity were increased, but the aspect ratio was decreased with time for the cells onto PDMS, similar to the time-dependent morphological alterations onto glass except of the case of time-independent cell circularity. Finally, stiffness dependence and orientation independence were found in cell morphology, consistent with our previous observations for MC3T3-E1 cells (Zhang et al., 2017).

Regulation of Cellular Mechanotransduction

The above-mentioned results indicated that optimizing substrate mechanics and orientation represents a critical step for maintaining the efficient longitudinal translocation of cell nucleus. To elucidate the mechanotransductive pathways involved in affecting rMSC nucleus longitudinal translocation, rMSCs grown typically at 90° on 2.5 MPa PDMS for 72 h were incubated with blocking mAbs against β1 integrin or F-actin depolymerizer cytochalasin D. The results indicated that β1 integrin expression was significantly decreased, and the fluorescence dots originally existing between rMSCs and the substrate in normal control (**Figure 7A**) disappeared after blocking (**Figure 7B**), which is positively correlated with the reduced nucleus longitudinal translocation (**Figure 7D**). Meanwhile, vinculin expression was lowered (**Figure 7C**), the number (**Figure 7E**) and area (**Figure 7F**) of total FACs were significantly reduced, and actin stress fibers became smaller and thinner (lower panels in **Figures 7A–C**). Similar observations were found by treating F-actin with cytochalasin D, resulting in remarkably reduced nucleus longitudinal translocation (**Figures 7G,H**).

To further test if the orientation effect is reversible, the rMSCs originally placed at 90° for 72 h were re-placed horizontally for an additional +24 or +72 h. The results indicated that the nucleus longitudinal translocation was still visualized at +24 h but significantly lower at +72 h, implying that, in addition to substrate stiffness, substrate orientation is also involved in maintaining the nuclear longitudinal translocation of rMSCs and that this orientation effect seemed to be reversible (**Figure 7I**). Taken together, these results suggested that the typical integrin–FACs–actin mechanotransductive axis plays a key role in regulating rMSC nucleus longitudinal translocation in response to substrate stiffness and orientation (**Figure 7J**).

DISCUSSION

In the current work, we attempted to elucidate the mechanical remodeling of rMSCs by integrating two biophysical factors of substrate stiffness and orientation. In contrast to those previous works designed for understanding their respective contributions, these combinations initiated the distinct nucleus longitudinal translocation in edge-on orientation at specific durations when the cells were placed onto stiffness-varied PDMS substrates. Not only these differences came from the global expressions of lowered actin and enhanced vimentin over the entire cell region onto PDMS but also they were attributed to the formation of isotropic tiny actin stress fibers on the softer PDMS substrates. Meanwhile, the low number or area of FACs was sufficient to anchor the cell stably onto the PDMS substrates. As a whole, significant differences were found among three PDMS stiffness, not only in nucleus translocation but also in CSK remodeling and FAC reorganization in their respective orientation-dependent patterns. On stiff PDMS, the orientation dependence of nuclear longitudinal translocation is presented *via* cytoskeletal remodeling and focal adhesion reorganization, while the soft PDMS tends to regulate cell morphology, spreading, and focal adhesion formation without visible orientation dependence of nuclear translocation (**Figure 8**).

Cellular mechanosensing to these combined microenvironments is biologically relevant since these stiffness and orientation are usually coupled together physiologically. *In vivo*, MSCs are required to be activated and transferred from the soft bone marrow to stiff, fibrotic regions for tissue regeneration and repair (Discher et al., 2009). In *in vitro* studies, substrate

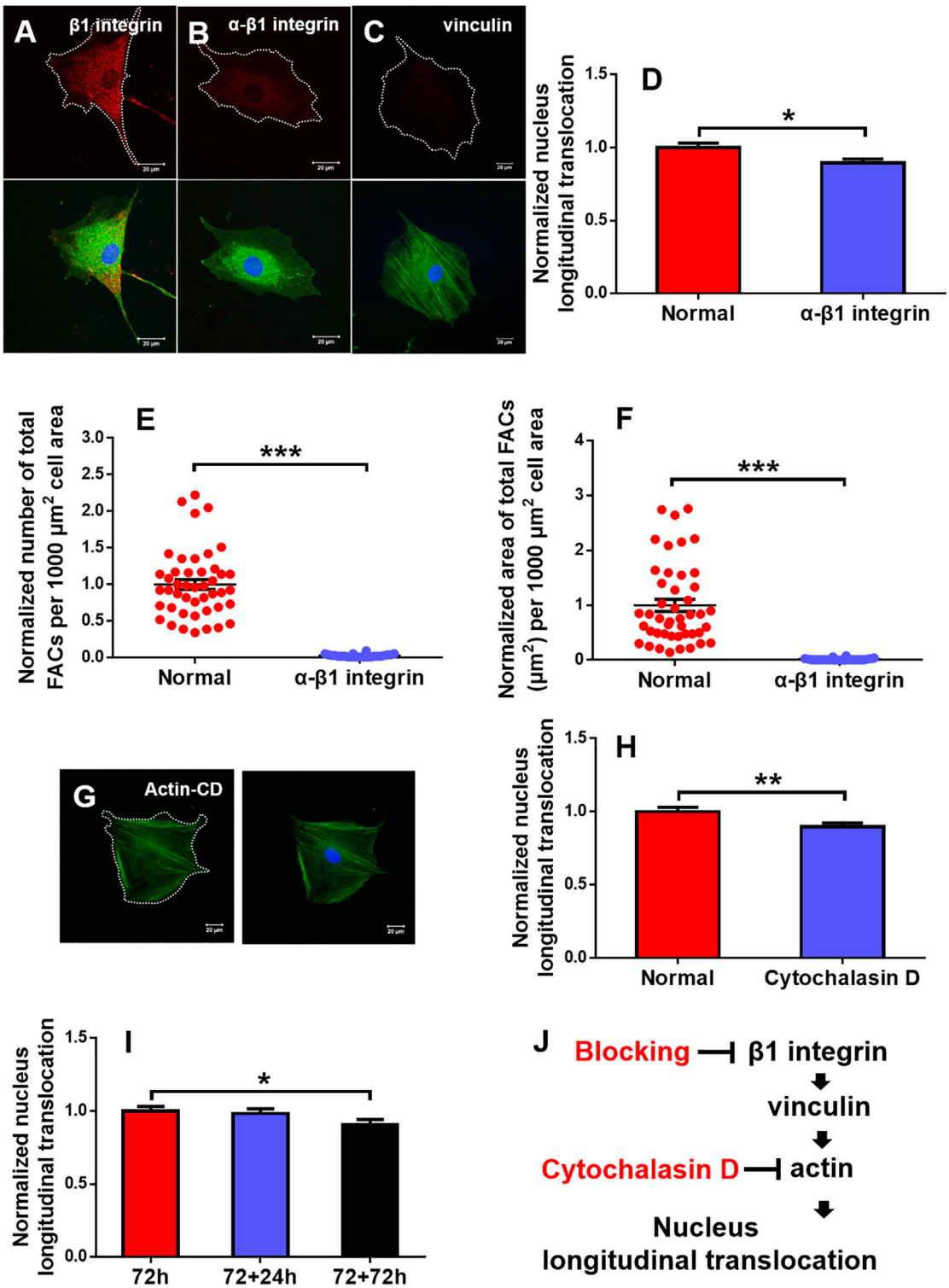

FIGURE 7 | Regulation of mechanotransductive pathways. Typical immunofluorescence staining of β1 integrin **(B)** (red), vinculin **(C)** (red), and actin **(G)** (green; blue, cell nucleus) was presented at 90° on 2.5 MPa poly-dimethylsiloxane (PDMS) for 72 h after β1 integrin function blocking when the one without β1 integrin blocking served as control **(A)** or cytochalasin D disturbed F-actin **(G)**. Bar = 20 μm. The nucleus longitudinal translocation was normalized to control for β1 integrin blocking **(D)** or cytochalasin D disturbed F-actin **(H)** at 90° on 2.5 MPa PDMS for 72 h. The number **(E)** and area **(F)** of total FACs were normalized to their respective controls. Nucleus longitudinal translocation for additional 24 and 72 h at 90° on 2.5 MPa PDMS was normalized to the one for 72 h **(I)**. *, **, or ***, *t*-test, $p < 0.05$, 0.01, or 0.001. Schematic of cellular mechanotransductive pathways. Inhibitors of key elements were depicted in red **(J)**. CD, cytochalasin D.

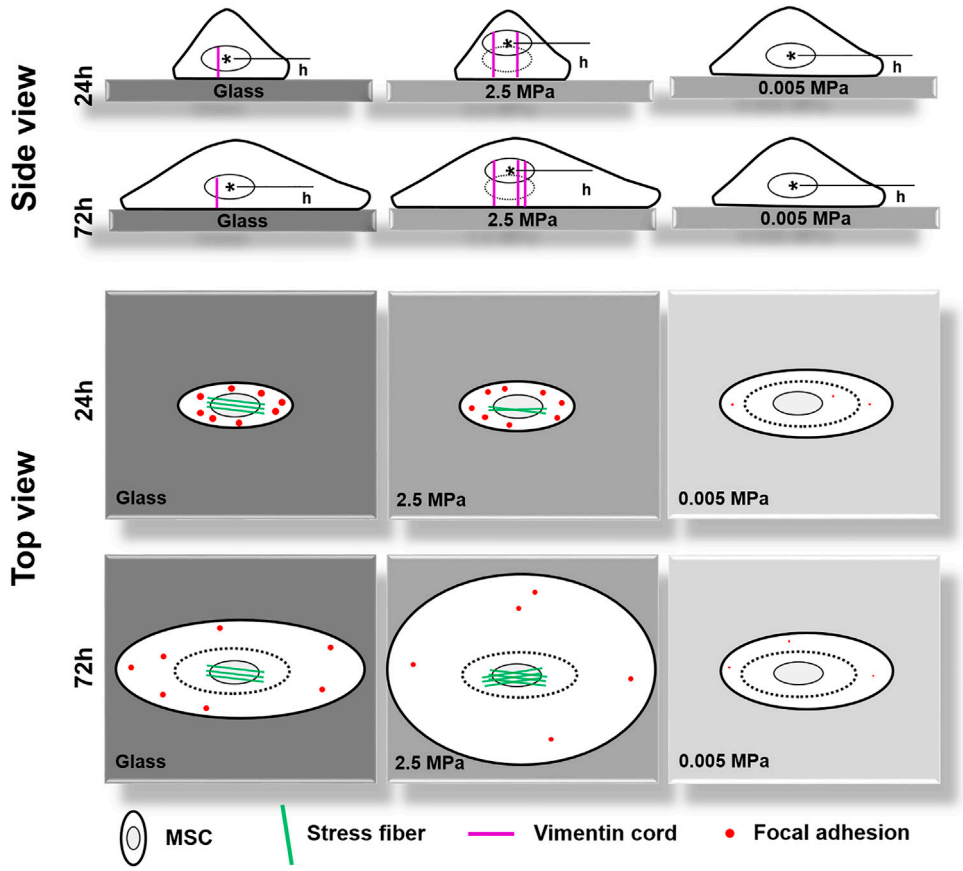

FIGURE 8 | A working model proposed for illustrating the coupled impacts of substrate stiffness and orientation on gravity-vector induced mechanical remodeling of rat bone marrow-derived stem cells. Here are two key points: (1) On stiff poly-dimethylsiloxane (PDMS), the orientation dependence of nuclear longitudinal translocation is similar to that onto glass *via* cytoskeletal remodeling and focal adhesion reorganization; (2) Soft PDMS tends to regulate cell morphology, spreading, and focal adhesion formation without a visible orientation dependence of nuclear translocation. Thick cycle, cell membrane; thin cycle, nucleus (also thin dotted cycle in the right panel); magenta lines, vimentin cords; green lines, actin fibers; red dots, large- or small-sized focal adhesions. *h*, the distance from the nuclear centroid (·) to the bottom surface of the substrate.

stiffness serves as a key factor to regulate the fate commitment of MSCs biomechanically (Engler et al., 2006; Discher et al., 2009; Li et al., 2011; Her et al., 2013; Wen et al., 2014; Mao et al., 2016). Meanwhile, the change in substrate orientation is not only associated with gravity vector-directed events but also correlated to mechanically induced cell remodeling in daily life. In fact, a stem cell anchored onto ECM varies its orientation frequently due to the posture change of the human body, and hence substrate orientation alteration *in vitro* could serve as a simple model to elucidate mechanosensing and mechanotransduction in a static state (Helmstetter, 1997; Li et al., 2010; Lü et al., 2014). Thus, the current work not only provided a platform to combine the substrate stiffness and orientation together but also it can be applied to elucidate mechanical remodeling of MSCs onto a physiologically mimicking substrate. A well-defined planar substrate with appropriate stiffness and orientation is crucial to define stem cell behaviors in stem cell biology and mechanotransduction.

Mechanical remodeling of MSCs upon the two combined factors is quite different from those found with their individualized factors. Physical or mechanical clues manipulate stem cell functions by altering the cell shape and re-organizing the cytoskeletal network (Dado et al., 2012). On one hand, a stiff matrix favors MSC proliferation by enhancing the expressions of cell-adhesive molecules to present counterbalancing forces to the substratum. Substrate stiffness also plays a key role in regulating MSC circularity and projected area upon distinct capacity of cell adhesion to a stiff or soft substrate. Moreover, the stiffness determines the fate commitment of MSCs by altering the cell traction force and changing the nuclear translocation of transcript factors (Kilian et al., 2010) or by inducing the specific biomarker expression of differentiated cells cooperatively with the substrate topography and dimension (Li et al., 2013). On the other hand, inversing or tilting the substrate cannot alter the number of attached osteoblasts but vary significantly the cell area and cycle in a time-dependent manner (Mitsiadis et al., 2007; Kordes and Haeussinger, 2013).

Meanwhile, orientation-dependent nucleus longitudinal translocation in a MC3T3-E1 cell in varied orientations is well correlated with the remodeling of perinuclear actin stress fibers and vimentin cords and the reorganization of FAC area and size (Zhang et al., 2017). In the current work, these gravity vector-directed orientation dependences were found to be altered. While the obvious nucleus translocation and cytoskeleton remodeling for rMSCs was presented onto 2.5 MPa PDMS, it disappeared for rMSCs onto softer PDMS substrates (**Figures 1**, **2**). These different patterns are presumably attributed to the different mechanically induced cell remodeling on stiffness-varied substrates. As a result, the slightly large, round cell shape was presented onto soft PDMS at long duration, which was supported by comparable FAC number with those onto glass (**Figures 5**, **6**). While these physical or mechanical signals are known to present the differential effects on rMSC functions, the underlying signaling pathways were associated with β1 integrin, FACs, and cytoskeleton (**Figure 7**).

Moreover, cell remodeling is specific when combining the two mechanical factors. For cytoskeleton remodeling, orientation-specific nucleus translocation at 0° for 24 h or at 90° for 72 h is positively correlated with the differential expressions of actin and vimentin between the two types of substrates (**Figures 1**, **2**). While perinuclear actin tends to form aligned, high-strength stress fibers onto glass that is consistent with those previous observations (Curran et al., 2005; Lipski et al., 2008), it is presented as isotropic and weak fibers onto stiff PDMS (**Figure 3**) that has not been observed before. Interestingly, this actin distribution is consistent with the differential cell morphology, that is, a large, circular shape onto 2.5 MPa PDMS and a small, circular shape onto softer PDMS (**Figure 6**). For MSCs placed onto either PDMS or glass substrate, vimentin tends to distribute over the entire cell and extends from the nucleus to the cell edge (**Figure 4**) as described previously (Murray et al., 2014). These clues suggested that vimentin can provide complementary support to cell stability in case of low actin expression (**Figure 2C**), which is consistent with previous observations that vimentin is key for protecting the cell against applied stress or stretch or to maintain cell integrity (Wang and Stamenović, 2000; Mendez et al., 2014; Chen et al., 2016). Meanwhile, this reasoning can also be used to explain why vimentin expression is upregulated dramatically on softer substratum in the current work (**Figure 2D**) or in the literature (Chen et al., 2016) where actin expression is low. Noticeably, vimentin is perinuclear distributed for MC3T3-E1 cells (Zhang et al., 2017) but dispersed over the entire cell for MSCs reported here, indicating the diversity of cytoskeleton remodeling in orientated substrates. Thus, future studies are required to elucidate the molecular mechanisms for these distinct distributions of actin and vimentin either for the same type of cells onto the two different substrates or for the different cell types onto the same substrate. For FAC reorganization, the normalized number or area on PDMS tends to be lower at short duration but comparable at long duration, implying the time-dependent mechanical reorganization of FACs (**Figure 5**). Nevertheless, the point-attached FACs are required to provide mechanical support for anchoring a cell on the substrate, and the β1 integrin–FACs–actin axis serves as one of the key mechanotransductive pathways in inducing nucleus longitudinal translocation (**Figure 7**).

CONCLUSION

Combining both substrate stiffness and orientation helps to analyze the underlying pathways of mechanical remodeling for a cell. The density difference between the nucleus and the cytosol induces accumulatively the differential nucleus translocation for MSCs onto PDMS or glass in distinct orientations. Actin and vimentin are major components to counter-balance the nucleus translocation in either a complementary or cooperative way. The cell is stabilized mechanically onto the substrate *via* β1 integrin–FACs–actin axis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal and Medical Ethics Committee, Institute of Mechanics, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

ML, CZ, DL, and SS designed the project. CZ, DL, FZ, YW, LZ, XZ, and ZL performed the study and analyzed the data. ML, CZ, and DL wrote the paper. All authors approved the final version of the manuscript.

FUNDING

This work was funded by the National Key Research and Development Program of China grant 2021YFA0719302, the National Natural Science Foundation of China grants 91642203, 31627804, and 31870931, and the Chinese Science Academy grant GJJSTD20220002.

ACKNOWLEDGMENTS

The authors thank Lüwen Zhou for his technical assistance in FACs counting by Matlab software.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.724101/full#supplementary-material

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frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 14 February 2022
doi: 10.3389/fbioe.2022.806761

Data Augmentation and Transfer Learning for Data Quality Assessment in Respiratory Monitoring

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Edited by:
Monica Monici, Università degli Studi di Firenze, Italy

Reviewed by:
Guanghao Sun, The University of Electro-Communications, Japan
Andrea Nicolò, Foro Italico University of Rome, Italy

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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *01 November 2021*
Accepted: *14 January 2022*
Published: *14 February 2022*

Citation:
Rozo A, Moeyersons J, Morales J, Garcia van der Westen R, Lijnen L, Smeets C, Jantzen S, Monpellier V, Ruttens D, Van Hoof C, Van Huffel S, Groenendaal W and Varon C (2022) Data Augmentation and Transfer Learning for Data Quality Assessment in Respiratory Monitoring. Front. Bioeng. Biotechnol. 10:806761. doi: 10.3389/fbioe.2022.806761

Changes in respiratory rate have been found to be one of the early signs of health deterioration in patients. In remote environments where diagnostic tools and medical attention are scarce, such as deep space exploration, the monitoring of the respiratory signal becomes crucial to timely detect life-threatening conditions. Nowadays, this signal can be measured using wearable technology; however, the use of such technology is often hampered by the low quality of the recordings, which leads more often to wrong diagnosis and conclusions. Therefore, to apply these data in diagnosis analysis, it is important to determine which parts of the signal are of sufficient quality. In this context, this study aims to evaluate the performance of a signal quality assessment framework, where two machine learning algorithms (support vector machine–SVM, and convolutional neural network–CNN) were used. The models were pre-trained using data of patients suffering from chronic obstructive pulmonary disease. The generalization capability of the models was evaluated by testing them on data from a different patient population, presenting normal and pathological breathing. The new patients underwent bariatric surgery and performed a controlled breathing protocol, displaying six different breathing patterns. Data augmentation (DA) and transfer learning (TL) were used to increase the size of the training set and to optimize the models for the new dataset. The effect of the different breathing patterns on the performance of the classifiers was also studied. The SVM did not improve when using DA, however, when using TL, the performance improved significantly ($p < 0.05$) compared to DA. The opposite effect was observed for CNN, where the biggest improvement was obtained using DA, while TL did not show a significant change. The models presented a low performance for shallow, slow and fast breathing patterns. These results suggest that it is possible to classify respiratory signals obtained with wearable technologies using pre-trained machine learning models. This will allow focusing on the relevant data and avoid misleading conclusions because of the noise, when designing bio-monitoring systems.

Keywords: respiratory monitoring, signal quality, machine learning, transfer learning, data augmentation

1 INTRODUCTION

Spaceflights impose many challenges to the well-being of astronauts due to their particular conditions, such as altered gravity, radiation, isolation and confinement (Vernikos, 1996; Tascher et al., 2019). The effects of these conditions on the health of the crew include affections to bone and muscle structures as well as deregulation of metabolic, cardiovascular, respiratory and immunologic systems, among others (Tascher et al., 2019; Crucian et al., 2013, 2015). Combined with the limited access to medical attention, these alterations can be a high risk to the crew members during the missions.

In addition to systemic alterations, the crew members can suffer from injuries, wounds, traumatic events and surgical emergencies. Illnesses and infections may also be acquired even though, prior to the start of the spaceflight, the crew undergoes a medical screening and a quarantine period. These events can be associated with external environmental conditions, such as first-degree burns as a result of ultraviolet light exposure, or with the internal atmosphere of the spacecraft, such as latent viruses reactivation, higher number of free-floating particles, chemicals, allergens, and microorganisms (e.g., bacteria, fungi and molds) (Crucian et al., 2016; Barratt et al., 2020; Barrila et al., 2021).

While in past and current space missions the incidence of these issues has been low, this might not be the case for longer missions like Mars or asteroid exploration. In such missions, health care *in-situ* becomes even more critical as delays in communications with the Earth are more common, and emergency extraction of crew members that could fall ill are harder. Under these new circumstances, wounds and infections require special attention regarding their management and monitoring.

It has been observed that in spaceflight conditions, the healing process of wounds is altered (Cialdai et al., 2020). This, in conjunction with the dysfunction of the immune system might cause complications. During the healing process, the continuous monitoring of vital signs can help to identify changes in the state of the patient and, in this way, allows to take the appropriate measures when deterioration is discovered (Brown et al., 2014).

Wearable technology presents a suitable alternative to traditional monitoring for continuous measurement of vital signs, because it does not interfere with the mobility and comfort of the patient. In the last years, this technology has been evaluated in clinical environments on Earth (Fieselmann et al., 1993; Prgomet et al., 2016; Weenk et al., 2020; Subbe and Kinsella, 2018). For the case of space exploration, joint efforts between space agencies (National Aeronautics and Space Administration—NASA, Canadian Space Agency—CSA) and industry have resulted in the design of wearable sensors to monitor the vital signs of the crew members during missions, which have been tested in the International Space Station (ISS) and in settings on ground to validate their performance (Mundt et al., 2005; Bellisle et al., 2020; Falker et al., 2015; Villa-Colín et al., 2018).

Even though the continuous monitoring works appropriately and the versatility of the wearable devices allows a wide range of movements, it has been found that this added flexibility makes the recorded signals more prone to noise sources (Orphanidou et al., 2015). As a consequence, the presence of artefacts prevents the extraction of reliable information, increasing the probability of false alarms and inaccurate measures. Some of the artefacts can be easily removed through filtering, but others are beyond repair, such as motion artefacts due to displacement of electrodes, for example, as observed in Villa-Colín et al. (2018).

In order to overcome the difficulties related with the removal of artefacts, some works have implemented signal quality indication approaches to identify the parts of the data that are useful for analysis (Orphanidou et al., 2015; Johnson et al., 2015; Castro et al., 2016; Castro et al., 2018; Charlton et al., 2021; Moeyersons et al., 2021). In general, the quality indication for electrocardiogram and photoplethysmogram signals, for the estimation of heart rate, has been investigated further than the quality indication of other signals, such as respiration.

The respiratory signal, however, is of great interest in health monitoring, given that it is one of the key markers that indicates deterioration in patients. Increasing respiratory rate (i.e., going above 20 breaths per minute) has been found to be an early predictor in different life-threatening conditions, such as cardiac arrest, respiratory adverse events and sepsis (Cretikos et al., 2008; Lee, 2016; Hotchkiss et al., 2016). Nevertheless, when the respiratory rate is not overlooked, its monitoring is often performed manually and at specific times during the day. The use of wearable devices for this task is not yet widely spread, because of the lack of evidence of accurate and reliable results. Given the useful characteristics of this signal for determining health adverse conditions (Nicolò et al., 2020) and the importance of its continuous monitoring in the early detection of these situations, this study is focused in the quality assessment of respiratory signals obtained from wearable sensors.

Previously, a quality index for respiratory signals was developed by Charlton et al. (2021), which then was compared to a machine learning framework for quality assessment by Moeyersons et al. (2021). Rozo et al. (2021) presented the results of applying transfer learning to the previous framework. In this context, this study extends the latter work including a data augmentation approach to improve the performance of the machine learning framework when applied to new data.

The presented framework consists of two machine learning models, which are used to classify segments of thoracic bio-impedance (BioZ) signals into clean or noisy (containing artefacts). The first classification model is a support vector machine (SVM), and the second one is a convolutional neural network (CNN). These models were designed for a dataset of patients suffering from chronic obstructive pulmonary disease (COPD).

Currently, there is a lack of available data from space exploration missions, in which health adverse situations have occurred, thanks to the extensive training and health screenings of the astronauts previous to the flight, combined with a relatively short time in the mission. Considering this, and the fact that the framework presented in this study could be used for the monitoring of astronauts' health during longer missions where

pathological breathing could be observed, a "worst case scenario" is presented. To this end, multiple respiratory patterns are included by testing the models on patients' data. As a consequence, the algorithms are tested not only on "normal" breathing patterns but they are also adequate for diseased conditions.

Considering this, the goal of this study is twofold. First, the models are pre-trained with the COPD data and their performance is evaluated when applied on a different patient population. Signals from patients undergoing bariatric surgery (BS) are used. These patients performed a controlled breathing protocol, which resulted in six different respiratory patterns. The use of these datasets for training and testing the models helps to obtain a general algorithm that is robust against changes in subjects population, and allows to generalize the classification performance for the use of normal and pathological respiration. Data augmentation (DA) and transfer learning (TL) are used to reduce the possible bias of the algorithms towards the class with the largest representation, and to optimize the models for the new, unseen data. Second, the effect of the properties of the respiration on the performance of the models is analyzed.

In this way, this study proposes a novel approach incorporating the use of DA and TL with machine learning algorithms for the quality assessment of respiratory signals from wearable devices. Also, the analysis of different breathing types using a single classification model is part of the novelty presented in this paper.

This paper is organized as follows: **Section 2** describes the datasets and methodologies used in this study. In **Section 3** the results are presented, and then discussed in **Section 4**. Concluding remarks are presented in **Section 5**.

2 METHODS

2.1 Datasets

Two datasets were used in this study. The first one was used to pre-train the classification models, and consists of the respiratory recordings of 47 COPD patients of the Ziekenhuis Oost-Limburg (ZOL), Belgium. From the patient population, 11 were female and the mean (±standard deviation) BMI was 26.2 ± 4.9 kg/m^2. Each patient was equipped with a wearable device to measure the BioZ, as well as a traditional wired acquisition system, which measures respiratory airflow with an airflow transducer, used as reference system. The recording of the data followed the World Medical Association's Declaration of Helsinki on Ethical Principles for Medical Research Involving Humans Subjects. More details about this dataset can be found in (Blanco-Almazan et al., 2021; Moeyersons et al., 2021).

The second dataset consists of 72 respiratory recordings of 20 patients who underwent bariatric surgery (BS), and were in treatment at the Nederlandse Obesitas Kliniek, Netherlands. There were 16 female and the mean (±standard deviation) BMI at inclusion was 42.5 ± 3.4 kg/m^2. The respiration of each patient was recorded using the same wearable device (BioZ) used for the COPD dataset, this time with a spirometer as the reference system. The recording of the data followed the World Medical Association's Declaration of Helsinki on Ethical Principles for Medical Research Involving Humans Subjects.

The wearable device (ROBIN, Stichting imec Netherlands) recorded the BioZ signals with a sampling frequency of 16 Hz. Stress test AG/AgCl electrodes (Kendall H92SG, Covidien Inc., Walpole, MA, United States) were placed on the thorax of the subject as shown in **Figure 1**. Using multiplexing, four different tetra-polar configurations were created. The amplitude of the excitation current was 110 μA at 80 kHz. The airflow for the COPD dataset was measured with a Biopac transducer (neumo-tach transducer TSD107B, Biopac Systems, Inc.), and digitalized at a sampling frequency of 10 kHz. The spirometer (TSD117A, Biopac Systems, Inc.) used a sampling frequency of 100 Hz. Further details on this equipment can be found in (Blanco-Almazan et al., 2019).

Each of the BS patients performed a controlled breathing protocol during the recording of their respiration. The protocol consisted of 1 minute of spontaneous breathing (Sp), followed by a period of breath holding and then five blocks of 30 seconds of chest (Ch), shallow (Sh), abdominal (Ab), slow (Sl) and fast (Fa) breathing. The pacing of the different breathing types was left to the patient's comfort. **Figure 2** shows an example of the respiratory signal of one of the patients during the followed protocol.

2.2 Preprocessing

Both datasets were preprocessed in the same way. First, the signals were band-pass filtered using a fourth-order Butterworth filter with cutoff frequencies at 0.05 Hz (3 breaths per minute) and 0.70 Hz (42 breaths per minute), removing the baseline changes and high frequency content not related to breathing.

The signals were then segmented as follows. For each recording, the first three and last seconds were removed due to loss of data when starting or stopping the measurements. Afterwards, the COPD recordings were divided into non-overlapping 1-min segments, resulting in 1,896 segments. In the case of the BS dataset, the breath holding period was localized and removed. After this, the recordings were divided into 30-s segments. Due to variations in the length of each respiration type, in some segments for the BS data two types of breathing were overlapped and the associated type of breathing is the one that is predominant. In total, 2,916 segments were obtained.

As one of the goals of this study was to observe the effect of different respiratory patterns on the performance of the classifiers, the segments of the BS dataset were grouped according to each of the six different breathing patterns.

2.3 Labeling

Supervised machine learning algorithms, such as the ones that are studied in this paper, require the ground truth of each sample for training and testing.

In this case, the ground truth of the recordings corresponds to one of the quality classes: clean or noisy. To obtain the class to which each signal belongs, four independent annotators were

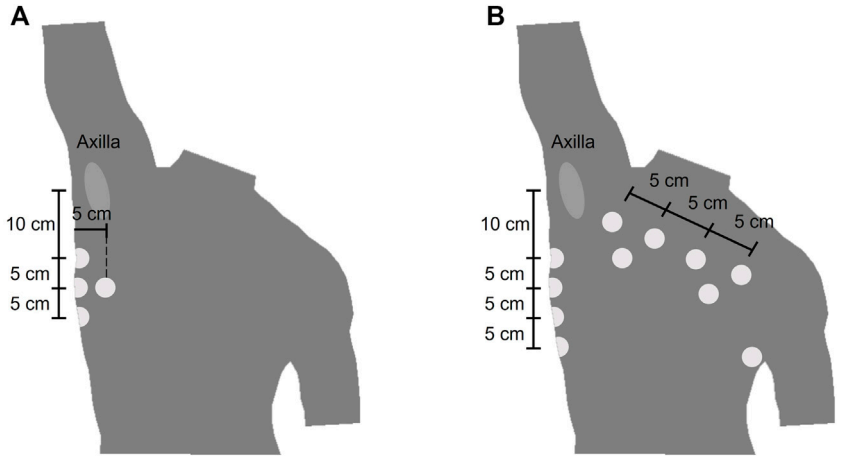

FIGURE 1 | Location of the electrodes of the wearable device **(A)** Electrode placement for the COPD dataset. The electrodes were placed symmetrically from the midsternal line and only the right side is represented **(B)** Electrode placement for the bariatric surgery dataset.

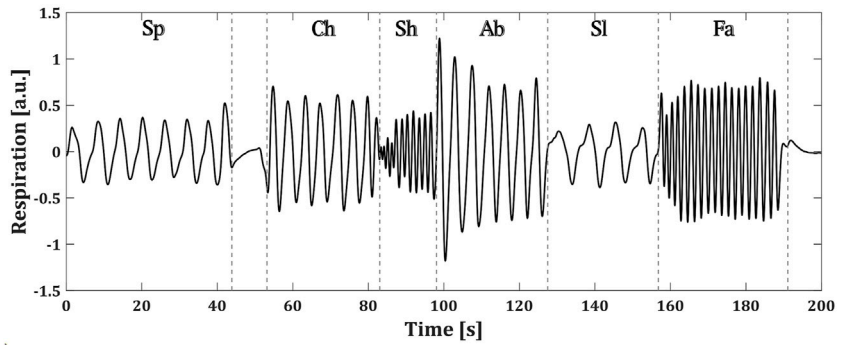

FIGURE 2 | Controlled breathing protocol followed by a BS patient. Each of the breathing types are specified as: spontaneous (Sp), chest (Ch), shallow (Sh), abdominal (Ab), slow (Sl) and fast (Fa) (a.u.) stands for arbitrary units.

asked to assign a label to them. For this, the graphical user interface and the five classes defined in (Moeyersons et al., 2021) were used. The classes 1 (Excellent signal quality), 2 (Good signal quality), 3 (Average signal quality) and 4 (Bad signal quality) refer to the BioZ signal with respect to the reference system. The class 5 (Bad reference quality) is reserved for the cases where the reference signal is of bad quality due to acquisition problems, motion artefacts or signal saturation. In **Figure 3** an example signal from each label is shown.

After the manual annotation of the signals, the labels for the BioZ signals were binarized, considering 1 and 2 as clean, while three and four were considered as noisy. Majority voting among annotators was performed to create a single label per signal, finding that the annotators fully agreed (4 annotators) on the 58.50% of the signals and the majority (3 annotators) agreed on the 28.09%. The Fleiss Kappa obtained for the labeling process was $\kappa = 0.58$, which suggested that the agreement was moderate and not at random. The segments in which no majority voting was achieved (13.41%) and the ones for which the majority voting resulted in label 5 (8.99% of the majority voting) were removed from further analysis. After this procedure, a total of 1,471 and 2,298 segments for the COPD and the BS datasets, respectively, remained.

2.4 Data Augmentation

It has been shown that when using machine learning approaches, the size of the train and test sets has an impact on the performance of the algorithm. The larger the training set the better the generalization of the model (Salamon and Bello, 2017; Lei et al., 2019). However, in many applications, especially those dealing with physiological data, collecting and labeling a large amount of data is not viable for various reasons, such as time limitations, ethical restrictions, or less population presenting a particular condition. One solution to this issue is to use DA (Simard et al., 2003). The principle behind DA is to generate synthetic data, using characteristics from the available data. Note that for supervised machine learning algorithms, the labels of the original data need to be preserved when generating the new data (Um et al., 2017).

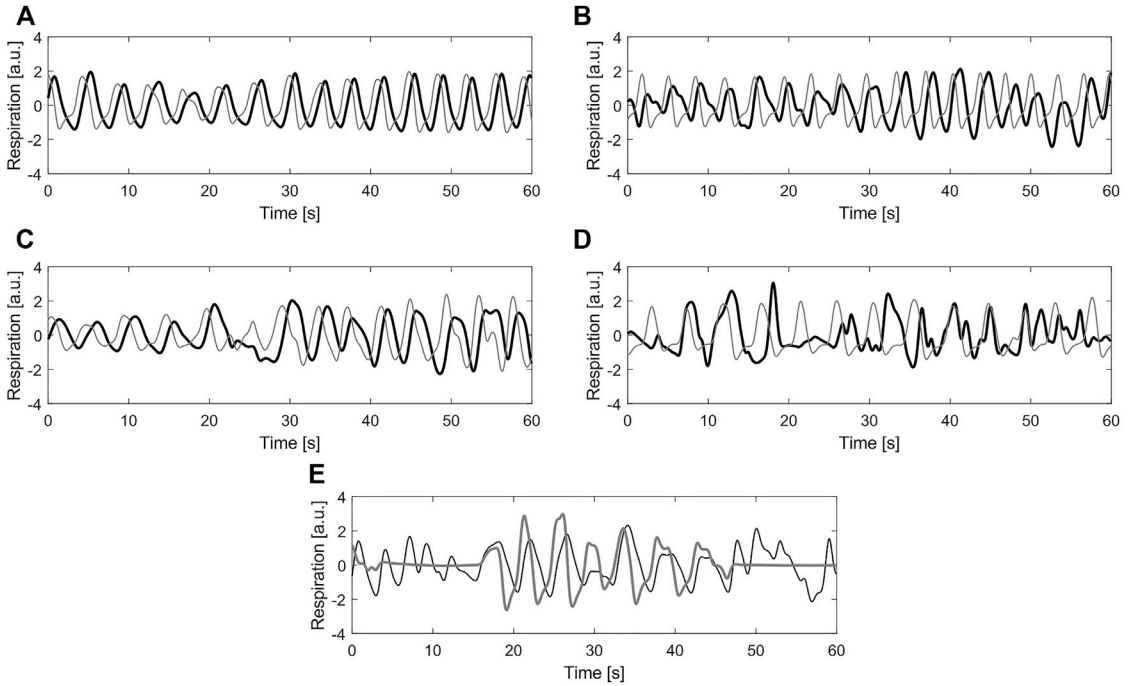

FIGURE 3 | Example of different labels for quality annotation **(A)** Label 1—Excellent signal quality **(B)** Label 2—Good signal quality **(C)** Label 3—Average signal quality **(D)** Label 4—Bad signal quality **(E)** Label 5—Bad reference quality. In all plots, black line corresponds to the bio-impedance signal and the gray line to the reference signal (a.u.) stands for arbitrary units.

In this study, four augmentation methods were applied to the BioZ signals after labeling, in order to maintain the same quality level that was originally assigned to them. The first augmentation was done by mirroring (flipping) the signal with respect to the *x*-axis and the *y*-axis. In this way, from one original signal, two new signals were obtained. This approach can be seen as an emulation of different placements of the electrodes in the skin, and does not affect the labels of the data.

The second augmentation was done by modulating the amplitude of the signals. For this, a sinusoidal modulating signal was defined with a period equal to twice the length of the original signal. In this way, the original data is modified in a way in which towards the both ends of the signal the amplitude is lower than the one at the center of the signal. In this case, when changing only the amplitude of the signal its general shape is maintained, which does not alter the labeling.

For the third method, the goal was to obtain a signal representing a slower breathing rate. To achieve this effect, the 10% of the points (5% at each end) of the original signal were removed. The time scale of the resulting segment was then assumed to be the same as the one for the original signal, meaning that if the original signal had a duration of 1 minute, the new segment was also supposed to be 1-min long. Considering this, and the fact that the new segment had less data points than the original one, it was assumed that it had a lower sampling frequency. Afterwards, it was resampled to the original sampling frequency (16 Hz), which results in a signal with a slower breathing rate. This transformation does not affect the labels of the data, given that in the guidelines for annotating the respiratory signals presented in (Moeyersons et al., 2021), the critical time frame to consider a signal of average or bad quality is the 16.6% (i.e. 10 and 5 s for the COPD and BS signals, respectively) of its length.

The goal of the final augmentation method was to obtain a signal representing a faster breathing rate. For this, two signals with the same label and from the same recording were concatenated. The resulting segment was assumed to have the same time scale than the original data but sampled at a higher frequency. Afterwards, this segment was resampled to the original sampling frequency obtaining a signal that corresponds to a faster breathing rate. The labeling of the signals is not altered, given that the concatenated signals had the same label and their general form was not altered.

In **Figure 4**, an example of the methods used for DA is presented.

The previous DA methods were applied to all the signals from the quality class with less data, while they were only applied to a randomly selected number of signals of the other class.

Considering that one of the goals of this study was to analyze the effect of the characteristics of the different breathing patterns on the performance of the classifiers, for the BS data only the first two augmentations were applied. In this way, it was possible to still discriminate between the different respiratory patterns.

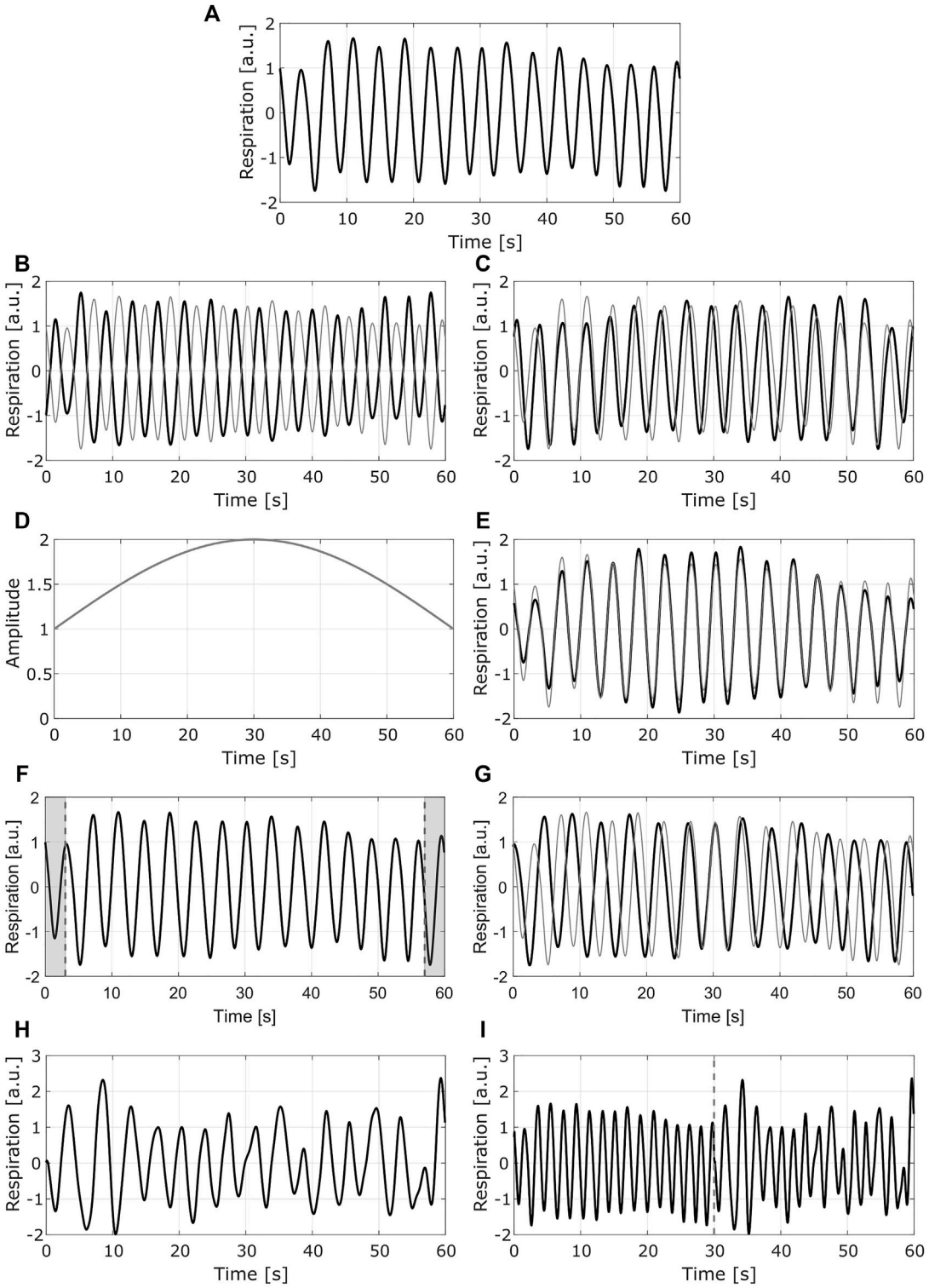

FIGURE 4 | Example of the data augmentation methods. Each row presents one method **(A)** Original signal **(B)** Mirrored signal with respect to *y* axis **(C)** mirrored signal with respect to *x* axis. **(D)** Modulating signal used for the second method **(E)** normalized modulated signal (**F**) Resulting segment after removing the 10% of the points (shaded areas at each end of the signal) **(G)** resampled signal with slower breathing rate **(H)** Second original signal to be concatenated to the first one **(I)** resampled signal with faster breathing rate. In **(B) (C) (E)** and **(G)**, the gray line shows the original signal. In **(I)** the dashed gray line corresponds to the point in which the signals are concatenated; the left side corresponds to the signal in **(A)**, the right side to the signal in **(H)** (a.u.) stands for arbitrary units.

2.5 Classification

The two machine learning approaches that were evaluated in this study are described below. The input of both classifiers corresponded to the normalized signals, after subtracting the mean and dividing by the standard deviation.

2.5.1 SVM

The first model was a feature-based SVM classifier. This classifier used a radial basis function kernel and its hyperparameters (i.e., box constraint and kernel scale) were selected with a Bayesian optimization technique along with five-fold cross-validation method, using the training data. The best pair of hyperparameters is obtained when the cross-validation loss is its lowest, and these are then used to train the optimal SVM model.

The features used with this model were computed from the whole segment and for 15-s sub-segments. In the case of the COPD data, the segments had a length of 60 s, while for the BS data the length of the signals was 30 s. The sub-segments of the COPD data were obtained without overlap. In contrast, the sub-segments of the BS data were obtained with a 10-s overlap.

The features were calculated from the auto-correlation function (ACF): amplitude of the first peak (Ap1), amplitude of the second peak (Ap2) and ratio between these two peaks (Ap1/Ap2); and from the power spectral density (PSD): bandwidth, frequency of the lower (f_{low}) and upper (f_{up}) bounds of the bandwidth, and the normalized power in this band. The five more informative features, common to all the signals, were selected as in (Moeyersons et al., 2021), using a maximum relevance minimum redundancy (MRMR) algorithm. From the ACF, the most relevant features were the Ap1 of the whole segment and the standard deviation of the Ap1 of the sub-segments. From the PSD, the most relevant features were: the f_{low} of the whole segments, the mean bandwidth of the sub-segments and the mean normalized power of the sub-segments.

More information on this model can be found in (Moeyersons et al., 2021).

2.5.2 CNN

The second classifier was a 1-dimensional CNN. The architecture of the network consisted of two blocks, each with two convolutional layers, followed by a global average pooling and ending with a fully-connected output layer with a softmax activation function.

Each of the two convolutional layers of the first block had 10 filters with a kernel size of 32 samples. The stride of these filters was of two samples and the padding in the borders was defined as the same end values. These layers had a ReLU activation function.

The output of the first block was then passed to the second block. The two convolutional layers of this block had the same hyperparameters of the ones from the previous block, but with only five filters instead of 10. This was done to ensure a resulting feature map with five features, analogous to the SVM approach.

This feature map was received by the average global pooling layer, which was used to generate a single feature vector by averaging the map over the temporal axis. Along with the characteristic behaviour of the convolutional layers, the robustness of this layer allowed to use the network with input signals of different sizes.

Finally, the feature vector was fed to the fully-connected layer, obtaining as output the classification probabilities for the two classes.

For more information regarding the architecture of this model, please refer to (Moeyersons et al., 2021).

2.6 Transfer Learning

One of the main assumptions of machine learning algorithms used for classification problems is that both the training and testing data have the same distribution and the same feature space. These assumptions, however, do not always hold in real life applications. An alternative to tackle this issue is TL (Weiss et al., 2016). With TL, a new classification problem is solved by using as a starting point an existing solution from a similar problem. Thus, the new classification problem requires less training data to obtain a robust solution.

In this study, the TL approach described in (De Cooman et al., 2020) was used for the SVM classifier. In this approach, an adapted model is generated by modifying the objective function of the SVM. For this, the classification error of the new data (BS signals) is minimized, as well as the dissimilarity between the original and the adapted models. One assumption of this approach is that the same features used for training the original model with the original data also describe the new data in which the adapted model is going to be applied. Considering this, the same features that were found to be the most relevant for the COPD data were computed and used with the BS data. Moreover, TL was applied to the original model in order to optimize the SVM for each breathing type.

For the CNN, the same principle presented in (Nanni et al., 2020) was used. In this approach, the adapted model is generated by copying the architecture and the weights of the pre-trained CNN. In a first step, the weights of all layers apart from the actual classification layer are fixed and then the classification layer is retrained. After, a fine-tuning (FT) step is added. In this step, the previously fixed weights are un-fixed and all the weights of the adapted model are retrained. The retraining is done setting a low learning rate and a small number of epochs to prevent modifying significantly the weights of the model. The only assumption of this approach is that the signals from the training and testing datasets should have the same sampling frequency. TL including FT was applied to the original CNN to obtain an adapted model for each breathing type.

2.7 Performance Evaluation

The models were tested with a cross-validation approach. The division was done by taking all the segments of the 70% of the recordings of the BS data for the training step of TL. The remaining 30% was used for testing the models. In order to assess the generalization capability of the models, this division was done 10 times at random. The same splits were used for both models with and without TL.

The area under the ROC curve (AUC) was used as the metric to evaluate and compare the performance of the models.

Significant differences between the models were evaluated with a Wilcoxon signed rank test (significant if $p < 0.05$) to assess the utility of TL and DA for the BS dataset.

2.8 Training and Testing

The classifiers were pre-trained with two strategies. The first one was to train the classifiers with all the available segments from the COPD data without DA. The second one was to train the classifiers with all the segments from the COPD data including DA.

The models obtained with these two strategies were tested on the BS data. Then, TL was applied to the models pre-trained with the COPD data with DA, using the BS with DA. This was done to ensure that when re-training the models during TL, the new data was sufficient for the task.

In this way, for each breathing type, three results were obtained, further referenced as Original (model pre-trained with COPD data without DA), DA (model pre-trained with COPD data including DA) and TL + DA (model pre-trained with COPD data including DA and TL).

3 RESULTS

The total number of segments after labeling and removing the ones with bad reference quality, for each dataset, is presented on the left side of **Table 1**. As can be observed, for the COPD there were more clean segments available, while for all the breathing types of the BS dataset there were more noisy segments. On the right side of the table the total number of segments for each data group after applying DA is presented.

The results obtained with both classification approaches are presented in **Table 2**. Results are indicated as median AUC [25th percentile - 75th percentile]. The values that are in bold correspond to the best results obtained with each classifier for each breathing type. The best results were defined as the ones with the higher AUC, which presented a significant ($p < 0.05$) improvement compared to the original results. In case of non-significant differences, the results with the smaller interquartile range were preferred. In **Figures 5**, **6**, the median of the ROC curves are presented for each of the classifiers, for the different breathing types. The original model is presented in black, the DA model in dark gray, and the TL + DA model in light gray. For the SVM, in all the breathing types it was observed that the DA model presented a worse behavior than the other models, and that the TL + DA model showed a similar behavior to the Original one. In contrast, for the CNN, the models DA and TL + DA showed a better behavior than the Original one, but they were similar to each other.

In the case of the SVM, in order to compare the results obtained with all the models, it was assumed that the best five features selected for the pre-training of the Original model were still the best for the new models. However, it can be seen that the performance of the DA model decreased in comparison to the Original one. Nevertheless, it can be noted that TL + DA improved significantly ($p < 0.05$) the performance compared to the DA model for all the breathing types.

TABLE 1 | Overview of the datasets, indicating the number of segments in each class. The suffix corresponds to the type of breathing imposed during the respiratory protocol. On the left, the total number of segments before data augmentation (DA). On the right, the segments after DA.

Group	Before DA			After DA		
	Clean	Noisy	Total	Clean	Noisy	Total
COPD	1,118	353	1,471	2072	2081	4,153
BS-Sp	202	240	442	808	807	1,615
BS-Ch	181	317	498	724	722	1,446
BS-Sh	181	305	486	724	722	1,446
BS-Ab	208	256	464	832	832	1,664
BS-Sl	83	216	299	332	330	662
BS-Fa	26	83	109	104	104	208

In contrast, in the case of the CNN the performance of the DA and TL + DA models improved significantly ($p < 0.05$) with respect to the Original model, with the only exception of Sh breathing. However, TL showed a significant improvement of the performance compared with the DA model only for the Sh breathing.

A comparison with the heuristic model presented by Charlton et al. (2021) was also performed to evaluate the advantages that using machine learning methods supposes when assessing the quality of respiratory signals. Charlton's signal quality index classify segments into high or low quality based on the variation on breath duration, the definition of peaks and troughs, and the similarity of the morphology of the breaths. The comparison of the accuracy, sensitivity and specificity of the classification of each breathing type are presented in **Tables 3–5**, respectively. Results are indicated as median [25th percentile—75th percentile]. The values that are in bold correspond to the best results obtained with each classifier for each breathing type, that were also higher than the heuristic model.

4 DISCUSSION

In this study, the performance of two pre-trained machine learning classifiers for the quality assessment of respiratory signals was evaluated on a dataset obtained from patients performing a respiratory protocol in which different breathing rates were imposed. To improve the performance of the classifiers, two techniques were used, namely Transfer Learning (TL) and Data Augmentation (DA).

It was noted that for the Slow (Sl) and Fast (Fa) breathing rates there were not as many data as for the other breathing types. In a first instance, there were less Fa data due to variations in the length of the raw data, which affected the segmentation of the last part of the signals, making it more difficult to obtain 30-s segments of the last breathing type. Second, for these two types of breathing the annotators found it more difficult to

TABLE 2 | Performance of the machine learning models for each of the sub-groups of the BS dataset. The results are presented as median AUC (25th percentile—75th percentile) (%). On the left side, the results for the SVM. On the right side, the results for the CNN. The best results (i.e., higher AUC) for each model for each breathing type are in bold.

—	SVM			CNN		
	Original	**DA**	**TL + DA**	**Original**	**DA**	**TL + DA**
Sp	91.03 [88.29–93.10]	66.92 [63.35–70.41]	**93.58^{++}** [91.03–94.85]	93.43 [92.49–93.81]	**98.32***** [96.69–98.81]	97.92**,* [97.24–98.21]
Ch	84.80 [83.24–91.88]	70.84 [67.31–73.18]	**89.32^{++}** [86.51–91.40]	90.56 [89.54–93.08]	**96.12***** [95.94–96.70]	96.10*** [95.68–96.29]
Sh	79.68 [73.89–83.04]	52.18 [49.47–59.64]	**84.75^{++}** [79.34–88.06]	83.86 [80.06–89.73]	84.84 [82.88–89.45]	**90.22$^{**,+}$** [87.89–92.82]
Ab	81.70 [77.78–86.18]	47.90 [43.55–51.21]	**84.80^{++}** [83.45–87.77]	91.74 [91.07–93.92]	94.74** [93.01–96.23]	**95.13*** [93.5–97.25]
Sl	85.65 [79.41–86.46]	71.73 [66.52–74.34]	**81.29^{++}** [79.20–83.92]	78.12 [72.89–86.83]	**90.54**** [87.77–93.51]	89.26** [87.23–93.05]
Fa	69.09 [59.64–75.69]	61.25 [45.86–70.56]	**74.05^{+}** [65.71–76.64]	73.02 [71.82–77.78]	**84.65*** [83.00–87.50]	83.41* [74.74–92.85]

Breathing patterns: Sp, spontaneous; Ch, chest; Sh, shallow; Ab, abdominal; Sl, slow; Fa, fast.
*Significant results compared to the Original model, *p < 0.05, **p < 0.01, ***p ≪ 0.01.*
Significant results compared to the DA, model, $^{+}$p < 0.05, $^{++}$p ≪ 0.01.

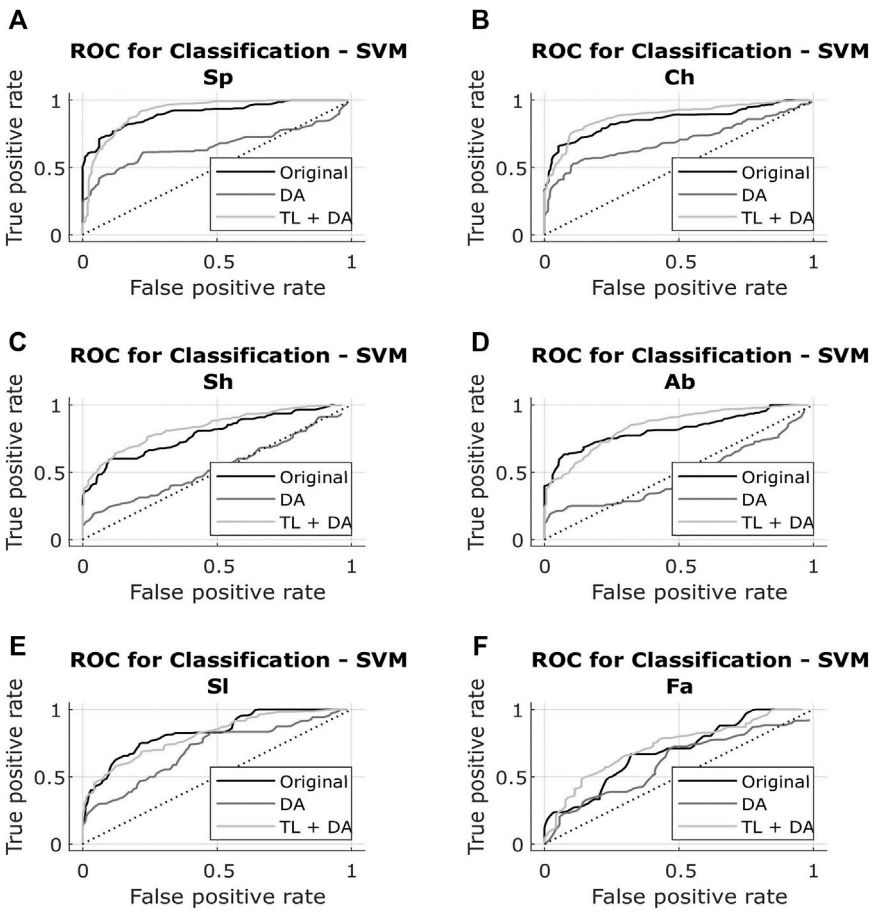

FIGURE 5 | ROC of the SVM models for each of the sub-groups of the BS dataset **(A)** Spontaneous breathing (Sp) **(B)** Chest breathing (Ch) **(C)** Shallow breathing (Sh) **(D)** Abdominal breathing (Ab) **(E)** Slow breathing (Sl) **(F)** Fast breathing (Fa). The curves present the median ROC for each model, in black the original, in dark gray the SVM-DA and in light gray the SVM-TL + DA. The dotted line corresponds to the random guess. It is observed that in all the breathing types the DA model presents a worse behavior than the other models, and that the TL + DA model shows a similar behavior to the Original one.

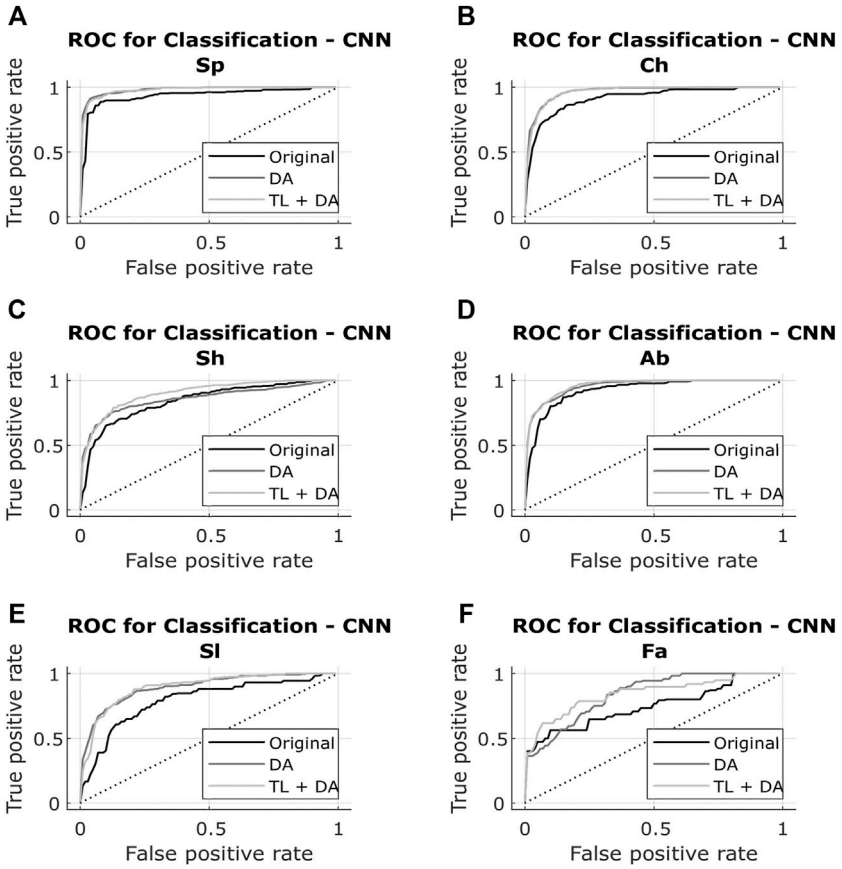

FIGURE 6 | ROC of the CNN models for each of the sub-groups of the BS dataset **(A)** Spontaneous breathing (Sp) **(B)** Chest breathing (Ch) **(C)** Shallow breathing (Sh) **(D)** Abdominal breathing (Ab) **(E)** Slow breathing (Sl) **(F)** Fast breathing (Fa). The curves present the median ROC for each model, in black the original, in dark gray the CNN-DA and in light gray the CNN-TL + DA. The dotted line corresponds to the random guess. It is observed that the models DA and TL + DA exhibit a better behavior than the Original one, while behaving similar to each other.

TABLE 3 | Performance of the machine learning models for each of the sub-groups of the BS dataset. The results are presented as median accuracy (25th percentile—75th percentile) (%). On the left side, the results for the SVM. On the middle, the results for the CNN. On the right side, the results using the heuristic method in Charlton et al. (2021). The best results of each model that performed better than the heuristic approach for each breathing type are in bold.

—	SVM			CNN			Heuristic
	Original	**DA**	**TL + DA**	**Original**	**DA**	**TL + DA**	
Sp	83.13 [81.82–84.91]	65.63 [58.57–70.59]	**87.23** [85.64–89.53]	87.41* [86.33–88.1]	81.21 [79.43–84.35]	**92.41*** [91.61–93.20]	84.03 [82.35–86.32]
Ch	82.10 [80.60–84.55]	66.25 [64.08–68.37]	**83.55** [81.87–85.20]	85.72* [82.43–87.41]	73.20 [71.59–74.88]	**89.06*** [88.32–89.68]	80.97 [79.05–83.58]
Sh	**80.18** [69.88–84.21]	70.31 [59.04–77.68]	77.71 [71.78–83.26]	74.66 [71.33–79.47]	65.01 [61.00–66.43]	**82.60** [80.04–85.00]	79.26 [71.08–87.50]
Ab	72.21 [69.49–76.79]	61.13 [57.63–66.27]	75.57 [72.00–76.76]	85.61* [83.46–87.23]	70.62 [69.12–73.01]	**87.33*** [85.66–89.62]	76.51 [68.18–79.52]
Sl	**80.19*** [77.33–85.14]	72.76 [67.39–82.43]	69.93 [67.26–72.48]	76.79 [73.03–80.21]	63.02 [60.98–66.50]	**82.96*** [79.07–86.19]	73.82 [72.55–78.57]
Fa	**72.67** [61.90–87.50]	69.62 [61.90–83.33]	72.35 [68.35–76.92]	**80.33** [71.43–89.29]	52.42 [42.11–61.70]	75.39 [63.89–83.64]	69.62 [61.90–83.33]

Breathing patterns: Sp, spontaneous; Ch, chest; Sh, shallow; Ab, abdominal; Sl, slow; Fa, fast.
*Significant results compared to the Heuristic method, *p < 0.05.*

reach an agreement in the labeling ($\kappa = 0.49$ for Sl, and $\kappa = 0.36$ for Fa). Besides, the quality of the reference signals was not good enough in the 18.97 and 32.30% of the data for which the majority voting was obtained for the Sl and Fa, respectively. The low quality of the reference signal was due to disconnection of the spirometer and breath holding periods during the protocol. This

TABLE 4 | Performance of the machine learning models for each of the sub-groups of the BS dataset. The results are presented as median sensitivity (25th percentile—75th percentile) (%). On the left side, the results for the SVM. On the middle, the results for the CNN. On the right side, the results using the heuristic method in Charlton et al. (2021). The best results of each model that performed better than the heuristic approach for each breathing type are in bold.

—	SVM			CNN			Heuristic
	Original	DA	TL + DA	Original	DA	TL + DA	
Sp	63.92 [61.11–68.75]	13.42 [8.57–19.44]	83.48 [80.43–89.42]	76.32 [71.43–76.81]	61.26 [57.65–67.86]	**91.69*** [89.13–93.37]	85.90 [81.58–91.30]
Ch	55.72 [46.67–59.52]	9.29 [6.82–11.11]	**85.87*** [80.00–88.89]	68.07 [63.64–74.14]	48.12 [40.00–49.60]	**89.86*** [87.22–90.69]	64.17 [57.78–70.37]
Sh	35.61 [33.33–47.22]	2.17 [0.00–5.56]	**81.41*** [74.22–87.14]	46.49 [41.94–48.48]	34.45 [31.05–35.00]	**81.57*** [74.48–86.57]	52.78 [45.71–66.67]
Ab	42.64 [40.91–43.75]	10.91 [0.00–12.50]	**78.84*** [71.88–81.58]	77.62* [74.32–82.09]	46.50 [44.64–49.32]	**86.46*** [84.33–90.82]	62.50 [55.56–64.00]
Sl	34.31 [28.57–42.86]	8.69 [0.00–17.65]	**64.99*** [58.33–68.75]	35.57 [33.33–42.11]	24.55 [18.42–28.41]	**79.56*** [76.14–83.62]	39.09 [33.33–41.18]
Fa	21.11* [14.29–33.33]	5.00 [0.00–13.33]	**77.68*** [75.00–100.00]	35.42 [28.57–40.00]	10.36 [10.00–12.50]	**61.88*** [47.50–84.38]	10.56 [0.00–14.29]

Breathing patterns: Sp, spontaneous; Ch, chest; Sh, shallow; Ab, abdominal; Sl, slow; Fa, fast.
*Significant results compared to the Heuristic method, *p < 0.05.*

TABLE 5 | Performance of the machine learning models for each of the sub-groups of the BS dataset. The results are presented as median specificity (25th percentile—75th percentile) (%). On the left side, the results for the SVM. On the middle, the results for the CNN. On the right side, the results using the heuristic method in Charlton et al. (2021). The best results of each model that performed better than the heuristic approach for each breathing type are in bold.

—	SVM			CNN			Heuristic
	Original	DA	TL + DA	Original	DA	TL + DA	
Sp	**95.81*** [91.89–98.33]	100.00 [100.00–100.00]	88.37* [84.62–94.62]	97.10* [96.30–97.59]	**99.57*** [99.29–99.61]	93.72* [92.53–95.24]	84.98 [78.13–85.42]
Ch	**97.40*** [96.88–98.39]	100.00 [100.00–100.00]	81.60 [79.75–84.38]	94.44* [93.91–96]	**98.86*** [98.47–100.00]	90.35 [87.94–93.51]	90.98 [88.71–92.19]
Sh	**99.43*** [95.00–100.00]	100.00 [98.73–100]	70.54 [67.57–74.32]	96.74* [95.71–97.41]	**99.08*** [98.9–99.62]	87.33 [85.61–88.5]	89.53 [88.68–92.31]
Ab	**98.44*** [96.30–100.00]	100.00 [100.00–100.00]	72.53 [70.10–77.65]	91.15* [90.16–94.20]	**98.87*** [97.17–99.61]	87.62* [86.57–90.71]	83.68 [81.25–88.89]
Sl	**98.01*** [97.14–100.00]	100.00 [100.00–100.00]	74.26 [68.82–81.82]	91.48 [89.58–93.90]	100.00 [100.00–100.00]	87.17 [83.53–89.36]	89.29 [88.24–95.24]
Fa	100.00 [100.00–100.00]	100.00 [100.00–100.00]	55.84 [50.00–73.91]	100.00 [96.30–100.00]	100.00 [100.00–100.00]	85.52 [78.26–92.59]	100.00 [100.00–100.00]

Breathing patterns: Sp, spontaneous; Ch, chest; Sh, shallow; Ab, abdominal; Sl, slow; Fa, fast.
*Significant results compared to the Heuristic method, *p < 0.05.*

might represent a challenge to future wearable monitoring developments, in particular if they are meant to be used in space and in early prediction of health deterioration, as they should ensure the good quality of signals with a wide range of breathing rates.

It was also observed that the distributions of noisy and clean segments in the BS and COPD datasets were different. While the BS dataset contained more noisy than clean signals, the opposite was observed in the COPD dataset. This behavior could be related with three factors. First, the higher BMI in the BS dataset has an impact in the impedance of the thorax making the signals more noisy. Second, the respiratory protocols followed by the patients could have an impact in this distribution. Third, as was shown in **Figure 1**, the electrode placement was different for each group, which could also affect the measurement of the impedance. However, it is suggested to investigate further the cause of these distributions differences as it can help to design a more robust device for vital signs monitoring.

When pre-training the models with the COPD data without using DA, it was found that for the Sh and Fa breathing types, the performance of the SVM was worse than for the other breathing types (median AUC <80%). In the case of the CNN, the performance was worse (median AUC <80%) for the Sl and Fa types. The reasons behind this behavior could be the different distribution of the classes in the training and testing sets, and the combination of morphological characteristics with the different breathing rates of the signals in the testing set in comparison with the training data. This is more evident when comparing the results obtained in the present study with the ones presented in (Moeyersons et al., 2021). In the latter, the SVM and CNN were trained and tested in the same COPD dataset. The average AUC obtained in that case is comparable to the results obtained for Sp, Ch and Ab data, which present a more similar breathing rate to the COPD data (normal breathing rate between 8 and 14 breaths per minute).

To overcome these challenges, it was decided to pre-train the models with the COPD data after using DA. The models then were tested in the original BS dataset. With this, the amount of clean and noisy signals in the COPD data was balanced, and different characteristics were included (different breathing rates and changes in amplitude). In this case, it was found that for the SVM, the performance of all breathing types decreased in comparison with the Original model. As mentioned before, the five more relevant features obtained from the original dataset were used as well for this analysis. However, the inclusion of the synthetic data to the training set could have altered the relevance of all the features and the best ones could have changed. The effect of changing the selection of the best five features was not explored in this study, and it is proposed as future research. The selection of the best features for the SVM classifier is a critical design variable in the development of decision support systems, as it can impact the performance of the model. It is also worth mentioning that in this study only five features were selected, however, as was shown in (Moeyersons et al., 2021), selecting more or less features also have an impact on the performance of the SVM. This effect should be also investigated further when working with TL, DA and more importantly with different types of signals for vital signs monitoring applications in extreme and remote environments.

In contrast, the performance of the CNN for the pre-training with the COPD data after DA improved significantly. It is known that the performance and the generalization capabilities of machine learning models are highly dependent on the quantity of data available for training (Salamon and Bello, 2017; Lei et al., 2019; Iwana and Uchida, 2021). In the case of the CNN, it performs an automatic feature selection based on the input signals, which implies that if a larger and more diverse set is available, the selection of the relevant features will be improved and the performance will be better. The notable difference between the SVM-DA and the CNN-DA is explained by the way in which the features for the classification are selected, being pre-defined for the SVM and automatically for the CNN.

As a final step to help improve the performance of the classifiers, TL was applied to the pre-trained models with the COPD data with DA. To ensure that the re-training set for TL was balanced, the BS data with DA were used. In this case, it was found that the performance after applying TL was consistently better than for the DA model for all the respiration types for SVM. However, it did not improve significantly with respect to the Original model. This could also be explained by the fact that when including synthetic data in the BS groups, the feature space changes and alters the selection of the best features. For CNN, the performance improved significantly compared with the Original model, but not compared with the DA model. One explanation for this could be that after including the DA in the training set, the inclusion of the DA in the BS data does not provide new and better information for the classification.

The effect of these two approaches is more noticeable when compared to the results presented by Rozo et al. (2021), where TL was applied to the models pre-trained with the COPD data without DA. In that case, SVM presented a higher improvement than CNN. The results obtained in the present study show that the inclusion of DA before applying TL reduces the improvement of performance in the SVM. In contrast, the inclusion of DA with and without applying TL have a bigger effect in the performance of the CNN, than only using TL.

In general, it could be seen that DA is a good alternative to improve the performance of machine learning models in which the features selection is done automatically. In contrast, when the features are computed a-priori and the best ones to be used are hand-picked, the best alternative could be the use of TL. Nevertheless, it is still important to research more in depth the dependencies of the performance of the classifier on DA and TL, as in this study only a limited patient population was used.

Despite the general improvement of both classifiers after using DA and TL, it could be seen that the specific performance for Sh, Ab, Sl and Fa breathing types is lower compared to Sp and Ch. This could be due to the fact that the changes of morphology and breathing rates included in the pre-training set after DA, were not enough to characterize the new signals in which the models were tested. More research is needed to characterize correctly the combinations of morphology and variation of breathing rates that consistently challenge the performance of the classifiers. In this way, it will be possible to have a better performance with a wider range of respiratory signals obtained with wearable devices for different applications. However, it is important for the model to achieve a balance between generalization capabilities and good performance, to fulfill the goal of using it with any new data. For this, it is proposed to collect and study a more diverse cohort of data in terms of length of the segments and protocols followed by the patients, to ensure that the models presented in this manuscript for the quality assessment of respiratory signals are sufficiently robust and general.

In addition, the performance of the machine learning models was compared with the performance of the heuristic method for signal quality index proposed by Charlton et al. (2021). It was found that, in general, the machine learning models presented a better performance, and it was improved even further when using DA and TL. This findings are in line with the comparison presented by Moeyersons et al. (2021), where it was found that for the COPD data quality assessment, the machine learning models performed significantly better than the heuristic model. This could serve as a basis for the selection of machine learning models over heuristic models when assessing the quality of respiratory signals obtained from wearable devices.

5 CONCLUSION

In this work, the quality assessment of respiratory signals obtained from wearable sensors was studied. The results presented in this study showed that with pre-trained machine learning classifiers in conjunction with data augmentation and transfer learning, it is possible to properly identify clean and noisy respiratory BioZ signals. CNN performed overall better than SVM when using DA, but the effect of TL was more noticeable in SVM.

For both classifiers, however, the results showed a lower performance for the breathing types whose morphology and

imposed breathing rates differed the most from the spontaneous breathing of the patients in which the models were pre-trained.

These findings could be beneficial for the steps of data processing and connection with decision support systems when designing new bio-monitoring devices for space exploration.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on reasonable request to the corresponding author. The data are not publicly available due to them containing information that could compromise research subject privacy.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ziekenhuis Oost-Limburg (Reference number:18/0047U). The study followed the World Medical Association's Declaration of Helsinki on Ethical Principles for Medical Research Involving Humans Subjects. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AR, JtM, CVH, SVH, WG, and CV designed the experiments. WG, CS, DR, RGW, VM, and SJ designed and supervised the data collection. RGW and LL performed the data collection. AR, JtM, JM, WG, and CV conducted a first analysis of the data. AR carried out the computational experiments. CV supervised the work. All authors contributed to manuscript revision, read, and approved the submitted version. The authors would like to thank Jeroen Buil for his contribution to the data collection.

FUNDING

Bijzonder Onderzoeksfonds KU Leuven (BOF): Prevalentie van epilepsie en slaapstoornissen in de ziekte van Alzheimer: C24/18/097. EIT 19 263—SeizeIT2: Discreet Personalized Epileptic Seizure Detection Device. KU Leuven STADIUS acknowledges the financial support of imec. This research received funding from the Flemish Government (AI Research Program). SH, AR, JtM and JM are affiliated to Leuven. AI-KU Leuven institute for AI, B-3000, Leuven, Belgium. AR and CV acknowledge the financial support of ESA, BELSPO.

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Conflict of Interest: Authors RGW and WG were employed by the company Imec The Netherlands/Holst Centre. Authors CS and DR, were employed by the company Ziekenhuis Oost-Limburg. Authors SJ and VM were employed by the company Nederlandse Obesitas Kliniek. Author CVH was employed by the companies Imec OnePlanet, Imec.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Bioengineering and Biotechnology

MINI REVIEW
published: 22 March 2022
doi: 10.3389/fbioe.2022.666434

The Epidermis in Microgravity and Unloading Conditions and Their Effects on Wound Healing

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The future objectives of human space flight are changing from low-term permanence in the International Space Station to missions beyond low Earth orbit to explore other planets. This implies that astronauts would remain exposed for long time to a micro-gravity environment with limited medical support available. This has sparkled medical research to investigate how tissues may adapt to such conditions and how wound repair may be influenced. This mini-review is focused on the effects of microgravity and unloading conditions on the epidermis and its keratinocytes. Previous studies, originally aimed at improving the *in vitro* protocols to generate skin substitutes for plastic surgery purposes, showed that epidermal stem cells cultured in simulated microgravity underwent enhanced proliferation and viability and reduced terminal differentiation than under normal gravity. In the meantime, microgravity also triggered epithelial-mesenchymal transition of keratinocytes, promoting a migratory behavior. The molecular mechanisms, only partially understood, involve mechano-trasduction signals and pathways whereby specific target genes are activated, i.e., those presiding to circadian rhythms, migration, and immune suppression, or inhibited, i.e., those involved in stress responses. However, despite the above *in vitro* studies suggest that microgravity would accelerate keratinocyte growth rate and migration, *in vivo* findings on animals in experimental set-ups to simulate low gravity rather suggest that prolonged mechanical unloading contributes to delayed and impaired epidermal repair. This is in keeping with the finding that microgravity interferes at multiple levels with the regulatory signals which coordinate the different cell types involved in the repair process, thereby negatively influencing skin wound healing.

Keywords: microgravity, keratinocytes, epidermis, epidermal stem cells, skin, wound healing

OPEN ACCESS

Edited by:
Carlo Saverio Iorio, Université libre de Bruxelles, Belgium

Reviewed by:
Tzu-Wei Wang, National Tsing Hua University, Taiwan
Michele Balsamo, Kayser (Italy), Italy

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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *10 February 2021*
Accepted: *08 March 2022*
Published: *22 March 2022*

Citation:
Bacci S and Bani D (2022) The Epidermis in Microgravity and Unloading Conditions and Their Effects on Wound Healing. Front. Bioeng. Biotechnol. 10:666434. doi: 10.3389/fbioe.2022.666434

INTRODUCTION

The new millennium has initiated a new chapter of space exploration. The many data gathered upon the unmanned missions to Mars have supported the feasibility of long-range space flights to bring astronauts to explore the closer planets, a perspective that may become something more than a screenplay for a science-fiction movie. To make this possible, many scientific issues have to be considered and managed, especially those related to the physical and mental health of crews exposed for unprecedented long times to the confined environment of a spaceship under low gravity conditions, and limited availability of medical resources. Studies on astronauts who performed

enduring missions in the International Space Station (ISS) have allowed to collect numerous medical data on the effects of low gravity on the human body, crucial to identify microgravity-induced diseases (Sekiguchi, 1994; Smith et al., 2014; Richter et al., 2017), as well as their pathogenic mechanisms at the cellular and molecular level (Pietsch et al., 2011). The best-known adverse effects of low gravity exposure, yet emerged upon short-term low-Earth-orbit permanence, consist in bone and muscle loss, reduction of cardiovascular capacity, delayed wound and bone fracture healing, and impaired immune function. Over the long-term, exposure to microgravity may impair stem cell-dependent tissue regeneration and homeostasis, adversely affecting bone formation and remodeling, and hemato/lymphopoiesis. In this context, long-term low gravity experiments on amphibians have demonstrated the loss of ability of stem cells of blastemata to regenerate the tail and ocular lens (Bizzarri et al., 2015). Another reason for concern is the susceptibility of astronauts to trauma due to peculiar working needs and conditions, since observational data suggest an impaired response to wounding and injury, such as the unusual behavior of hemorrhage, microbiologic flora, and wound healing (Kirkpatrick et al., 1997). This has sparkled medical research to investigate how tissues, particularly the skin, may adapt to such conditions, and how wound repair may be influenced. This mini-review will be focused on the effects of microgravity and unloading conditions on the epidermis and its keratinocytes, viewed in the context of their contribution to the wound healing process of the skin.

KERATINOCYTES, STEM CELLS AND EPIDERMAL HOMEOSTASIS

Keratinocytes are directly involved in several functions of the epidermis relevant for healing of skin wounds: first, they proliferate to maintain epidermal tissue homeostasis and repair tissue losses; second, they produce an array of cytokines and growth factors involved in the autocrine regulation of keratinocyte proliferation, migration, and differentiation, as well as in paracrine effects on stromal, inflammatory and immune cells (Yang et al., 2020). Epidermal cell homeostasis results from the continuing activity of the so-called epidermal proliferative unit (EPU), which encompasses undifferentiated epidermal stem cells (ESCs), transit amplifying (TA) cells and committed keratinocytes which, in normal conditions, evolve in terminally differentiated corneocytes within 20–30 days. Approximately, a single ESC yields by asymmetric mitosis 2 siblings, another ESC and a rapidly dividing TA from which approx. 32 terminal keratinocytes arise (Jones, 1997). Typically, ESCs are mainly harbored in stem cell niches located in hair follicle bulges, from which they can settle in the basal layers of interfollicular epidermis and sebaceous glands (Jones, 1997). Interestingly, evidence has emerged that ESCs from the different sites follow their own differentiation paths in normal homeostasis of the epidermis and its annexes, whereas all of them can synergize to give rise to any differentiated epidermal/annexal cell type in response to skin injury (Ito et al., 2005; Watt & Jensen, 2009). Obviously, these populations of ESCs and TA keratinocytes are the most susceptible to regulatory signals, including micro-mechanical stimuli: hence, their disruption in altered gravity conditions can have an impact on homeostasis of the epidermis and its ability to respond to injuries.

KERATINOCYTES AND MICROGRAVITY: EFFECTS AND POSSIBLE MECHANISMS

How microgravity influences the morpho-functional features of keratinocytes and their contribution to skin wound healing is a poorly explored field. Most cell types are able to respond to mechanical cues that activate specific sensor molecules, chiefly integrin-extracellular matrix (ECM) pairings, intercellular adhesion molecules and junctions, ion channels, α/βcatenins, and cytoskeletal components which transduce them into molecular signals modulating cell morphology, proliferation, differentiation and migration (Janmey and McCulloch, 2007; Farahani and DiPietro, 2008). Therefore, it is conceivable that abnormal micro-mechanical stimuli, as those operating in microgravity, may have an impact on keratinocyte behavior, especially when resting cells are aroused to the dynamic condition needed for wound healing.

Previous *in vitro* studies, originally aimed at improving the *in vitro* protocols to generate skin substitutes for plastic surgery purposes, showed that human ESCs cultured in simulated microgravity underwent enhanced proliferation and viability and reduced terminal differentiation as compared with those cultured under normal gravity condition, albeit they retained the capability to form a multi-layered epidermis-like tissue (Lei et al., 2011). Later investigations with human immortalized keratinocytes exposed for up to 60 h to simulated microgravity by a random-positioning machine have revealed changes in the expression and periodicity of *Bmal1* gene involved in the regulation of circadian rhythm, not accompanied by substantial changes in overall cell morphology, proliferation and apoptosis rates, and at least at the explored experimental times (Ranieri et al., 2015). Concurrently, exposure of human keratinocytes to the same simulated microgravity conditions was shown to trigger epithelial-to-mesenchymal transition (EMT), mediated by over-expression of specific transcription factors and markers, such as Snail1, Snail2, *ZEB2*, MMPs, and ECM adhesion molecules, as well as by re-arrangement of cytoskeletal components in a pro-motile pattern (Ranieri et al., 2017). As will be discussed in more detail in a following section, EMT is a first key step required to promote a migratory behavior of keratinocytes whereby they accelerate re-epithelization during wound healing.

The molecular signals and mechanisms involved in these cellular effects of microgravity are only partially understood. Since epidermal growth factor (EGF) and other molecules of the EGF family, which also include TGF-α and heparin-binding EGF, are major regulators of epithelial cell growth and differentiation and are known to play a pivotal role in re-epithelization during the early steps of wound healing (Steed, 1998) it appeared conceivable that these could be altered under low gravity conditions. Indeed, exposure of a human squamous

cell line to low gravity was shown to decrease the expression of key genes (*c-fos*, *c-jun*) downstream EGF activation involved in cell cycle progression, likely through an interference with protein kinase C-dependent signal transduction and actin cytoskeleton (Rijken et al., 1994; Boonstra, 1999).

In view of a possible translation of the results of *in vitro* microgravity experiments to space medicine, studies were performed to ascertain whether the microgravity-induced changes of keratinocytes were reversible upon restoration of normal gravity. Of note, human keratinocytes are capable to recover a static epithelial phenotype, as assessed by re-establishment of intercellular adherent junctions and normal cytoskeletal features, mirrored by reduction of their mesenchyme-like migratory phenotype (Ricci et al., 2021). On the other hand, microarray studies on gene expression patterns by human keratinocytes exposed to microgravity for up to 10 days have shown that the longer the exposure to low gravity, the slower and less complete the return to a normal cell morphology and gene expression profile (Clement et al., 2008), In partial agreement with these findings, recent data from cultured endothelial cells suggested that short-term microgravity post-transcriptionally modulated the expression of several genes involved in angiogenesis and vascular patterning (Kasiviswanathan et al., 2020). Other studies have shown that cells in cultures recovered from spaceflight did not migrate normally, as occurs during epithelial wound closure (Almeida, 2011) and that their cytokine, and growth factor secretion pattern is altered (Huang et al., 2020). These results suggest that the migratory and paracrine ability of microgravity-exposed precursor cells is impaired and support the hypothesis that the tissue regenerative potential of stem cells, including ESCs, and may be decreased during spaceflight (Blaber et al., 2014). The positive aspect is that chromosomal, DNA damage, and tumorigenicity assays performed upon return of cell cultures to Earth showed no signs of damage which could be related to malignant transformation (Huang et al., 2020).

A major limitation of the above findings consists in the fact that they have been obtained by *in vitro* cell culture experiments, in which cells are distanced from the complex network of signals they would receive in their physiological tissue environment. This is especially true for skin wound healing, characterized by a functional co-ordination between different player cells which, besides epidermal keratinocytes, also include platelets, inflammatory cells, mesenchymal stem cells, fibroblasts, and endothelial cells (Martin and Nunan, 2015; Dekoninck and Blanpain, 2019). Nonetheless, the above *in vitro* data can rise concerns about the adverse effects of long-lasting space missions on epidermal integrity. Additional background to concerns was provided by *in vivo* studies on skin wound healing using the tail-suspended hindlimb-unloaded rat model to induce functional disuse and intended to simulate microgravity. These studies demonstrated that keratinocyte migration and wound closure were delayed in the mechanically unloaded rats. These effects were accompanied by lower density of dermal microvessels, which also lacked the directional growth toward the epidermis typical of normal skin (Radek et al., 2008). These results collectively indicate that both keratinocyte and endothelial cell function are impaired during wound healing in unloading conditions, likely because of alterations of the complex interplay of regulatory signals exchanged between the epidermis and dermis in the skin as a whole. However, due to the extreme complexity in tuning up reliable microgravity animal models, not to mention true spaceflight *in vivo* experiments (Globus and Morey-Holton, 2016), the scientific data required to better understand how epidermal cells behave during healing of skin wounds in low gravity and the exact mechanisms involved are limited yet.

WOUND HEALING: THE ROLES OF KERATINOCYTES AND THE EFFECTS OF MICROGRAVITY

Wound healing is the process that makes organisms resilient to injuries, allowing survival. Being of fundamental importance for life, it basic pathways and mechanisms have been substantially conserved throughout evolution (Eming et al., 2014), although the final outcome diverges from lower vertebrates—like fishes and amphibians—which retain throughout life the embryonic capability to regenerate the missing tissues and organs, and upper vertebrates, which rather heal by reparative scarring (Odelberg, 2005; Bani and Nistri, 2014). As mentioned above, wound healing results from an interplay of diverse cells involved and is classically divided into three phases: inflammation, proliferation, and remodeling, whose mechanisms are partially overlapped both spatially and temporally. Detailed analysis of the complex events and molecular mechanisms of wound healing is outside the scope of this mini-review; here, we will limit to recapitulate the key points. After an injury, clotting suddenly takes place due to immediate interactions among endothelial cells and platelets and activation of the coagulation cascade. Mediators released during this early process trigger an inflammatory reaction summoning neutrophils and macrophages from the bloodstream: in turn, these cells produce pro-inflammatory cytokines and growth factors, resulting in recruitment of stromal cells and their differentiation into myofibroblasts, which are responsible for wound contraction and ECM deposition, and stimulation of endothelial and epithelial cell proliferation in the wound site to induce neoangiogenesis and re-epithelialization, respectively. Clot and tissue debris are eventually removed by macrophages and extracellular hydrolases (matrix metalloproteases MMPs, elastase and plasmin) and tissue repair proceeds towards and terminates with scarring (Singer and Clark, 1999; Li and Kirsner, 2005; Eming et al., 2014; Martin and Nunan, 2015; Thiruvoth et al., 2015).

Many factors can impair wound healing. A crucial one is tissue ischemia, which may be caused by primary vascular diseases, diabetes and persistent local pressure. Another adverse factor is persistence of inflammation, as occurs in necrotic and chronically infected wounds and in burns, which leads to inactivation of growth factors and other molecular stimuli required for tissue repair, trapped by ECM molecules or degraded by extracellular proteases (Han and Ceiley, 2017). A further factor is persistence

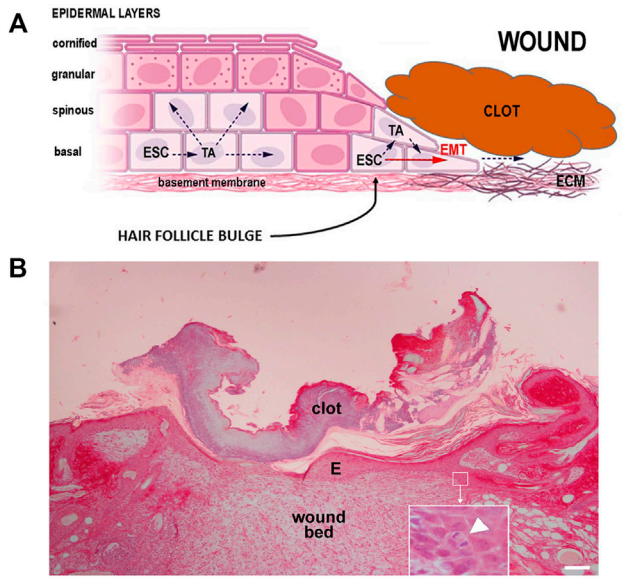

FIGURE 1 | (A) Schematic diagram of epidermal keratinocyte proliferation and migration during wound closure: ESC, epidermal stem cells; TA transit amplifying cells; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix. **(B)** Wound healing in rat skin: a thin, flattened layer of newly formed epidermis (E) lies over the wound bed and under the clot; the inset shows a detail of an ESC undergoing mitosis (arrowhead). Hematoxylin, picrosirius red, scale bar = 100 µm (courtesy Dr. P. Nardini).

of myofibroblast activation, leading to hypertrophic scars, or keloids (Martin and Nunan, 2015; Berman et al., 2017). Complicated wound healing represent a major public health issue, as it requires complex and lengthy treatments, prolonged hospitalization and an increasing burden on healthcare expenses. These problems become even more challenging when transposed to space medicine, considering the limited therapeutic options available to astronauts during long-lasting space flights (Cialdai and Monici, 2013).

Keratinocytes play a major role in wound healing since they are activated during the inflammatory phase to secrete several cytokines and growth factors (Barrientos et al., 2008). The activated phenotype is marked by changes in the cytoskeleton (i.e., expression of prekeratins K6 and K16) and plasma membrane receptors essential for re-epithelialization, allowing keratinocytes to migrate towards the wound to fill the defect (Coulombe, 1997). For successful wound healing, keratinocytes should be able to not only detach from the underlying basal lamina but also to move and migrate through fibrin and ECM of the wound, a process facilitated by MMP-1, which is expressed at high levels at the wound edges (Pastar et al., 2014) (**Figure 1**).

Keratinocyte migration from the free edges of the wound takes place within 24 h. As reported in *Keratinocytes, Stem Cells and Epidermal Homeostasis* above, the major contribution to the new cells needed to close the wound is given by ESCs located in stem niches of hair follicle bulges, whereas ESCs of the basal layers of the interfollicular epidermis likely play a minor role (Jones, 1997; Ito et al., 2005). Migration is accompanied by morpho-functional changes of the keratinocytes: in resting phase they appear as cuboid-shaped basal cells reciprocally connected by desmosomes and fixed over their basement membrane by hemidesmosomes. A few hours after wounding, keratinocytes become flattened and elongated, lose their cell-cell and cell-matrix junctions, detach cytoskeletal intermediate filaments from the inner aspect of plasma membrane at the junctional level, show a thick network of contractile filaments in the cortical cytoplasm extending into newly formed lamellipodia, all typical features of EMT occurring during embryo development. While keratinocytes are migrating, their proliferative potential is inhibited. Migrating basal cells are thought to express specific surface markers such as CD44, at variance with resting basal cells (Lü et al., 2013; Pastar et al., 2014; Michopoulou and Rousselle, 2015).

The mechanisms of wound re-epithelialization have not been completely unveiled. The most commonly accepted model is the "leap frog" theory, whereby keratinocytes migrate two or three cell lengths from their initial position and slide or roll over the similar cells previously implanted in the wound (**Figure 1**). In this way, the epidermal border progressively advances and closes the defect. Such movement depend on surface integrins interacting with fibronectin and newly formed collagen molecules in the wound bed. Keratinocytes are also capable to slide under the scab by exploiting the underlying moist environment (this may also explain the success of occlusive dressings in speeding wound healing). Among the stimuli required for re-epithelialization, TGF-α, keratinocyte growth factor (KGF), and EGF have been identified. Migrating keratinocytes can also produce MMPs to remove damaged matrix: of note, keratinocytes secrete MMP-1/collagenase when in contact with fibrillar collagens of the wound bed, but this secretion is stopped as soon as a new basement membrane is formed and the wound is re-epithelialized. (Steffensen et al., 2001; Li and Kirsner, 2005; Lü et al., 2013; Pastar et al., 2014; Michopoulou and Rousselle, 2015). Taken together, these data suggest that keratinocytes require proper micro-mechanical stimuli to activate their wound closure abilities, which may be altered or absent in low gravity conditions.

During wound healing, keratinocytes can also modulate the functional activity of stromal cells. *In vitro* experiments on skin-equivalent models show that keratinocyte conditioned medium downregulates the production of the profibrotic cytokines TGF-ß and connective tissue growth factor (CTGF) by dermal fibroblasts. Of note, while normal keratinocytes increase fibroblast proliferation but simultaneously reduce collagen production and increase MMP-1 expression and collagen breakdown, thereby promoting normal wound healing, keratinocytes from keloids show higher, and persistent proliferation rates and induce an abnormal, pro-fibrotic phenotype of dermal fibroblasts (Limandjaja et al., 2018).

CONCLUSION

The literature on skin wound healing in weightlessness is relatively poor. *In vitro* studies on immune cells, fibroblasts, endothelial, and epithelial cells cultured both in real and modeled micro-gravity conditions show alterations of

functions involved in wound healing, such as phagocytosis, adhesion/migration, apoptosis, proliferation, intercellular cross-talking, production of inflammatory mediators, ECM molecules, and growth factors, etc. On the other hand, the studies on animal models in unloading conditions are scanty and insufficient to get definite conclusions. In astronauts, impaired immune function, signs of chronic inflammation, metabolic alterations and skin atrophy have been observed, all factors capable to jeopardize the known skin repair mechanisms (Farahani and DiPietro, 2008; Cialdai and Monici, 2013). The space biology community is aware that only a few wound healing studies have been performed in a real microgravity environment. To fill this gap, specific experiments have been scheduled for being carried out at the ISS in the next months: these have been designed by an international multi-disciplinary research team with the aim to provide further insight into the effects of real unloading conditions on surgically wounded and sutured human skin tissues (Monici et al., 2019; 2021). Despite this uncertain scenario, careful appraisal of the literature suggests a possible general response to microgravity across various cell, tissue, and organ experimental models, consisting of a partial inhibition of the transition of stem cells towards TA cells and terminally differentiated adult cells (Blaber et al., 2014). Thus, gravity-related micro-mechanical stimulation appears a fundamental need for tissues to maintain their regenerative potential and overall health and emerges as a basic feature of mammalian life on Earth under normal gravity load. This notion also suggests that long-lasting microgravity may result in compromised tissue homeostasis and wound healing capability. For this reason, management of wounds during enduring space missions represents a very challenging issue, especially considering the limited availability of diagnostic and therapeutic tools and, likely, the lack of a specialized on-board medical staff. In low-orbit missions, the management of traumatic and surgical emergencies consists of patient stabilization and rapid return to Earth. In future interplanetary missions, timely medical evacuation to Earth would not be possible, nor would telemedicine by either surgical robots or remote guidance of crew medical actions because of communication lag. Nonetheless, the rapid advancements in robotics allow to foresee that the medic/paramedic crew could be assisted by specifically designed robots, programmed to perform basic medical interventions, such as surgical sutures, anaesthesia and vital signs monitoring, as well as diagnostic procedures by ultrasound, computed tomography scan or magnetic resonance imaging, thus improving the capability of on-board assistance to severely ill or injured astronauts (Pantalone et al., 2021). This bio-engineering challenge also underscores the need for further studies on wound healing in space to better understand the problems and define adequate countermeasures.

AUTHOR CONTRIBUTIONS

Conceptualization, resources, writing, review, and editing: SB and DB.

FUNDING

This research was funded by the Italian Space Agency (Wound Healing and Sutures in Unloading Conditions ASI N. 2018-14-U.0).

ACKNOWLEDGMENTS

The authors are indebted with Monica Monici for her skillful assistance during the drafting of this document.

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frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 25 March 2022
doi: 10.3389/fbioe.2022.825339

A Composite Hydrogel Containing Mesoporous Silica Nanoparticles Loaded With *Artemisia argyi* Extract for Improving Chronic Wound Healing

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *30 November 2021*
Accepted: *28 February 2022*
Published: *25 March 2022*

Citation:
Xue L, Deng T, Guo R, Peng L, Guo J, Tang F, Lin J, Jiang S, Lu H, Liu X and Deng L (2022) A Composite Hydrogel Containing Mesoporous Silica Nanoparticles Loaded With Artemisia argyi Extract for Improving Chronic Wound Healing. Front. Bioeng. Biotechnol. 10:825339. doi: 10.3389/fbioe.2022.825339

Chronic wounds are a major health problem with increasing global prevalence, which endangers the physical and mental health of those affected and is a heavy burden to healthcare providers. *Artemisia argyi* extract (AE) has excellent antibacterial and anti-inflammatory properties. In this research, we developed AE loaded composite hydrogel scaffold based on methacrylate gelatin (GelMA)/methacrylate hyaluronic acid (HAMA) and mesoporous silica nanoparticle (MSN) as sustained-release drug carrier vehicles for the treatment of chronic wounds. The presented GelMA/1%HAMA hydrogel possessed stable rheological properties, suitable mechanical properties, appropriate biodegradability, swelling, sustained-release AE capacity. *In vitro* antibacterial and cell experiments showed that the GelMA/HAMA/MSN@AE hydrogel had excellent antibacterial activity and biocompatibility and induced macrophages to differentiate into M2 phenotype. *In vivo* wound healing of rat full-thickness cutaneous wounds further demonstrated that the prepared GelMA/HAMA/MSN@AE hydrogel could significantly promote chronic wound healing by upregulating the expression of IL-4, TGF-β1, CD31, and α-SMA but downregulating the expression of TNF-α and IFN-γ and promoting M1-M2 macrophages polarization. Altogether, we believe that the GelMA/HAMA/MSN@AE hydrogel will have wide application prospects in healing chronic wounds.

Keywords: *Artemisia argyi* extract (AE), mesoporous silica nanoparticle (MSN), methacrylate gelatin (GelMA), methacrylate hyaluronic acid (HAMA), chronic wounds

INTRODUCTION

Chronic wounds have been regarded as a "silent epidemic," presenting a major challenge for the healthcare system (Ferriol and Moran, 2021), among which the most common ones are varicose ulcers, diabetic foot ulcers, and pressure ulcers (Deptuła et al., 2021; Osi et al., 2021). The prevalence of chronic diseases has also increased annually, especially in developed countries, as a result of the improvement in quality of life and the increase in risk factors such as diabetes and obesity (Milho et al., 2019). In addition, due to the progress in healthcare, the continuous improvement of the

survival rate of the population and the emergence of an aging population, the incidence and prevalence of chronic wounds will continue to rise in the coming decades (Wang et al., 2019).

The impact of chronic nonhealing wounds is enormous, resulting in prolongation of hospitalization and economic burden on patients with the use of expensive wound care products, which further increases the burden on the national healthcare system (Jennings et al., 2019; Rodrigues et al., 2019). Meanwhile, it also endangers the physical and mental health of patients, even leading to amputation or sepsis, increasing the disability rate, and reducing their quality of life (Liu et al., 2020). In addition, patients may feel self-blame for the wound and feel powerless for the prognosis, but positive and correct treatment can show the hope of healing (Kapp et al., 2018). Additionally, the possibility of wound healing is related to wound duration. The possibility of successfully healing the wound will significantly reduce with the extension of the time of wound appearance, which will increase the difficulty of wound healing (Borda et al., 2018). Therefore, the demand for wound treatment and care is increasing. The effective treatments of promoting wound healing and reducing the risk of wound infection and inflammation are still urgently required in chronic infectious wounds.

As a commonly used Chinese herbal medicine, *Artemisia argyi* has been widely used in promoting wound healing. The active compounds from *Artemisia argyi* leaves can easily permeate the skin and have the effects of activating blood circulation, transforming qi, dispelling dampness, dissipating cold, eliminating swelling, resolving blood stasis, relieving pain, and promoting necrotic tissue shedding (Hou et al., 2019). Moreover, *Artemisia argyi* extract (AE) has a variety of pharmacological activities, such as anti-allergic, anticoagulant, complement activation, anti-inflammatory, antibacterial, antiviral, and sedation, which can reduce tissue edema, promote granulation growth for wound tissue, and avoid adverse consequences of long-term antibiotic treatment (Bao et al., 2013; Yun et al., 2016; Wang et al., 2019; Yang et al., 2020). However, there are some limitations of AE application, such as poor aqueous solubility and easy volatilization, resulting in low bioavailability and thus making its application difficult in practice. Prior study has been demonstrated that mesoporous silica nanoparticles (MSN) are excellent candidates for drug loading owing to their good biocompatibility, biochemical and physicochemical stability, large specific surface area and pore volume, and strong loading capacity, which can improve the low bioavailability of drugs (Wang et al., 2021).

Recently, natural hydrogels have been applied to the field of biomedicine due to unique physical and biological characteristics, which are similar to the extracellular matrix (Elkhoury et al., 2021). Meanwhile, hydrogels not only can be used as drug release carriers of antimicrobial substances but also kill bacteria through their inherent antimicrobial properties (Feng et al., 2021). As an inexpensive and easily obtained natural biomaterial, gelatin can be obtained by partial hydrolysis of collagen. It has been widely used in 3D bioprinting and biomaterials (Yue et al., 2017; Garcia-Cruz et al., 2021), especially in repairing skin tissue. However, pure methacrylated gelatin (GelMA) hydrogel presents low mechanical modulus, and thermal stability is relatively poor. Therefore, we added methacrylated hyaluronic acid (HAMA) to form an interpenetrating polymer network (IPN) to improve the viscosity and maintain its gel stability at higher temperatures (Schuurman et al., 2013).

The principal purpose of this study was to prepare a suitable hydrogel system carrying AE to treat chronic wounds, which have antibacterial properties, can promote M2 macrophage polarization, and enhance collagen deposition and angiogenesis, thus effectively promoting wound healing. In this study, GelMA and HAMA were synthesized, characterized, and then mixed with MSN-loaded AE. The GelMA/HAMA/MSN@AE hydrogel was prepared under an ultraviolet (UV) light condition. The porous structure, physical properties, drug release, and antibacterial properties of this hydrogel were characterized. The slow and sustained release of AE in the hydrogel can be realized by a large specific surface area and large pore volume of MSN. In addition, the cytocompatibility in hydrogels was studied in detail by CCK-8 staining and living/dead cell staining. Moreover, an excellent therapeutic effect for promoting the repair of skin defects was shown in *in vivo* experiments. In summary, all of these results demonstrated that the GelMA/HAMA/MSN@AE hydrogel could improve patients' feeling of medication, prolong the efficacy time of AE, and facilitate chronic wound healing.

EXPERIMENTAL SECTION

Materials

Artemisia argyi extract (AE) was purchased from Anhui Chinature Biological Co., Ltd. (Anhui, China). Gelatin (Gel, derived from cold water fish skin, adhesive strength ~500 g bloom) was obtained from the Sigma-Aldrich Chemical Company (Shanghai, China). Hyaluronic acid (HA, Mw = 4–10 kDa) and methacrylic anhydride (MA) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was obtained from Yinchang New Material Co., Ltd. (Shanghai, China). Live/dead cell staining kits and Cell Counting Kit-8 (CCK-8) were obtained from BestBio Co., Ltd. (Shanghai, China). The bacteria strains of *Escherichia coli* (*E. coli*, ATCC-8739) and *Staphylococcus aureus* (*S. aureus*, ATCC-14458) were obtained from Luwei microbial Sci&Tech Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were obtained from Gibco (Waltham, MA, United States).

Synthesis of Methacrylated Gelatin (GelMA)

GelMA was prepared according to the previous method (Zandi et al., 2021; Zhang et al., 2021). Briefly, 20 g of gelatin was dissolved in 250 ml ultrapure water at 60°C. Then, 12 ml of MA was added dropwise into the gelatin solution. After stirring 12 h at 37°C, the solution was dialyzed in a dialysis bag (3,500 Da molecular weight cutoff) against ultrapure water for 3 days. Then, the solution was freeze-dried to obtain GelMA polymer and stored at −20°C for future use.

Synthesis of Methacrylated Hyaluronic Acid (HAMA)

HAMA was prepared as previously described with slight modification (Fan et al., 2020; Chen et al., 2021). Briefly, 1 g of HA was stirred in 100 ml of ultra-pure water until completely dissolved. Then, 3 ml of MA was added dropwise into the hyaluronic acid solution and stirred for 8 h at room temperature (pH was kept at about 8.5 by adding 5 mol/L sodium hydroxide). Finally, the reaction mixture was dialyzed in a dialysis bag (MWCO 8–14 kDa) against ultra-pure water for 3 days. The resulting solution was freeze-dried to attain HAMA polymer and stored at −20°C for further use.

Synthesis of Mesoporous Silica Nanoparticle (MSN)

Uniform-sized MSN was synthesized according to a previously reported method (Wu et al., 2018; Xie et al., 2019). Totally, 2 g of hexadecyl trimethyl ammonium chloride (CTAC) and 0.07 g of triethanolamine (TEA) were dissolved in 20 ml ultra-pure water under vigorous stirring at 95°C. After the solution stabilized for 1 h, 1.5 ml of tetraethyl orthosilicate (TEOS) was dropped into the resulting solution within 2 min, and the reaction continued to stir for 1 h at 95°C. The resulting MSN was collected by centrifuged at 15,000 r/min for 5 min and washed three times with ethanol to remove the residual reactants. The final sample was dialyzed in a dialysis bag (MWCO 3500 Da) against 1% (wt %) NaCl/methanol solution for 3 h to remove the template agent CTAC and centrifuged at 15,000 r/min. Morphological analysis of MSN was visualized under a transmission electron microscope (TEM, Zeiss LIBRA 200 FEG, Oberkochen, Germany).

In order to prepare AE-loaded MSN (MSN@AE), 10 mg of AE and 50 mg MSN were added into 5 ml of 25% ethanol solution and stirred for 24 h at 37°C. The mixture was centrifuged, and the AE concentrations of residues in the supernatant were measured by a UV spectrophotometer to determine the MSN loading capacity (UV-5200, Metash Instruments, Shanghai, China).

Preparation of Composite Hydrogels

The freeze-dried GelMA and HAMA were dissolved in PBS at 60°C to make the final GelMA concentrations of 10% (w/v) with different HAMA concentrations of 0.5% (w/v), 1% (w/v), and 2% (w/v), named GelMA, GelMA/0.5%HAMA, GelMA/1%HAMA, and GelMA/2%HAMA, respectively. Then, 0.1% (w/v) photo-initiator LAP was added, and the prepolymer solution then exposed to the UV light (365 nm) for 30 s. For the preparation of GelMA/HAMA/MSN and GelMA/HAMA/MSN@AE, the MSN 0.5% (w/v) and MSN@AE 0.5% (w/v) were added indirectly into above GelMA10% (w/v)/HAMA 2% (w/v) mixed solutions, respectively.

Characterization of Polymers

The chemical structure of GelMA and HAMA were characterized by ^{1}H NMR using a nuclear magnetic resonance spectrometer (DR×500, Bruker, Germany). Fourier transform infrared spectroscopy (FTIR) spectra of Gel, GelMA, MSN, MSN/AE, GelMA/HAMA/MSN, and GelMA/HAMA/MSN@AE were measured by Fourier transform infrared spectrometer (Spectrum One, Perkin Elmer, Norwalk, United States).

The surface area, pore size, and pore volume of MSN and MSN/AE were measured by the BET method using Micromeritics ASAP2460 instrument (Norcross, GE, United States). The micromorphology of the composite hydrogels was analyzed by scanning electron microscopy (SEM; S-3400, Hitachi, Japan). The average pore size of hydrogels was calculated by Nanomeasure software. Thermal gravimetric analysis (TGA) measurements of MSN, MSN/AE, GelMA/HAMA/MSN, and GelMA/HAMA/MSN@AE were performed with a TGA thermogravimetric analyzer (Netzsch Instruments, Selb, Germany).

Physical Properties of Hydrogels

Swelling Ratio

The swelling ratio of the composite hydrogel was investigated using a gravimetric method (Osi et al., 2021). The test hydrogels were placed in PBS solution. At given time points, the samples were weighed after wiping off the surface excess water by a weighing paper. The swelling ratio of hydrogels was calculated according to the following formula:

$$\text{Swelling ratio} = (W_t - W_0)/W_0 \times 100\%,$$

where W_t and W_0 mean the weight of hydrogel at times t and 0, respectively.

Porosity

The hydrogel was soaked in PBS for 24 h to reach swelling equilibrium and then freeze-dried. The volume (V) of the freeze-dried hydrogel was accurately measured with a Vernier caliper. The mass of the dry sample (W_1) and the mass of the dry sample immersed in anhydrous ethanol (density = ρ) for 2 h (W_2) were weighed. The porosity (P) was calculated according to the following formula:

$$P(\%) = \frac{W_2 - W_1}{\rho \times V} \times 100.$$

Rheological Measurements

Dynamic strain scanning was carried out with a TA rheometer instrument (MCR 301, Graz, Austria). The change curves of the storage modulus (G′) and loss modulus (G″) of the hydrogels were detected. In order to assess the shear viscosity behavior, the flow sweep assay was tested with a shear frequency range of 0.1–10 Hz at room temperature. Moreover, the viscoelastic region with a fixed frequency of 1 Hz was recorded over time performed at a strain of 1%.

Compression Test

The compression modulus of GelMA/HAMA hydrogels was recorded by a universal testing machine (ELF3200, Bose, United States). The 500 μL hydrogels were prepared as a cylindrical shape (height = 6 mm and diameter = 12 mm). The compressive strain rate was fixed at 0.05 mm/s, and the strain level reached 60% of the maximum. Meanwhile, Young's

modulus could be calculated as the slope at the initial linear region.

In Vitro Biodegradation

The 300 μL hydrogel samples were immersed in a PBS solution containing either 0 or 1000 U/ml lysozyme at 37°C. At given time points, the samples were taken out from the solution and rinsed three times with ultra-pure water. The dry weight of samples was weighed after freeze-drying. The weight loss ratio was calculated according to the following equation:

$$\text{Weight loss ratio } (\%) = W_t/W_0 \times 100,$$

where W_t and W_0 corresponded to the weight of lyophilized hydrogel at times t and 0, respectively.

In Vitro Drug Release

The composite hydrogels were immersed in 10 ml PBS and incubated at 100 rpm at 37°C. At given time points, 1 ml supernatant was collected and replaced with 1 ml fresh PBS. The AE concentration was measured using a UV spectrophotometer at λ = 345 nm. The concentration of AE released from hydrogels was back-calculated using a standard curve.

In Vitro Antibacterial Behavior

Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) were cultured in a sterilized LB liquid medium and diluted to an optical density of 600 nm of 0.1. Then, 400 μL tested hydrogels were prepared in a 48-well plate. Each sample was cocultured with 1.8 ml bacterial suspension and incubated at 37°C for 4 h under constant shaking. The resulting bacterial liquid (100 μL) was plated on an LB agar plate after a series of dilutions and incubator for 24 h. In order to determine the antibacterial activity, the antibacterial rate was determined according to the equation as follows:

$$\text{Antibacterial rate}\,(\%) = \frac{(N_{control} - N_{sample})}{N_{control}} \times 100,$$

where $N_{control}$ and N_{sample} are the numbers of bacterial colonies of the GelMA/HAMA hydrogel sample and the hydrogel with different concentrations of AE, respectively.

Assessment of Cytocompatibility

Mouse fibroblast cells (L929 cells) were cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in an incubator containing 5% CO_2.

The CCK-8 and live/dead cell staining methods were used to assess the cytotoxicity of hydrogels. Briefly, 500 μL hydrogels were prepared in 48-well plates and irradiated by UV light for 30 s. Then, 400 μL cell suspension (1×10^4 cells/ml) was added to the hydrogel surface. After incubation for 1, 2, and 3 days, each well was immersed in 300 μL CCK-8 and incubated in a 5% CO_2 humidified incubator at 37°C for 1 h. The absorbance of each well was measured at 450 nm using a microplate reader (SH1000, Corona, Japan).

For the live/dead staining, each well was added 100 μL live/dead stock solution and incubated for 20 min. Cells were observed under an inverted fluorescence microscope (Olympus FV3000, Nikon, Japan). Meanwhile, the tested hydrogels were fixed to evaluate L929 cells morphology. TRITC Phalloidin was used to counterstain cytoskeleton, and diamidino-2-phenylindole (DAPI) was used to counterstain cell nuclei.

The Transformative Effect on Macrophage Phenotype

The RAW 264.7 cells were seeded in a 6-well plate containing DMEM at densities of 1×10^4 cells/well and cultivated overnight at 37°C in an incubator containing 5% CO_2 for later use. Secondly, the sterilized hydrogel was immersed in the DMEM (containing LPS); Then, the hydrogel extract was collected after culturing at 37°C for 24 h and cocultured with cells for 48 h. The sample without the hydrogel extract was used as the control group. Characterization of M1/M2-polarized macrophages was determined by the WB technique. Raw 264.7 macrophages were lysed using RIPA lysis buffer supplemented with 1% PMSF on ice for 20 min and subjected to Western blot analysis. Each protein sample was subjected to electrophorese by 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Then, the protein was transferred to polyvinylidene difluoride (PVDF) membrane, further incubated with different primary antibodies, anti-rabbit iNOS (Abcam, Ab178945, 1: 1,000), and anti-rabbit CD206 (Novus NBP1-90020, 1: 1,000), overnight at 4°C and treated with horseradish peroxidase- (HRP-) conjugated secondary antibodies for 1 h at 22°C. Blots were developed using an enhanced chemiluminescent reagent (Beyotime, Jiangsu, China), and the signals were detected *via* X-ray films. The expression levels of protein were quantified by IPP 6.0 software.

Evaluation of *In Vivo* Wound Healing

Rat Wound Model

All animal experimental protocols have been reviewed and approved by the Animal Protection and Use Committee of Jinan University. Sprague Dawley female rats, 200–250 g, were used in this study. Rats were anesthetized by 3% pentobarbital *via* intraperitoneal injection (45–60 mg/kg) prior to surgery. The backs of rats were depilated with a depilatory cream and disinfected with iodophor. Four 12 mm diameter circular full-thickness skin injuries were created on the dorsum of rats by excising the dorsum with a distance of 2 cm. Then, the wound was coated with gauze, GelMA/HAMA hydrogel (200 μL), GelMA/HAMA/MSN@AE hydrogel (200 μL), commercially available hydrocolloid dressing, respectively. The hydrogels were fixed by Tegaderm (3M) to cover and protect the wound area. Sterile medical cotton gauze was served as the control group. Following surgery, each rat was housed in a single cage, and the wound hydrogels were replaced every 3 days.

Wound Closure Evaluation

The physical appearance was photographed, and the area of wounds was calculated using the IPP 6.0 software on days 3,

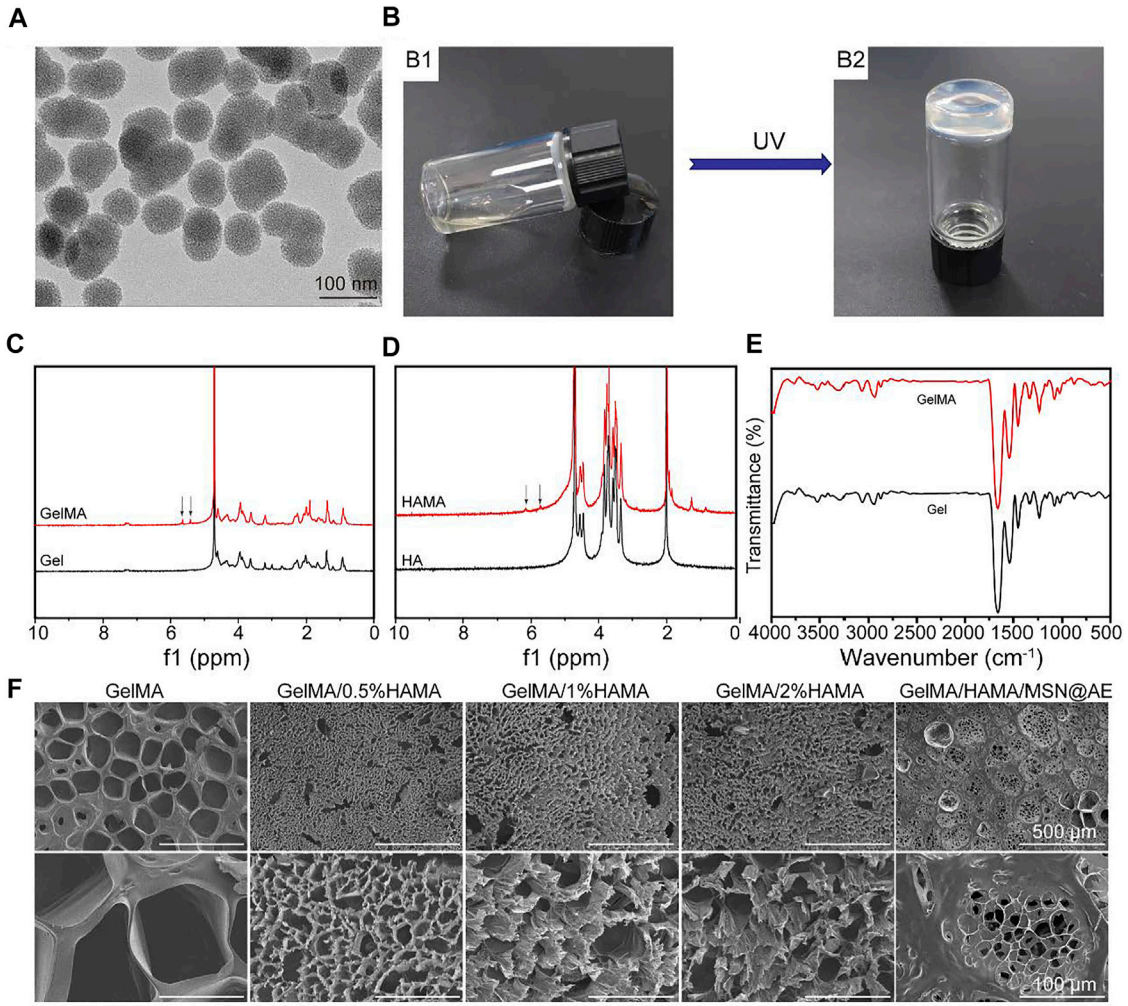

FIGURE 1 | (A) TEM images of MSN. **(B)** Photograph of the GelMA/HAMA hydrogel through a UV cross-linking. **(C)** ^{1}H NMR spectra of Gel and GelMA. **(D)** ^{1}H NMR spectra of HA and HAMA. **(E)** FTIR spectra of Gel and GelMA. **(F)** SEM images of GelMA/HAMA hydrogels with different HAMA concentrations.

7, 10, and 14 after surgery. The wound closure rate was expressed as the percentage values of the day 0 measurements. The wound tissues, including the wound site and unwounded area, were collected for the following experiment.

Histological Examination

Each wound tissue was isolated and fixed in 4% paraformaldehyde solution for 24 h, then, dehydrated through a graded series of ethanol, transferred into dimethylbenzene, and embedded in paraffin. Serial sections (4 μm thick) were cut and stained with hematoxylin and eosin (H&E) and Masson's trichrome staining. Images of the stained sections were captured by the microtome (RM 2016, Leica, Shanghai, China).

Immunofluorescence

After paraffin sections were rehydrated, slices were incubated in an antigen retrieval solution and blocking serum. Then, the primary antibodies, α-SMA (Servicebio, GB13044, 1:300), CD31 (Servicebio, GB113151, 1: 1,000), transforming growth factor-β_1 (TGF-β_1, Servicebio, GB13028, 1: 200), tumor necrosis factor-α (TNF-α, Servicebio, GB13452, 1: 200), interleukin-4 (IL-4, Bioss, bs-0581r, 1: 200), interferon-γ (IFN-γ, Proteintech, 15365-1-AP, 1: 2000), iNOS (Proteintech, 18985-I-AP, 1: 1,000), and CD206 (Novus, NBP1-90020, 1: 1,000), were added at 4°C overnight. Next, these sections were incubated with HRP-labeled goat anti-rabbit IgG secondary antibody for 50 min at room temperature. Subsequently, the sections were reacted with DAB solution after being washed in PBS, and the nuclei were counterstained with DAPI. Images of smear specimens were also collected by the inverted fluorescence microscope.

Statistical Analysis

All quantitative experimental values from the studies were presented as means ± standard deviation. Analysis of variance (one-way ANOVA statistical test) was used to determine significant differences between two groups. A value of $p < 0.05$ was defined as statistically significant: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS AND DISCUSSION

Characterization of the Hydrogel

We have developed a UV-cross-linked biocompatible hydrogel composed of photosensitive GelMA and HAMA. GelMA and HAMA were combined by cross-linking to improve mechanical properties and biological stability. Moreover, AE was used as an anti-inflammatory, antimicrobial agent, which was encapsulated by MSN to improve its bioavailability and loaded into the GelMA/HAMA hydrogel for application in faster healing of chronic wounds. These hydrogels also exhibited good antibacterial properties and comparable modulus to human soft tissue (Chen, 2017; Chen et al., 2017).

TEM imaging of MSN showed a uniform spherical shape with average diameters of ~70 nm (**Figure 1A**). The BET analysis showed that the surface area of MSN and MSN@AE was 841.6659 and 793.2698 m^2/g, the adsorption cumulative pore volume was 1.488,417 cm^3/g and 1.377,355 cm^3/g, and the adsorption average pore width was 6.9999 and 6.9297 nm, respectively. N_2 adsorption/desorption isotherms of MSN and MSN@AE are shown in **Supplementary Figure S1**. As shown in **Figure 1B**, the synthesized GelMA/HAMA mixture hydrogel was flowing liquid before cross-linking and gradually changed into a solid phase after the UV curing. Photographs of samples containing HAMA with different concentrations are shown in **Supplementary Figure S2**. All the composite hydrogel is colorless and transparent, with a smooth surface.

The chemical structure of GelMA and HAMA was investigated by ^{1}H-NMR. The distinctive double peaks (δ = 5.4 and 5.6 ppm) were observed in GelMA (**Figure 1C**), which verified that the gelatin had been chemically linked with methacrylate-related motifs (Yi et al., 2018). Meanwhile, the distinctive double peaks (δ = 5.7 and 6.1 ppm) were observed in HAMA (**Figure 1D**), which verified that the methacrylic group was successfully grafted onto the molecular backbone of hyaluronic acid (Eke et al., 2017).

As shown in **Figure 1E**, FTIR was used to characterize the presence of specific peak distribution of the infrared spectrum of polymers before and after modification. The spectrum of GelMA displayed the characteristic hydroxyl group peaks at 3,298 cm^{-1} (N-H stretching). Bands were observed at 1,656, 1,539, and 1,448 cm^{-1} belonging to the CH_2 wagging vibrations of amide I, plane bending of N–H bond of amide II, and C–H stretching vibrations of amide III, respectively. Meanwhile, the characteristic peak intensities of amide III, amide II, and C=O in GelMA had a partial shift and change compared with gelatin, which demonstrated the newly formed amide bond. As shown in **Supplementary Figure S3**, the broad absorption band at 3,440 cm^{-1} is related to the stretching vibration of the OH group. The strong absorption band at 1,083 cm^{-1} is the characteristic absorption band of Si-O-Si antisymmetric stretching vibration. However, there are no other absorption peaks related to C-H, which indicates that the organic guides in the MSN have been removed at high temperatures. Therefore, FTIR showed that the composition of the MSN should be silica. There was a large wide peak in 3,500–3,700 cm^{-1}, representing the characteristic absorption peak of hydroxyl in the three materials.

To further understand the thermal stability of MSN and hydrogels, we carried out their TG analysis. Meanwhile, in order to confirm the influence of *Artemisia argyi*, the TG curves of MSN and MSN@AE were almost identical (**Supplementary Figure S4**). Thermal degradation was carried out in three steps. The first step was related to free water loss in the hydrogel. GelMA/HAMA/MSN and GelMA/HAMA/MSN@AE hydrogels had a slight weight loss due to the loss of free water, usually below 200°C. The second step occurred between 200°C and 400°C, which was due to protein degradation. The third step occurred between 400°C and 600°C, which was due to the carbonization of the polymer material. The sample was no longer weightless after 600°C. It showed that water and other organic components had been completely volatilized, and the rest was pure silica powder.

Furthermore, SEM was conducted to visualize the changes in micromorphology of the hydrogels after freeze-drying. All hydrogels possessed a uniform porous network structure, which played an important role in the effective diffusion of nutrients and gases and provided a suitable moisture environment for the wound (Zhang et al., 2021). **Figure 1F** revealed that HAMA-free GelMA hydrogel consisted of large and nonhomogeneous pores, while the GelMA/HAMA hydrogels exhibited smaller and more uniform porous structures. These variations in the internal structure of the hydrogel were primarily caused by the enhancement of cross-linking degree in the GelMA/HAMA hydrogel. This three-dimensional porous structure was similar to other natural polymer hydrogel scaffolds (Chen et al., 2017), which could absorb secretions of the wound, avoiding soft tissue maceration and promoting wound healing. As shown in **Supplementary Figure S5**, the average pore sizes of GelMA, GelMA/0.5%HAMA, GelMA/1% HAMA, GelMA/2%HAMA, and GelMA/HAMA/MSN@AE hydrogels were 138.0 ± 20.1 μm, 14.0 ± 5.0 μm, 35.6 ± 9.4 μm, 31.8 ± 6.0 μm, and 26.1 ± 0.9 μm, respectively.

Swelling Ratio and Porosity Assay

The swelling ability of GelMA/HAMA hydrogels with different HAMA concentrations is shown in **Figure 2A**. The swelling ability of all hydrogels became almost saturated after 5 h, reaching their equilibrium swelling. The swelling ratios of HAMA-free GelMA, GelMA/0.5%HAMA, GelMA/1%HAMA, and GelMA/2%HAMA hydrogels were 5.7 ± 0.5%, 9.0 ± 0.4%, 12.6 ± 0.7%, and 16.0 ± 0.9%, respectively. GelMA/HAMA mixture exhibited an excellent water absorption capacity.

Usually, hydrogel dressings have excellent liquid absorption ability, which can maintain a moist wound environment and slow down the speed of liquid evaporation (Qu et al., 2018). Meanwhile, these unique characteristics have a great impact on drug release and practical applications, which endowed hydrogel with a good ability to transport nutrients and wastes (Wang et al., 2021; Yang et al., 2021). As shown in **Supplementary Figure S6**, the porosities of GelMA, GelMA/0.5%HAMA, GelMA/1%HAMA, and GelMA/2%HAMA hydrogels were 74.7 ± 6.1%, 80.4 ± 6.4%, 86.1 ± 5.0%, and

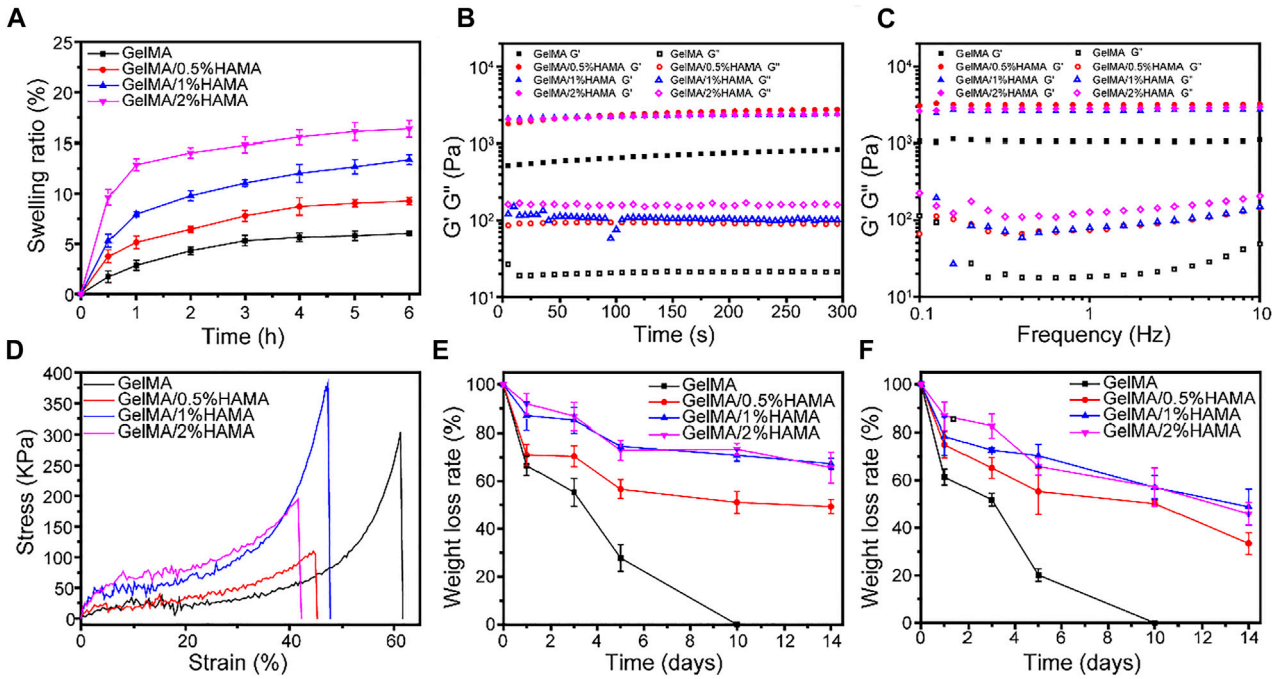

FIGURE 2 | (A) The swelling ratio of hydrogels. **(B)** The rheological properties. **(C)** The viscosity of hydrogels with frequency ranging from 0.1 to 10 Hz. **(D)** Compressive stress-strain curves of hydrogels. **(E)** The weight loss curve of hydrogels in PBS. **(F)** The weight loss curve of hydrogels in PBS containing 1000 U/mL lysozyme.

92.5 ± 8.8%, respectively. A prior study reported that the high porosity could provide channels for nutrient supply and metabolic exchange for internal cells (Li et al., 2016).

Rheological and Compression Analysis

In order to evaluate the durability and integrity, the effect of HAMA concentration on the mechanical properties, the rheological properties were studied. For rheological analysis, the viscoelastic properties of hydrogels are shown in **Figure 2B**. When the oscillatory shear strain was fixed at 1%, it could be seen that the storage modulus (G′) of all samples was much larger than the loss modulus (G″), suggesting that the hydrogel gels rapidly. With the frequency varying from 0.1 to 10 Hz, the change of G′ was almost constant and was still larger than that of G″, indicating the elastic solid properties and good stability of hydrogels.

A wound hydrogel dressing with appropriate mechanical properties can facilitate wound healing, which is conducive to maintaining hydrogel integrity and protecting the wound from external impact. The differences of compression modulus between GelMA hydrogel with different concentrations of HAMA under compressive stress are displayed in **Figure 2D**. The result showed that about 60% of strain could break the GelMA hydrogel, but further increasing HAMA concentration will increase brittleness. The compression modulus of GelMA/1% HAMA hydrogel is significantly highest, up to 381.9 kPa, with about 1.3-fold higher than that of simple GelMA hydrogel, which was similar to the dermis of human skin (Lu et al., 2021). As shown in **Supplementary Figure S7**, Young's moduli of GelMA, GelMA/0.5%HAMA, GelMA/1%HAMA, and GelMA/2%HAMA hydrogels were 3.3 ± 0.7, 14.5 ± 3.6, 42.7 ± 14.1, and 27.9 ± 6.8 kDa, respectively. It is seen that the GelMA/1%HAMA hydrogel has a more compact network than other hydrogels. In addition, although the failure strain of the GelMA/1%HAMA hydrogel decreased to 47%, it could still compress to 40% without breaking. In summary, 1% HAMA was selected as a suitable concentration for obtaining a composite hydrogel.

In Vitro Degradation and AE Release Studies

The degradation behavior is an essential property to evaluate the ability to close tissues and support tissue regeneration (Tavafoghi et al., 2020). As shown in **Figure 2E**, all hydrogels exhibited obvious mass losses with increasing the incubation time. Besides, the degradation rate of samples quality in PBS without lysozyme was not as fast as in lysozyme. The GelMA hydrogel was completely degraded at 10 days. With the increase in the HAMA content, the degradation rate decreased gradually, which was likely related to the formation of a higher cross-linking-density network and denser porous structures in the hydrogel. The SEM images of freeze-dried GelMA and GelMA/HAMA hydrogels after degradation at day 3 are shown in **Supplementary Figure S8**. After degradation, the pore sizes of the hydrogel were increased and the porous structure was destroyed, which indicated that hydrogel was degraded with incubation. In the presence of lysozyme, the pore size of hydrogel was larger and the degradation was

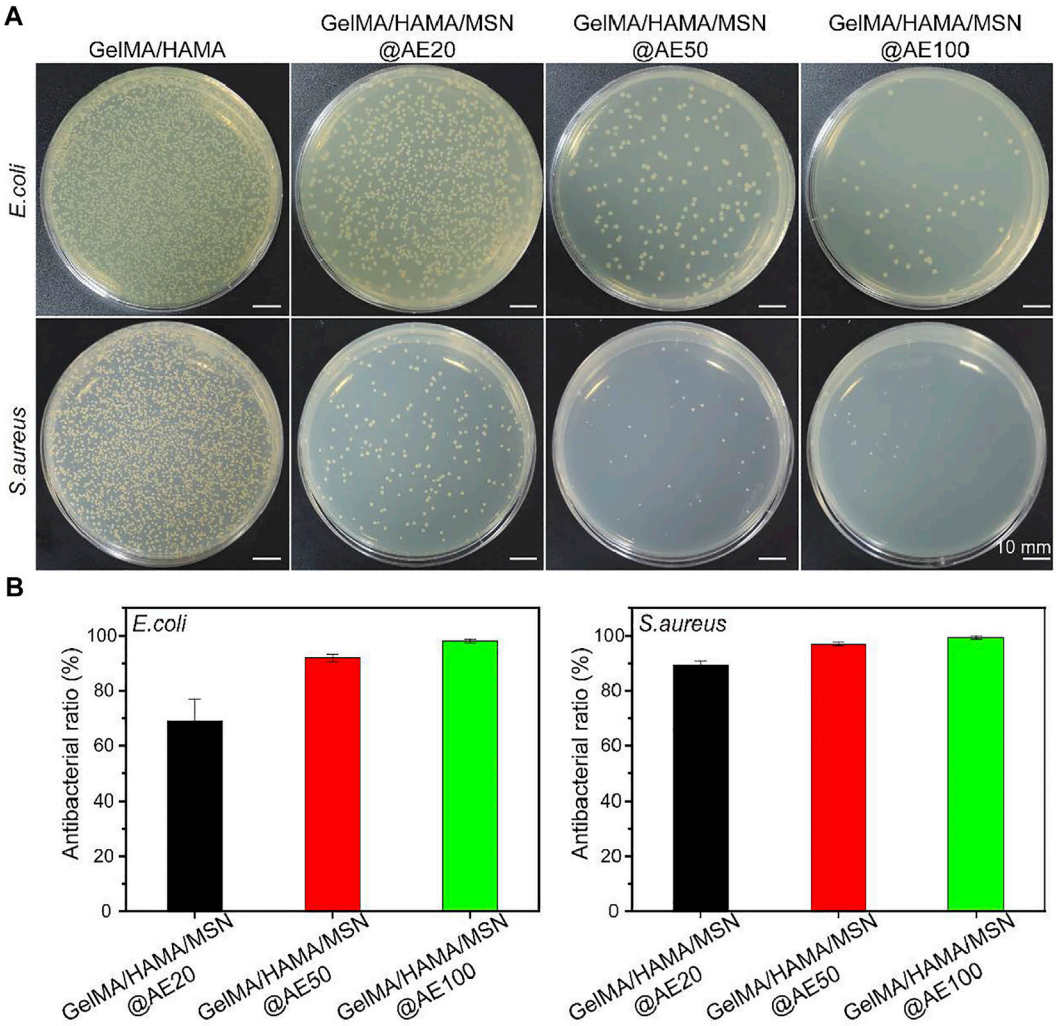

FIGURE 3 | (A) Bacterial colonies of *E. coli* and *S. aureus* after coculture with GelMA/HAMA/MSN@AE hydrogel with different AE concentrations. **(B)** Antibacterial rate of *E. coli* and *S. aureus*.

faster. The cracks and fragments' porous structure of GelMA hydrogel containing 1%HAMA was less than that of pure GelMA hydrogel, which demonstrated that adding HAMA could slow down the degradation rate of hydrogel and maintain the porous structure.

The traditional ways of medication administration were directly mixing the drug with sterile gauze or hydrogel, but the encapsulated function drugs were quickly released or absorbed by gauze, resulting in the inability of drug release. Moreover, the action time of the drugs was greatly shortened, which could not meet the demand for long-term drug therapy during the process of tissue regeneration. In this study, the release curve of AE was measured to confirm whether the GelMA/HAMA hydrogel and MSN can reduce the initial burst release and realize sustained release of AE. **Supplementary Figure S9A** showed the standard curve of AE. **Supplementary Figure S9B** shows that AE from GelMA/HAMA/AE hydrogel experienced a burst release. Approximately 48.1% of the loaded AE was released at the beginning 8 h. In the following 48 h, AE was released slowly and reached a plateau, with a cumulative release of about 93.9%. In contrast, the release of AE from GelMA/HAMA/MSN@AE hydrogel was significantly prolonged. About 9.3% of AE was sustainably released at 8 h, and the AE-releasing curve was close to zero-order kinetic drug-releasing profile. These phenomena were mainly because the AE adsorbed on the outer surface and pore surface of MSN was directly dissolved into PBS without the diffusion process, resulting in a sudden release. After that, when PBS (the release medium) permeated into the MSN channels, AE diffused from the mesoporous structure of MSN to the hydrogel structure first and then escaped from the porous structure of the hydrogel, producing a concentration gradient across the surrounding fluid to achieve the sustained release of AE.

Antibacterial Activity Evaluation

To further evaluated the antibacterial activity of hydrogels *in vitro*, the tested hydrogels with different AE concentrations

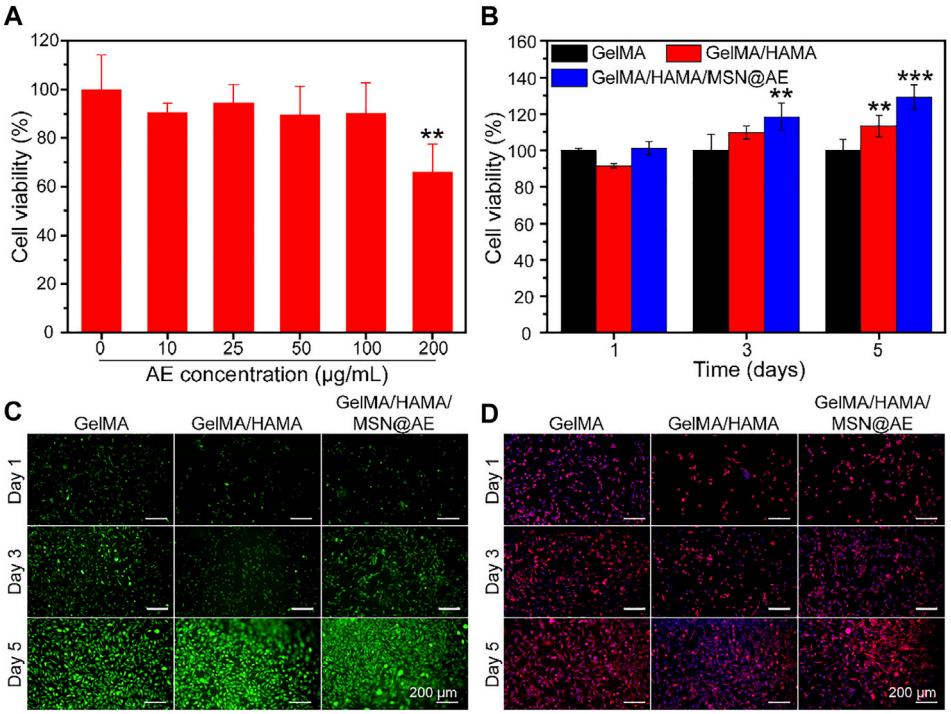

FIGURE 4 | (A) Cell viability of MSN@AE with different AE concentrations. **(B)** Cell viability of L929 cells grown on GelMA, GelMA/HAMA, and GelMA/HAMA/MSN@AE hydrogels at days 1, 3, and 5. **(C)** Live/dead staining images of L929 cells grown on the hydrogels. **(D)** Cytoskeletal staining images of L929 cells grown on the hydrogels. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

ranging from 20 to 100 μg/ml were cocultured with two bacterial strains, *S. aureus* and *E. coli*, which are involved in most infections (Huang et al., 2020; Lin et al., 2021). As shown in **Figure 3A**, the GelMA/HAMA/MSN@AE hydrogel exhibits good antibacterial properties compared to GelMA/HAMA hydrogels. After incubation in a medium for 4 h, the number of *S. aureus* and *E. coli* decreased significantly with the increase in AE concentration, showing a dose-dependent behavior. However, until the concentration of AE reached 50 μg/ml, its antibacterial activity increased only slightly and began to stabilize. As shown in **Figure 3B**, the antibacterial rates, up to 99.9 ± 0.7% of *S. aureus* and 91.9 ± 1.4% of *E. coli*, were killed by the GelMA/HAMA/MSN@AE (50) hydrogels, which demonstrated the outstanding potential for antibacterial performance of GelMA/HAMA/MSN@AE. The excellent antibacterial properties of the GelMA/HAMA/MSN@AE hydrogel were attributed to the AE, which could increase the permeability of bacterial cell membranes and inhibit the synthesis of bacterial nucleic acid, resulting in killing bacteria. We believed that GelMA/HAMA/MSN@AE had excellent antibacterial properties through the sustained release of the AE decomposing from MSN@AE, which could effectively eliminate the risk of bacterial infections in skin wounds.

Biocompatibility of Cells in the Hydrogel

The biocompatibility of composite hydrogel is a crucial factor for functional tissue regeneration and a standard for safe application in the biomedical field (Huang et al., 2021). The cytocompatibility of MSN and hydrogels was examined using CCK-8 and live/dead cell staining, respectively. As shown in **Figure 4A**, MSN@AE showed no cytotoxicity at AE concentrations ranging between 0 and 100 μg/ml. Based on the results of antibacterial activity evaluation and cell cytotoxicity, 50 μg/ml was selected as a suitable concentration for obtaining a composite hydrogel. As shown in **Figure 4B**, all hydrogel groups of cells present a high viability and proliferation activity. Especially on day 5, the GelMA/HAMA/MSN@AE group showed high cell viability (129.3 ± 6.8%), which significantly stimulated the proliferation of L929 cells. Live/dead cell staining results showed that the L929 cells seeded in GelMA/HAMA/MSN@AE hydrogel exhibited green fluorescence, indicating that most of the cells were alive (**Figure 4C**). As shown in **Figure 4D**, the morphology of L929 grown on GelMA/HAMA/MSN@AE hydrogel showed more elongation and thicker actin filaments at day 5 compared to GelMA hydrogel.

It has been recognized that the inflammatory microenvironment plays a critical role in regulating wound healing (Zhang et al., 2018). Macrophage polarization was evaluated by WB to assess the *in vitro* anti-inflammatory effect of the GelMA/HAMA/MSN@AE hydrogel. As shown in **Supplementary Figures S10A,B**, WB analysis showed that the expression of inducible nitric oxide synthase (iNOS), an M1 marker, was significantly downregulated in the GelMA/HAMA/MSN@AE hydrogel treated groups compared to the hydrogel-free treatment and GelMA/HAMA group, while the

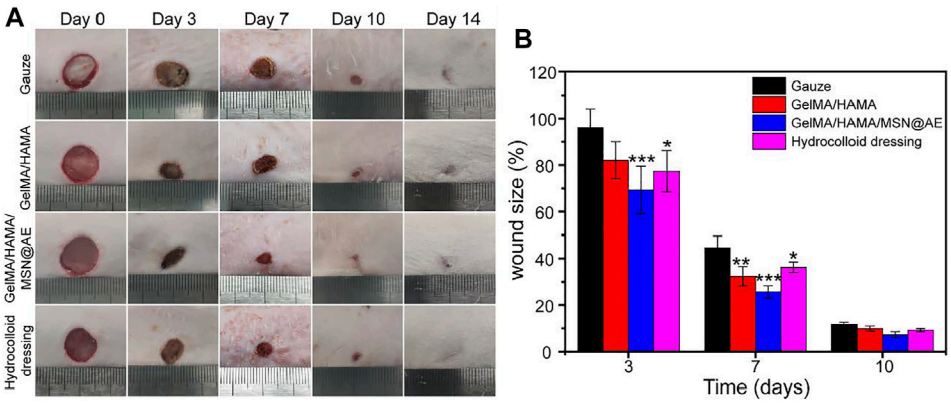

FIGURE 5 | (A) Representative photograph of wounds treated with different materials, including Gauze, GelMA/HAMA hydrogel, GelMA/HAMA/MSN@AE hydrogel, commercially available hydrocolloid dressing at days 0, 3, 7, 10, and 14. **(B)** The changes of wound size of each group.

expression of M2 phenotype markers of anti-inflammatory macrophage (CD206) was relatively upregulated. The results demonstrated that GelMA/HAMA/MSN@AE hydrogel could help promote the resolution of the inflammatory response by facilitating the macrophages phenotype transformation from M1 to M2 and had the potential to inhibit inflammatory reactions in the process of wound healing.

In Vivo Wound Healing Evaluation

Wound Healing Examination

To further verify the clinical potential of hydrogel, a full-thickness cutaneous wound model in rats was applied to assess the wound repair capability of the GelMA/HAMA/MSN@AE hydrogel. As shown in **Figure 5A**, the skin defect in all groups shrank over time. Interestingly, the GelMA/HAMA hydrogel, GelMA/HAMA/MSN@AE hydrogel, and commercially available hydrocolloid groups significantly promoted wound healing, but the gauze group presented a weak effect on the acceleration of wound healing. In particular, the full-thickness cutaneous defects for the GelMA/HAMA/MSN@AE group were almost closed at day 14, followed by GelMA/HAMA and commercially available hydrocolloid groups, and the slowest in gauze group, as depicted by the quantification of the wound area in **Figure 5B**. The rapid wound closure may be attributed to the good skin permeability of AE percutaneous administration, which has various physiological and pharmaceutical effects, such as invigorating blood circulation, reducing swelling, relieving pain, relieving itching, and antibacterial.

On day 3, the wound size of the gauze, GelMA/HAMA hydrogel, GelMA/HAMA/MSN@AE hydrogel, and commercially available hydrocolloid were 96.1 ± 8.1%, 82.1 ± 8.0%, 69.3 ± 10.2%, and 77.4 ± 8.9%, respectively. After 7 days of operation, the wound size of the GelMA/HAMA/MSN@AE group was 25.7 ± 2.6%, which was significantly lower than that of the control group (44.6 ± 25.0%). On day 10, it can be observed that GelMA/HAMA/MSN@AE hydrogels displayed superiority in promoting wound healing and the wound was nearly closed, while the gauze group, GelMA/HAMA, and commercially available hydrocolloid group still had a wound area of 11.7 ± 0.8%, 9.9 ± 1.1%, and 9.2 ± 0.7%, respectively. In summary, GelMA/HAMA/MSN@AE hydrogels showed the smallest wound size, which was attributed to the antibacterial and anti-inflammatory properties of AE, gelatin, and hyaluronic acid's desirable function in the promotion of wound healing, and the moist wound environment provided by the hydrogel dressing. AE could accelerate different wound healing phases (inflammation, proliferation, and remodeling), as it could effectively scavenge reactive oxygen species (ROS) and exert anti-oxidant effects during the inflammatory phase, promote fibroblast migration, collagen formation, and epithelialization in the proliferation phase, and increase the number of cytokines in the remodeling phase.

Histological Analysis

Wound tissue regeneration and re-epithelialization is one of the evaluation criteria in the process of wound healing (Xiong et al., 2021). H&E staining was carried out to observe the morphological changes of skin layer reconstruction on days 3, 7, 10, and 14 *in vivo*. As shown in **Figure 6A**, a large number of inflammatory cell infiltration could be seen in gauze, while GelMA/HAMA and commercially available hydrocolloid groups showed a mild inflammatory infiltration in the GelMA/HAMA/MSN@AE group on day 3. The control group showed a large number of inflammatory cells at day 7, and the new epidermal cells were relatively few, which were still in the inflammatory stage. However, the number of inflammatory cells in the GelMA/HAMA/MSN@AE group was reduced and the epidermis was thickened at day 7, which was attributed to the well anti-oxidant action and anti-inflammation capacity of AE (Jaradat, 2021). On day 3, loose granulation tissue could be seen in the wound treated with GelMA/HAMA/MSN@AE. On day 7, granulation tissue in GelMA/HAMA/MSN@AE group became compact and thicker than that of other groups. Additionally, the treated wounds also presented a closer dermal-epidermal junction and restored the aesthetic function at day 14. What is more, intact neoepidermis could be observed in the wound tissue, while the epidermis of the control group was not evenly covered.

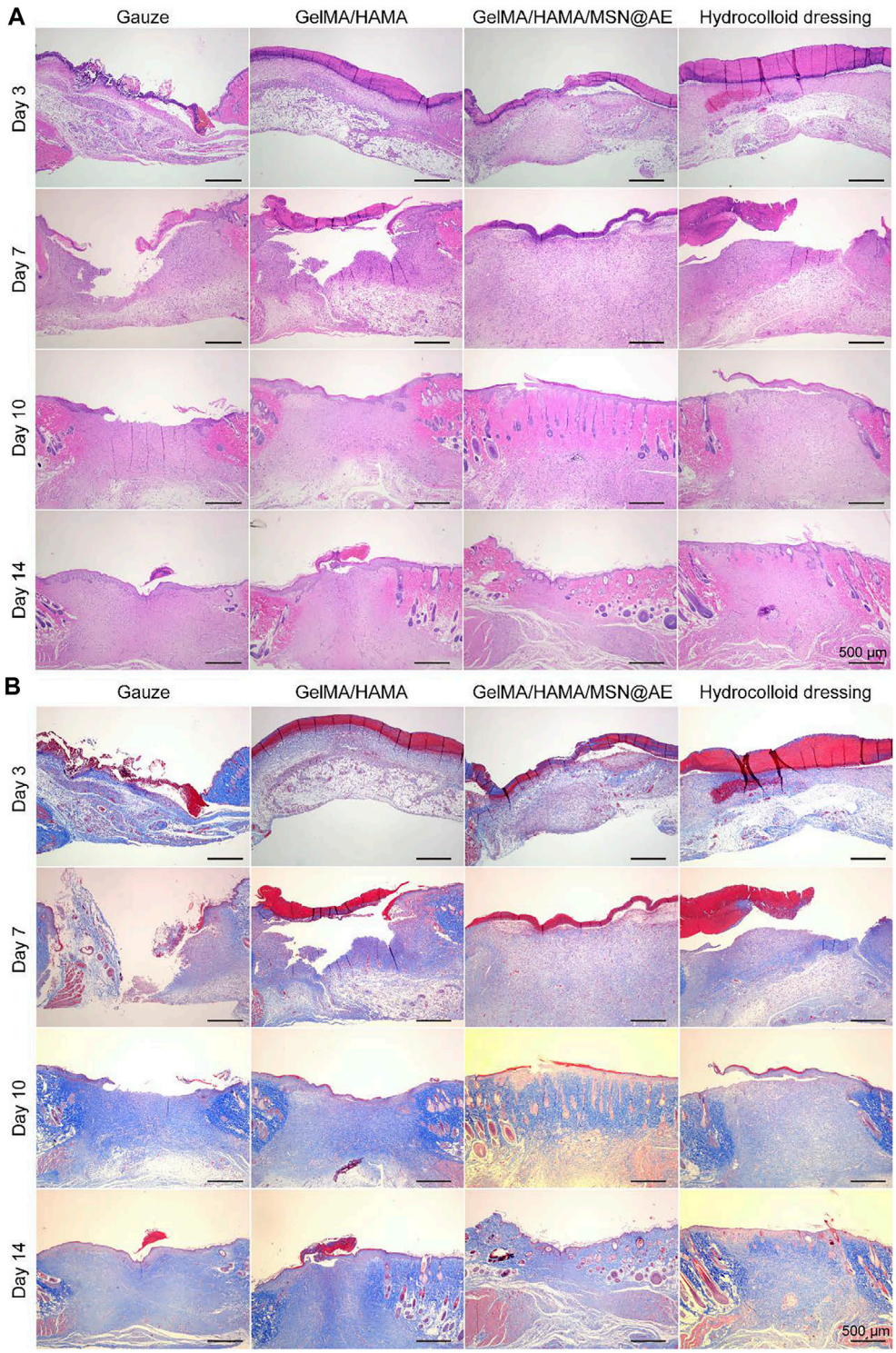

FIGURE 6 | (A) Representative H&E staining images acquired on days 3, 7, 10, and 14 after surgery. **(B)** Representative Masson's trichrome staining images acquired on days 3, 7, 10, and 14 after surgery.

In addition, collagen deposition plays a significant role in the remodeling period of wound healing and is beneficial to wound contraction. Masson staining was carried out to evaluate the collagen deposition in the regenerated skin tissue. As shown in **Figure 6B**, the staining depth of all groups increased with time, indicating the increase in collagen content. On day 3, there were

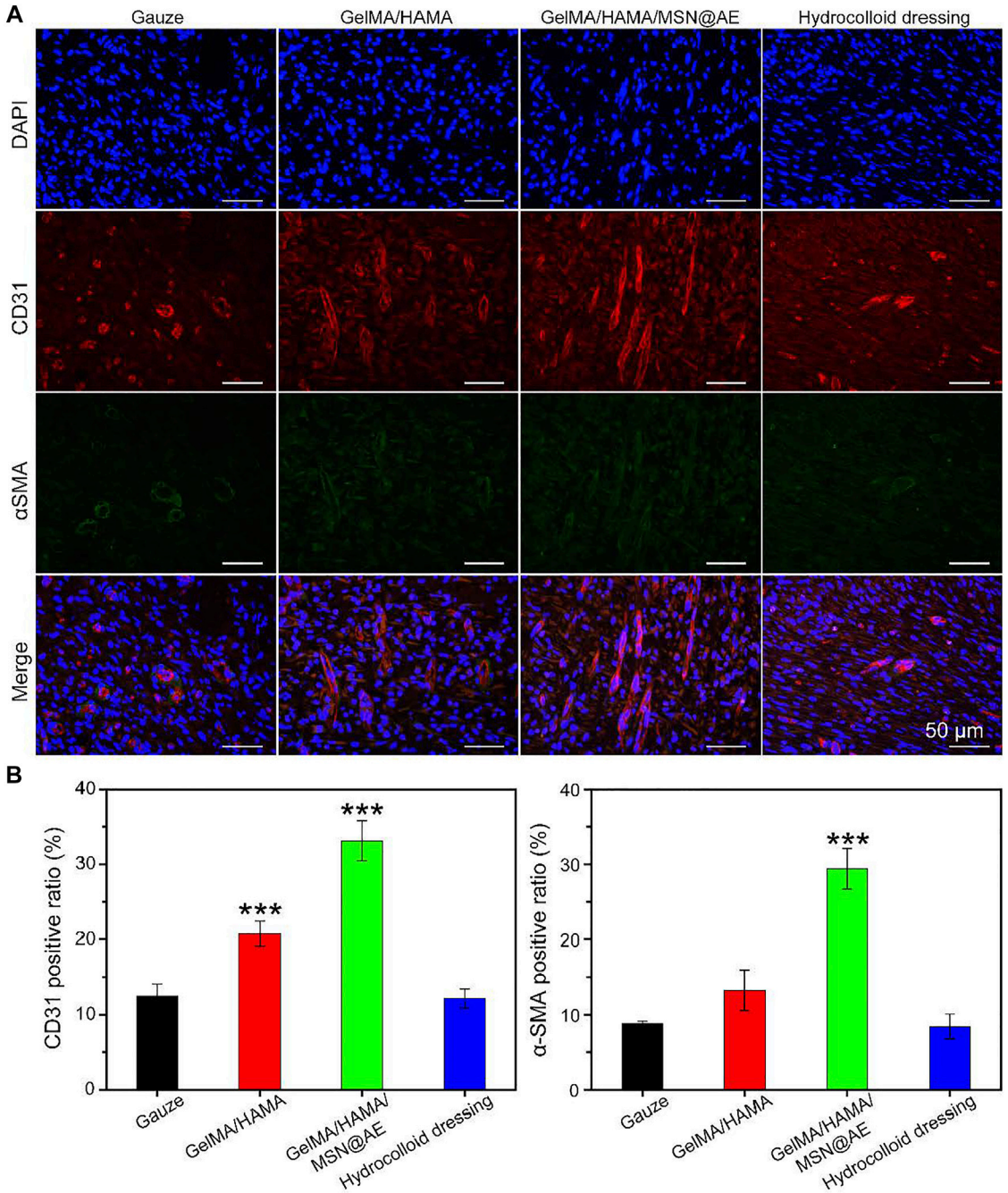

FIGURE 7 | (A) Representative CD31/α-SMA images of immunofluorescence staining of wound sections treated with each group at day 7. **(B)** Quantitative analysis of newly formed (CD31, red) and mature blood vessels (α-SMA, green) (*p < 0.05, **p < 0.01, and ***p < 0.001).

fewer collagen fibers in the control group, while the GelMA/HAMA/MSN@AE group presented more collagen fibers, and the immature collagen deposition could be seen. On day 10, the collagen fibers in GelMA/HAMA/MSN@AE group became compact and orderly, showing the basketweave arrangement of collagen bundles, while those in the control group were sparse and disordered. The arrangement characteristics of collagen fibers at day 14 in the GelMA/HAMA/MSN@AE group tended to the normal skin tissue more than other groups. Thus, the GelMA/HAMA/MSN@AE hydrogel with slow release ability of AE could facilitate wound healing through promoting re-epithelialization and collagen deposition.

Angiogenesis Analysis

Neovascularization is one of the key factors to promote wound healing, which reflects the degree of skin tissue regeneration and

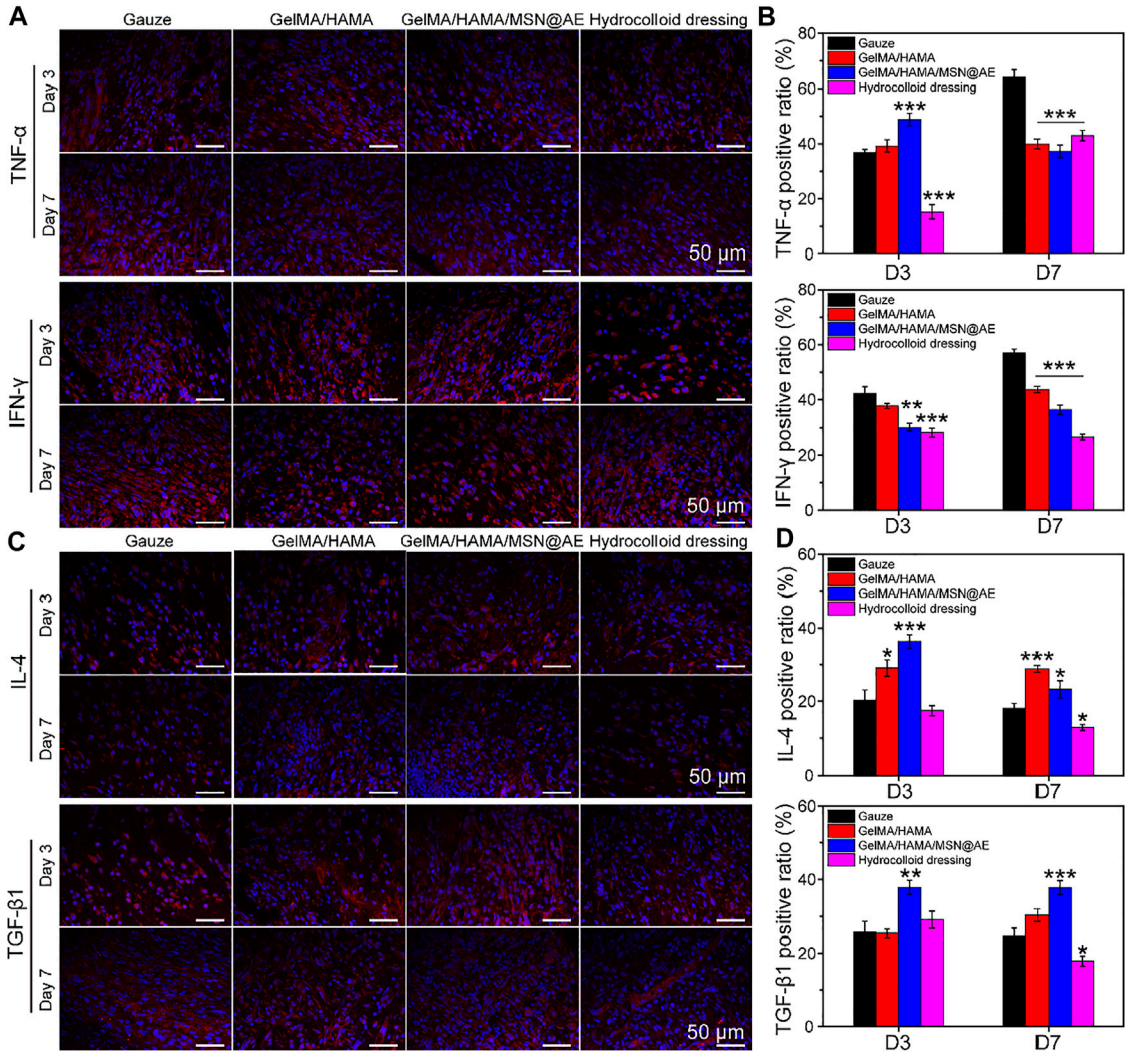

FIGURE 8 | (A) Representative TNF-α and IFN-γ images of immunofluorescence staining of wound sections treated with each group at day 3 and day 7. **(B)** Quantitative analysis of TNF-α positive ratio and IFN-γ positive ratio. **(C)** Representative IL-4 and TGF-β1 images of immunofluorescence staining of wound sections treated with each group at day 3 and day 7. **(D)** Quantitative analysis of IL-4 positive ratio and TGF-β1 positive ratio (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

functional recovery (Yao et al., 2021). The mechanism of angiogenesis induced by GelMA/HAMA/MSN@AE hydrogel was studied by immunofluorescent staining, in which CD31 and α-smooth muscle actin (α-SMA) were the markers of neovascularization and mature blood vessels, respectively. As shown in **Figures 7A,B**, except for the lower density of CD31 positive vascular endothelial cells in the gauze group, the indexes of other groups increased significantly at day 7, especially in the GelMA/HAMA/MSN@AE group. In addition, the density and area of α-SMA in the subcutaneous tissue around the wound were the highest in the GelMA/HAMA/MSN@AE group, indicating that the degree of myofibroblasts activation was high in the process of wound healing (Mao et al., 2021). Thus, the GelMA/HAMA/MSN@AE group was conducted to the production of vascular endothelial cells, the synthesis of actin in the vascular wall, and promoting vascularization, which demonstrated that GelMA/HAMA/MSN@AE Hydrogel could upregulate the expression of CD31 and α-SMA and promote myofibroblasts to accelerate the wound closure.

Inflammatory Response Analysis

Inflammation will hinder the healing process of the wound bed, but anti-inflammatory properties of AE have been unequivocally established in previous studies (Yun et al., 2016). To further explore the mechanism of hydrogel in controlling wound infection, immunofluorescence staining of four typical inflammatory cytokines, anti-inflammatory factors (IL-4 and TGF-β1), and pro-inflammatory factors (TNF-α and IFN-γ) were performed on day 3 and day 7.

As shown in **Figures 8A,B**, elevated levels of TNF-α and IFN-γ production could be monitored in each group on day 3, indicating a severe inflammatory response. However, the levels of TNF-α

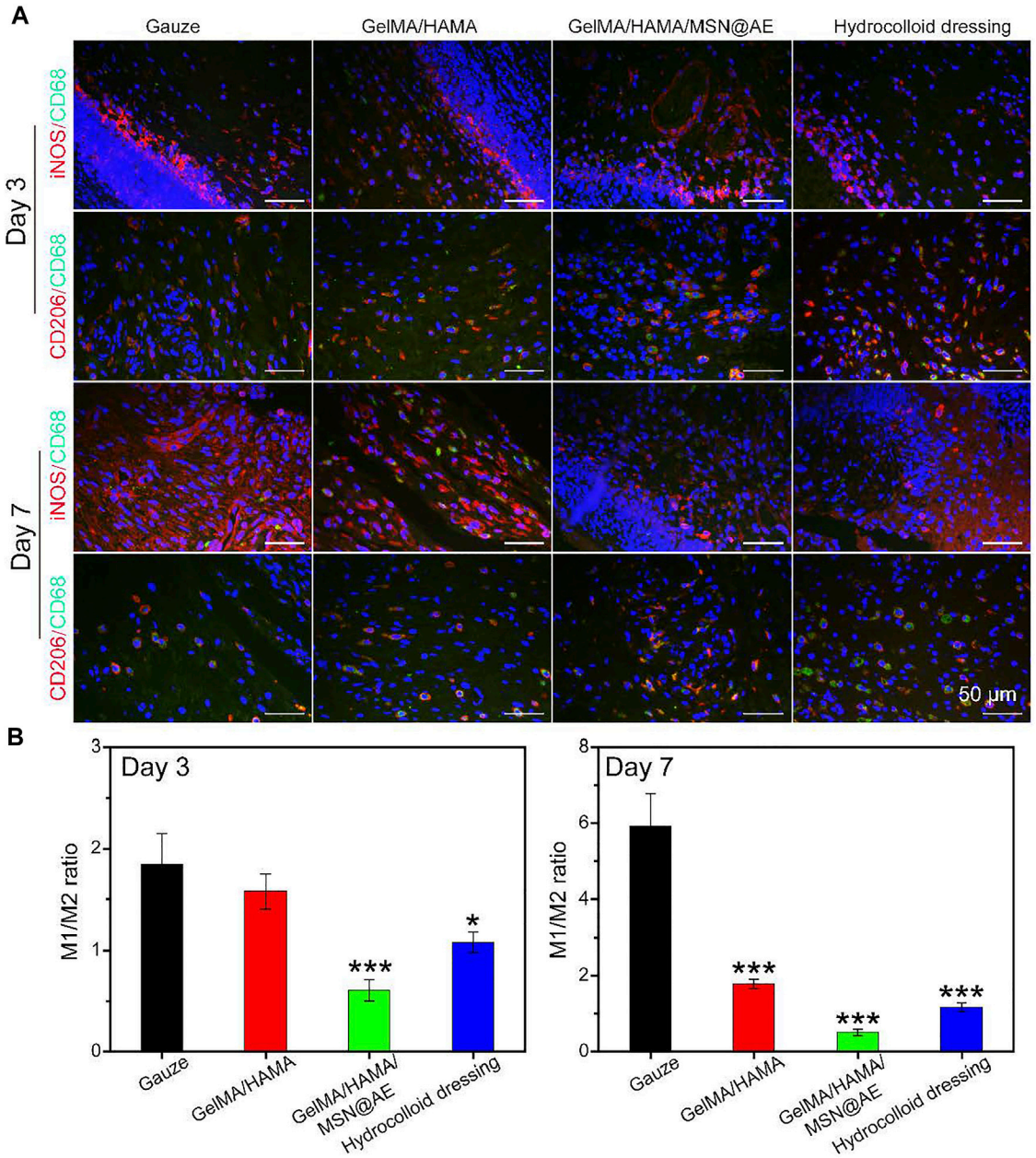

FIGURE 9 | (A) Representative iNOS (red), CD206 (red) and CD68 (green) images of immunofluorescence staining of wound sections treated with each group at day 3 and day 7. **(B)** Ratio of M1 to M2 analysis of each group (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

and IFN-γ in the GelMA/HAMA/MSN@AE group were lower than those in other groups on day 7, and a large amount of IL-4 and TGF-β1 were observed (**Figures 8C,D**). Meanwhile, GelMA/HAMA and commercially available hydrocolloid group also exhibited low expression compared to the control group, representing that the sustainable release of AE significantly prevented the increase in these pro-inflammatory cytokines and upregulated the expression of anti-inflammatory factors to some extent. On day 7, little expression of TNF-α and IFN-γ was seen in the GelMA/HAMA/MSN@AE group compared to other groups. Inflammation of the wounds plays an important role in the clearance of necrotic tissue and resistance to bacterial infection, thereby of a higher level of inflammation in the early stage. If the wound remains in the inflammatory stage, the wound healing rate will be delayed. The GelMA/HAMA/MSN@AE group has the immunomodulatory effect, which accelerates the transition from the inflammatory phase to the proliferation phase, thereby accelerating the wound healing process, resulting in decreased levels of TNF-α on day 7. Meanwhile, the gauze groups maintained high levels of

inflammation, resulting in slower extracellular matrix deposition and slower wound healing (Larouche J et al., 2018). The statistical results revealed that GelMA/HAMA/MSN@AE hydrogel could greatly diminish the inflammation at the wound sites. All results suggested that GelMA/HAMA/MSN@AE hydrogel could upregulate the expression of IL-4 and TGF-β1 but downregulate the expression of TNF-α and IFN-γ, which exert anti-inflammatory effects.

Meanwhile, macrophages can coordinate the complex processes of cell proliferation, infection, inflammation, and functional tissue regeneration in the wound (Freund et al., 2020), responding to the wound microenvironment and driving their conversion from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype (Zhao et al., 2021). Hence, hydrogels with the capability of promoting M2 macrophage polarization can accelerate the progression from the inflammatory stage to the proliferative and remodeling stage of wound healing. To further elucidate the anti-inflammatory pathways of GelMA/HAMA/MSN@AE hydrogel, macrophage polarization was evaluated by immunofluorescence. As shown in **Figures 9A,B**, the GelMA/HAMA/MSN@AE group showed some expressions of the M2 phenotype macrophages maker (CD206), while the macrophages in the gauze group were still in the M1 phenotype, indicating the initial stage of inflammation. The inflammatory microenvironment is crucial in the wound healing phase, and the inflammatory phase of chronic wounds is severely prolonged, which fails to transition from the inflammation phase to the proliferation phase (Liu et al., 2020).

Additionally, although anti-inflammatory M2 phenotype macrophages maker (CD206) was also detected in the GelMA/HAMA and commercially available hydrocolloid groups, the GelMA/HAMA/MSN@AE group showed the most expression of CD206. All results demonstrated that GelMA/HAMA/MSN@AE hydrogel could upregulate the expression of CD206 but downregulate the expression of iNOS, which promoted the transformation of macrophages from inflammatory phenotype to a reparative phenotype and induced the wound to enter the proliferative phase. Summary, all results indicated that compared to other groups, the GelMA/HAMA/MSN@AE hydrogel group exerted anti-inflammatory effects in the chronic wound through enhanced expression of CD31, α-SMA, IL-4, and TGF-β1, reduced the expression of TNF-α and IFN-γ, and facilitated the M1-to-M2 transition of macrophages in the early stage of wound healing, playing a beneficial role in wound closure.

CONCLUSION

The purpose of this work is to prepare hydrogels with antibacterial properties, immune regulation, anti-inflammation, and good biocompatibility for the treatment of wounds. Then, GelMA/HAMA hydrogel with MSN-loaded *Artemisia argyi* extract (AE) was successfully prepared. GelMA/HAMA/MSN@AE hydrogel has stable rheological properties, suitable mechanical properties, appropriate biodegradability, swelling ratio, and sustained-release AE capacity. AE could increase the permeability of bacterial cell membrane and inhibit the synthesis of bacterial nucleic acid, so GelMA/HAMA/MSN@AE hydrogel had excellent antibacterial activity. AE could regulate inflammation, and the synthetic materials of GelMA and HAMA are natural cytoplasmic matrix materials, so GelMA/HAMA/MSN@AE hydrogel had good biocompatibility and promoted M1-M2 transformation of macrophages. AE could promote the growth of collagen fibers after full-thickness skin trauma and inhibit inflammation. Hydrogel could protect the wound from external stimulation and provide a moist environment. GelMA/HAMA/MSN@AE hydrogel could increase collagen deposition, promote angiogenesis, and regulate inflammation in chronic wound healing of rats. Overall, our results concluded that GelMA/HAMA/MSN@AE hydrogel with the sustainable release ability of AE exhibited an excellent property in promoting chronic wound healing.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Protection and Use Committee of Jinan University.

AUTHOR CONTRIBUTIONS

LX, LD, and XL contributed to the conception and design of the study. LX, TD, RG, and SJ performed the experiences. LP, JG, FT, JL, and HL performed the statistical analysis. LX wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

FUNDING

This research is supported by the 2020 Guangdong Provincial Science and Technology Innovation Strategy Special Fund (Guangdong-Hong Kong-Macau Joint Lab) (no. 2020B1212030006) and the Promotion Project of Clinical Efficacy Evaluation of Traditional Chinese Medicine Based on Dominant Diseases in Clinical Research base of Traditional Chinese Medicine (Chronic Kidney disease) (no. 0103041001).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.825339/full#supplementary-material

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frontiers | Frontiers in Bioengineering and Biotechnology

PERSPECTIVE
published: 29 April 2022
doi: 10.3389/fbioe.2022.873384

Exploitation of Skin Microbiota in Wound Healing: Perspectives During Space Missions

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Wound healing is slowed in Space. Microgravity and possible physical factors associated with Space affect alterations in fibroblast, matrix formation, dysregulation in apoptosis and inflammation. The microbial populations settled on skin, space modules, in space suits, are also playing a pivotal role, as wound healing is also affected by the microbial community. We propose a perspective that includes four domines for the application of human skin microbiota for wound healing in Space: The natural antimicrobial properties of the skin microbiota, the crosstalk of the skin microbiota with the immune system during wound healing, the contribution of the microbiota in precision medicine, and the role of gut-skin and gut-brain axes. A stronger understanding of the connections and metabolic network among bacteria, fungi, the host's immune system and the host metabolism will support the basis for a better wound healing in Space.

Keywords: microbiome and dysbiosis, wound healing, space mission, skin, wound

OPEN ACCESS

Edited by:
Mona Kamal Marei,
Alexandria University, Egypt

Reviewed by:
Guy K. German,
Binghamton University, United States
Maria A. Mariggiò,
University of Studies G. d'Annunzio Chieti and Pescara, Italy

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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *10 February 2022*
Accepted: *11 April 2022*
Published: *29 April 2022*

Citation:
Marvasi M, Monici M, Pantalone D and Cavalieri D (2022) Exploitation of Skin Microbiota in Wound Healing: Perspectives During Space Missions. Front. Bioeng. Biotechnol. 10:873384. doi: 10.3389/fbioe.2022.873384

INTRODUCTION

Wounding can happen during Space exploration (Gontcharov et al., 2005; Tronnier et al., 2008; Crucian et al., 2016; Farkas and Farkas, 2021). Astronauts are exposed to skin erythema, peeling, dryness, burning, pruritus, sensitivity, thinning, therefore the physiology of the healing process of cutaneous injuries is hence affected (Farahani and DiPietro, 2008; Cubo-Mateo and Gelinsky, 2021). Recent studies have confirmed that wound healing is altered during Space missions (Cubo-Mateo and Gelinsky, 2021). During the normal wound healing process and under normal gravity (on earth), the body can repair wounds, trauma, burns by recreating the skin barrier (Reinke and Sorg, 2012; Singh et al., 2017; Maheswary et al., 2021). The physiology of wound healing under normal conditions is achieved *via* several defined steps: inflammation, proliferation, epithelialization, maturation, and remodelling phases (Reinke and Sorg, 2012; Singh et al., 2017; Maheswary et al., 2021). Chronic wounds extend the inflammatory phase, with immune cells continually degrading collagen and extracellular matrix (Blaber et al., 2010; Dam and Paller, 2018; Jemison and Olabisi, 2021). The delayed wound healing induced by microgravity differs in aetiology from that of chronic wounds on earth (Jemison and Olabisi, 2021). Microgravity affects matrix formation (Blaber et al., 2010), the alterations in fibroblast and dysregulation in apoptosis (Cialdai and Monici, 2013), inflammation (Blaber et al., 2010), delayed cellular proliferation of the basal skin layer and a thinning of the upper layer of the epidermis (this study is based on a single crew member) (Tronnier et al., 2008). Space radiation including solar particle events, galactic cosmic radiation and intra-vehicular secondary radiation may also compromise the physiology of the skin (Onorato et al., 2020). Space

radiations cause damage to the DNA, through direct interaction or production of free radicals (Moreno-Villanueva et al., 2017; Afshinnekoo et al., 2020). Non-ionizing UV radiations also damage the skin as well, but this is not an issue in Space due to extensive protection provided to the astronauts (Tyrrell, 1995; Gasperini et al., 2017; Lipsky and German, 2019). UV exposure is under strict control inside the International Space Station (ISS) also in case of use of UV lamps.

Physical factors are not the only factors that affect the physiology of skin. The microbial populations settled on skin, space modules, in space suits, are also playing a pivotal role. The skin hosts an immense number of microorganisms adapted to utilize the nutrients available. The skin microbiota of healthy adults remains stable over time, despite environmental perturbations—at least on earth (Byrd et al., 2018). It is well known that skin diseases and disorders, are associated with an altered microbial state (Lunjani et al., 2019). Space missions occur in non-sterile, extreme confined environments, where air pressure, temperature, humidity, limited water supply are kept under strict control (Gentry and Cover, 2015). In this environment, astronauts are not able to take proper shower keeping their body clean by using wet tissues, using rinseless shampoo, and they do not change their clothes often (Farkas and Farkas, 2021). It is therefore important to understand the astronauts' skin microbiomes and their fluctuation over time.

The skin microbiota has been studied by using both cultivable microbes and metagenomic profiles. The application of targeted and untargeted Next Generation Sequencing (NGS) has been used to further differentiate strains and functional variability (Tomida et al., 2013), e.g., the association of microorganisms with sweet and sebaceous glands, hair follicle, sebum and the stratum corneum (Byrd et al., 2018). Bacteria may variate according with the site of the body, in contrast fungal community composition was in general similar across core body sites regardless the physiology (Findley et al., 2013; Byrd et al., 2018).

The ISS is not a sterile environment and its microbial community is routinely monitored on various surfaces, as part of standard operations and maintenance requirement procedures (NASA, 2005). Influx of new microbes from travels at the ISS may quickly resemble astronaut skin microbiomes: it is transient (following crew exchanges), and some can settle permanently (Voorhies et al., 2019), showing that there is shift of the microbial communities, but also a small proportion of ubiquitous bacteria are long-term residents (in dust, Actinobacteria, Proteobacteria, and Firmicutes) (Checinska et al., 2015). A survey of cultivable bacterial and fungal populations from surface wipes over 14 months from the ISS surfaces, showed a range of 10^4–10^9 CFU/m^2 changing over time but remained similar between locations. With reference to the phyla, the bacteria Actinobacteria, Firmicutes, and Proteobacteria were the most represented, while Ascomycota and Basidiomycota phyla represented the fungal domine. The dominant organisms are associated with the human microbiome and may include opportunistic pathogens. Methylobacteriaceae were also dominant across the ISS (as well as in some hospitals) (Checinska Sielaff et al., 2019), and Staphylococcaceae and Enterobacteriaceae were the most predominant organisms on the US module, very similar to fitness centres and, again, hospitals (Mukherjee et al., 2014; Lax et al., 2017). Interestingly, 46% of viable bacteria and 40% of viable fungi from the overall meta-taxonomical 16S were culturable, reflecting that a high percentage of possible opportunistic pathogens are present and alive, suggesting that ISS is like other built environments (Checinska Sielaff et al., 2019). Recent findings suggest that possible thinning of the upper layer of the epidermis and a significant loss of elasticity of the skin (Tronnier et al., 2008) could increase the exposure of the microbial communities that reside in the deeper layers of the skin, including the stratum corneum (Zeeuwen et al., 2012; Lipsky et al., 2020). In this conditions the skin of astronauts is continuously exposed to different microbial communities with relatively low biodiversity (Checinska Sielaff et al., 2019; Voorhies et al., 2019).

A crosstalk in Space: application of the knowledge on skin microbiota during wound healing.

The skin microbiome is protective against pathogens, nevertheless in certain conditions the microorganisms that are ordinarily beneficial to their host can become pathogenic (dysbiosis) (Byrd et al., 2018). Skin microorganisms are important in educating the innate and adaptive arms of the cutaneous immune system (Byrd et al., 2018), and the commensal skin microbiome during healing is essential for the regulation of the cutaneous immune system (Tomic-Canic et al., 2020). Skin microorganisms can provide protection from pathogens through modulation of antimicrobials (Tomic-Canic et al., 2020). It is urgent to better understand the crosstalk of the skin microbiota and the immune system in Space, as it is difficult to disentangle the components of microgravity, space radiations, stress, and the effect of the microbiota itself (Siddiqui et al., 2021). Exposure to microgravity during Space flights produces immunosuppression, and this can lead to dysbiosis or ill-states (Spatz et al., 2021).

The Skin Microbiota has Natural Antimicrobial Properties

The role of microorganisms in chronic wounds provides important insight on how to handle would healing in Space (**Figure 1**). The role of the microbiome in chronic wound is not fully understood and the importance of topical antimicrobial agents in their treatment is continuously debated (Tomic-Canic et al., 2020). Pathogens or pathobionts are suspected to delay the healing process (Eming et al., 2014; Misic et al., 2014). Alterations in the skin microbiome might contribute to the high frequency of skin rashes/hypersensitivity episodes experienced by astronauts in Space (Voorhies et al., 2019). Microorganisms can act competitively to exclude one another or synergistically for mutual benefits depending by the interaction between the skin surface and other microorganisms that live on it (Cleary et al., 2018).

Microgravity can affect the biology of microorganisms. Under simulated microgravity, the liquid phase of a culture of *Lactobacillus acidophilus* exhibited higher antibacterial activity against *Staphylococcus aureus* in a time-dependent manner (Shao et al., 2017). Targeting *S. aureus* and *Staphylococcus epidermidis*

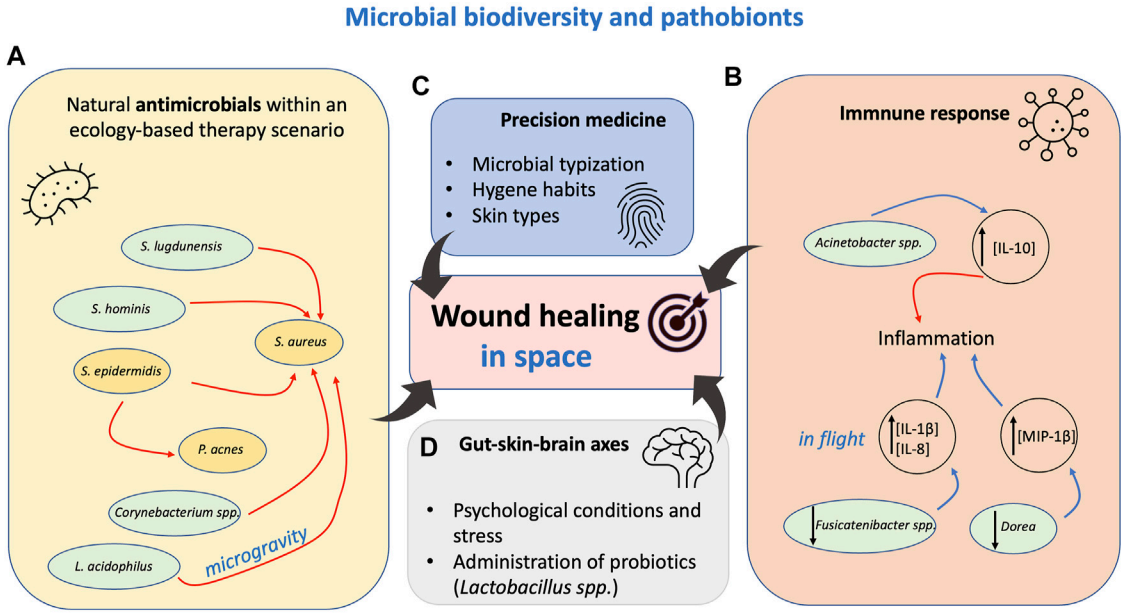

FIGURE 1 | Perspectives for the application of human skin microbiota for wound healing in Space. Four possible domines for intervention developed from studies aimed to improve medical applications on Earth. These solutions can also find application in Space. **(A)** The ecological network among microorganisms is also regulated by natural antimicrobial. Few examples are shown as reported in the main narrative. *S*, *Staphylococcus* spp. *L*, *Lactobacillus. P, Propionibacterium.* Red arrows represent inhibition. Gold boxes are common pathobionts. Green boxes are other organisms. **(B)** Crosstalk of the skin microbiota with the immune system during wound healing. Microorganisms can support cytokine interleukin regulation. Up and down black arrows mean activation and reduction, respectively. Blue arrow represents IL expression. **(C,D)** The typization of the skin microbiota can lead to a better treatment of wound and prevention of complications. Gut-skin, gut-brain axes can be crucial for healing: in mice the administration of probiotics has shown better regulation of healing. Please refer to the text for all the details.

in wounds is important. *S. epidermidis* can either help or hurt the skin barrier being strain-dependent in disease (Brown and Horswill, 2020). *Staphylococcus lugdunensis* inhibited *S. aureus* growth through the production of the antibiotic lugdunin, a thiazolidine-containing cyclic peptide (Zipperer et al., 2016). In another study, *S. epidermidis* strains were capable of inhibiting *Propionibacterium acnes* grown *in-vitro* (Christensen et al., 2016). *P. acnes* is involved in developing cutaneous inflammation. The peptidoglycan of *P. acnes* can activate monocytes to produce cytokines such as IL-1β, IL-8, and TNF-α, which cause granulomatous responses in inflammatory skin disorder (Kistowska et al., 2014; Lee et al., 2019).

In a different study, multiple coagulase-negative *Staphylococcus* spp, *S. epidermidis* and *Staphylococcus hominis* were shown to produce lantibiotics that were able to synergize with the human antimicrobial peptide cathelicidin and to inhibit the growth of *S. aureus* (Nakatsuji et al., 2017; Byrd et al., 2018). Strains producing lantibiotics were depleted in individuals with atopic dermatitis, who are frequently colonized with *S. aureus* (Byrd et al., 2018). Other studies have shown that *S. aureus* shifts toward commensalism in response to *Corynebacterium* species, affecting *S. aureus* behaviour and fitness, leading to a reduced virulence of *S. aureus* (Ramsey et al., 2016). *In vitro* mono-vs. co-culture of commensal *Corynebacterium striatum* increased transcription of genes related to human nasal colonization and decreased transcription of virulence genes of *S. aureus* (Ramsey et al., 2016). Biotechnological application of *Corynebacterium*'s metabolic products could lead to develop anti-virulence therapies against *S. aureus* (Ramsey et al., 2016).

It must be mentioned that the direct influences of changes of the microbial community should be carefully considered, since changes in abundance may not be solely beneficial but leading to opposite results, For example, *Propionibacterium* species induced *S. aureus* aggregation and biofilm formation in a manner dependent on dose, growth phase and pH (Wollenberg et al., 2014).

The discovery, characterization and production of the antimicrobials or elicitors involved in quorum sensing or signalling could find important biotechnological applications to control pathobionts. The interactions among microorganisms in wounds are common but poorly characterized.

Bacterial community instability was associated with faster healing and more positive clinical outcomes (Loesche et al., 2017). One hundred subjects diagnosed with diabetic foot ulcers were enrolled into a prospective, longitudinal cohort study to analyse the temporal dynamics of the bacterial community of the ulcers. In this study the bacterial community stability reflected a delayed healing phenotype (Loesche et al., 2017). This observation may apparently be counterintuitive, as in many other pathologies bacterial community instability was associated with disease (Martinez et al., 2008). Nevertheless, as proposed by Loesche and collaborators "instability in the microbiome is a reflection of

effective control of wound bacteria, which prevents any community structure from stabilizing" (Loesche et al., 2017). Stabilization could proceed on the trajectory of the progressive ulceration. Temporal stability of the microbiota of wound should be further studied in Space.

In another similar study, the mycobiome (the fungal microbial community) in chronic wounds is predictive of healing time, associated with poor outcomes when forming mixed fungal-bacterial biofilms (Kalan et al., 2016). *Cladosporium herbarum* and *Candida albicans* have been identified as the most abundant species in chronic wounds (Kalan et al., 2016). More information must be acquired from the microbiome of chronic wounds, which is believed to play an important role in impaired healing and the development of infection-related complications (Loesche et al., 2017). It is not known to what extent the mycobiome composition in Space provides little predictive value of wound outcomes, and culture-independent studies have been limited by cross-sectional design and small cohort size. An interesting approach adopted by Loesche and collaborators was to define the outcome of the diabetic foot ulcers by classifying the microbiota in different community types (Loesche et al., 2017). In this disease the transition patterns and frequencies of microbial populations were associated with healing time, and this can be extended to the prognosis. Similar experimental approaches could be done in microgravity scenarios.

Crosstalk of the Skin Microbiota With the Immune System During wound Healing

The immune system is interconnected with the skin microbiota, especially by targeting pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs). For example, *Propionibacterium acnes* and the lipopolysaccharides induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes contributing to the host defence and skin inflammation (Nagy et al., 2006; Naik et al., 2012). Microorganisms could be beneficial if able to regulate the immune response toward a normal healing process. For example, the lack of interleukin-10 (IL-10) (a key anti-inflammatory cytokine in immunologic tolerance) results in a strong inflammatory response. IL-10 is a key mediator of the pro-to anti-inflammatory transition that counters collagen deposition (Singampalli et al., 2020). In healthy individuals, IL-10 expression was positively correlated with the abundance of the gammaproteobacterial genus *Acinetobacter* on the skin (Hanski et al., 2012) (**Figure 1**). Abundance of *Acinetobacter* could be monitored in astronauts, and further biotechnological elicitors released by *Acinetobacter* could be used as food additive to support wound healing in microgravity, as well as on earth.

It is well known that environmental biodiversity, human microbiota, and allergy are correlated (Hanski et al., 2012). Pro-inflammatory cytokines MCP-1, IL-8, IL-1b and MIP-1β showed a significant increase in their concentration during flight (flight day, FD 180), while TNFa had a near significant rise. Conversely, a decrease in OTU of *Fusicatenibacter* spp. and *Dorea* spp. was measured when IL-8, IL-1 β and MIP-1β increased during flight (**Figure 1**). Cytokine concentrations reverted to pre-flight levels within 2 months of returning to earth (Voorhies et al., 2019). It would be interesting to further understand the association of the astronauts' immune dysregulation with the skin microbiome. The main picture is still far to be resolved. The analysis of skin microbiota forehead and forearm (Voorhies et al., 2019) (Voorhies et al., 2019) (Voorhies et al., 2019) (Voorhies et al., 2019) during inflight showed a significant reduction of Proteobacteria, mainly, Gamma and Betaproteobacteria, with an associated increase in Firmicutes, including Staphylococcal, and Streptococcal species (Voorhies et al., 2019) (**Figure 1**). A better regulation of Proteobacteria on skin could also provide further details on how the dynamics of the microbiota can regulate pro-inflammatory cytokines and wound healing in Space. Voorhies and co-authors proposed that it is possible that the constant filtration of environmental air in the ISS contributes to the overall reduction of skin Proteobacteria (Voorhies et al., 2019). Recent studies conducted in children showed the importance of "green" areas around the homes. Children living nearby green areas showed reduced atopic sensitization, the same absence of "green" resources (possibly "green" areas) could reduce Proteobacteria on the skin to a pathological level (Ruokolainen et al., 2015). Absence of "green" resources (or better, reservoir of similar microbial communities) could control the incidence of skin clinical symptoms during long-duration orbital spaceflight (Crucian et al., 2016; Voorhies et al., 2019). It is reasonable to assume that skin clinical symptoms and changes in skin structure may facilitate the establishment of skin infections, inflammation, leading to reduced wound healing during spaceflight. Further research needs to be addressed.

Precision Medicine, Personal Skin Features in Wound Healing

The microbial typing for each individual is ideal for precision medicine approaches, leading treatment of wounds (Hülpüsch et al., 2021) (**Figure 1**). This important field of research needs to be further explored in Space: it would be important to understand to what extent the skin microbiota changes in astronauts according with each personal skin moisture, pH, personal hygiene habits. It is not clear how these factors affect wound healing and it would need further investigations (Voorhies et al., 2019). In this context it would be interesting to stimulate *ad hoc* skin probiotic communities in an ecology-based therapy scenario, by limiting dysbiosis that leads to cutaneous disorders (Zhou et al., 2020).

Gut-Skin, Gut-Brain Axes can be Crucial for Wound Healing in Space

The crosstalk between the immune system and the skin microbiota can lead to normal healing (Byrd et al., 2018). In this context the gut-brain/skin axis can play a role in wound healing (**Figure 1**). The gut microbiome influences many domains of the human body: e.g., the central nervous system (Ma et al., 2019), endocrine control (Régnier et al., 2021), immune fitness (Balikji et al., 2021). New biotechnological approaches should identify microbial elicitors that could be

used to control inflammatory cytokines and be administered as probiotics or as additive in food. Probiotics actively can crosstalk between the immune system and the skin microbiota. From the biotechnological potential a few examples have been proposed: in mice, the administration of *Lactobacillus reuteri* enhanced wound-healing properties through the up-regulation of the neuropeptide hormone oxytocin (Poutahidis et al., 2013). In mice, diet control has been proved to alter the formation of chronic wounds, as a diet with kefir products or the administration of *Lactobacillus johnsonii* showed to support some benefits (Guéniche et al., 2006; Huseini et al., 2012). In another murine model, preparations of orally administered *Lactobacillus acidophilus*, showed enhanced wound contraction and fast epithelization when compared with the not treated. The treatment with *L. acidophilus* also increased breaking strength in sutured incision wound, increased granuloma dry weight and marked increase in collagen content indicating wound healing (Gudadappanavar et al., 2017). The use of *Lactobacillus* spp. in food biotechnology is well known, it has essentially negligible biological risk, and it is known for balancing gut microbial population (Bernardeau et al., 2006; Widyastuti et al., 2021).

It is reasonable to assume that the gut-skin and gut-brain axes biochemical signalling could also affect wound healing in Space, having a profound influence on astronaut health (Siddiqui et al., 2021). Further studies must be completed. For example, depression and chronic wounds were demonstrated to share common pathologic features, which included dysregulated inflammation and altered microbiome (Hadian et al., 2020). For this reason, it should not be excluded that isolation and stress associated with the fluctuation of the microbiome can originate delay in healing. Recently, in a questionary survey about the irritable bowel syndrome symptoms, the perceived immune fitness (subjective judgment), and impaired wound healing were studied in a cohort of 1942 Dutch students (Balikji et al., 2021). The assessment showed that impaired wound healing (self-reported) was significantly associated with irritable bowel syndrome, showing that when both conditions occurred, complaints were significantly more severe (Balikji et al., 2021).

CONCLUSION

The last frontiers on the association of the skin microbiome and wound healing during short and long mission in Space show that further understanding is needed. The perspectives for further research range from studies on microbial dynamics, natural antimicrobial properties, understating how personal skin features affect healing, the crosstalk of the microbiome with the immune system, to the skin-gut-brain axes.

Further biotechnological applications will help to understand better the skin microbiome and the healing process: for example, the three-dimensional bioprinting of skin utilizing different cell types (fibroblasts and keratinocytes) as bio-inks for tissue engineering (Jemison and Olabisi, 2021). This technology can help in studying the healing process associated with specific probiotic microbial communities (mediated by elicitors), or the deposition of different cell types along with specific biomaterials (Cubo-Mateo and Gelinsky, 2021). This is particularly important for travels to Mars, where also surgery can be a possible scenario (Kirkpatrick et al., 1997; Farahani and DiPietro, 2008; Cubo-Mateo and Gelinsky, 2021).

In this context, human skin-associated microorganisms provide a pivotal role to the skin-microbe ecosystems that still need to be fully understood. A stronger understanding of the connections and metabolic network among bacteria, fungi, the host's immune system and the host metabolism will support the basis for a better wound healing in Space. Improving our knowledge in this field of the biomedicine not only can be important in the perspective of ensuring adequate medical assistance in future space exploration missions beyond LEO, but it can have important repercussions on earth. In fact, chronic ulcers, which are continually at risk of infection, are a huge health problem, with high social costs and poor quality of patients's life.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MMar and DC prepared the manuscript. DP and MMon revised the manuscript.

FUNDING

WHISPER—"Wound Healing In Space: problems and Perspectives for tissue Regeneration and engineering," Sci Space Microgravity Application Promotion Program, ESA Contract Number 4000130928/20/NL/PG/pt.

ACKNOWLEDGMENTS

This perspective was written within the activities of ESA Topical Team on "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration".

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frontiers | Frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 12 May 2022
doi: 10.3389/fbioe.2022.806362

Thermotropic Liquid Crystals for Temperature Mapping

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Wound management in Space is an important factor to be considered in future Human Space Exploration. It demands the development of reliable wound monitoring systems that will facilitate the assessment and proper care of wounds in isolated environments, such as Space. One possible system could be developed using liquid crystal films, which have been a promising solution for real-time *in-situ* temperature monitoring in healthcare, but they are not yet implemented in clinical practice. To progress in the latter, the goal of this study is twofold. First, it provides a full characterization of a sensing element composed of thermotropic liquid crystals arrays embedded between two elastomer layers, and second, it discusses how such a system compares against non-local infrared measurements. The sensing element evaluated here has an operating temperature range of 34–38°C, and a quick response time of approximately 0.25 s. The temperature distribution of surfaces obtained using this system was compared to the one obtained using the infrared thermography, a technique commonly used to measure temperature distributions at the wound site. This comparison was done on a mimicked wound, and results indicate that the proposed sensing element can reproduce the temperature distributions, similar to the ones obtained using infrared imaging. Although there is a long way to go before implementing the liquid crystal sensing element into clinical practice, the results of this work demonstrate that such sensors can be suitable for future wound monitoring systems.

Keywords: temperature sensing, infrared thermography, colorimetric sensor, temperature distribution, thermotropic liquid crystals

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *31 October 2021*
Accepted: *24 March 2022*
Published: *12 May 2022*

Citation:
Miskovic V, Malafronte E, Minetti C, Machrafi H, Varon C and Iorio CS (2022) Thermotropic Liquid Crystals for Temperature Mapping. Front. Bioeng. Biotechnol. 10:806362. doi: 10.3389/fbioe.2022.806362

1 INTRODUCTION

Although injuries, traumatic events, and surgical emergencies have been unlikely during the current space missions, their occurrence must be considered for future human space explorations. During long-lasting Space-missions, wound treatment and monitoring could become a fundamental problem, demanding more research in this area (Barratt and Pool, 2008; Cialdai et al., 2020). For example, first-degree burns can occur as a result of ultraviolet (UV) light exposure through unfiltered spacecraft windows (Barratt and Pool, 2008). Space represents a very special remote environment, and solutions developed for Space applications could inspire the creation of a better healthcare system in remote areas on-ground.

When a wound occurs, the first step is to assess its severity, which will form the basis for the following treatment. Clinical assessment of the wound is still the most common and cost-efficient method to assess wound severity. This method relies on a subjective evaluation of the wounds'

external features, such as size of the wound, wound edges, site of wound, wound bed (colour, amount of granulation tissue), presence of necrotic tissue, wound's depth, level of exudate, and pain caused by the wound (Grey et al., 2006). The advantages of this method are that it is a rapid method and does not require specialized equipment. However, it is subjective. For instance, a nurse and clinician may have different evaluation and assessment criteria depending on their prior experiences (Nagle et al., 2020). Seeing that wound severity is usually diagnosed by a specialist, a problem appears when wound injury happens in places that lack wound care specialists, a situation common in remote areas. One way to face these problems is to develop diagnostic tools and utilities that are cost-efficient, easy-to-use, and that could support medical workers in wound diagnosis and evaluation of the healing by monitoring one or more biomarkers.

Among many clinical biomarkers, special attention has been given to pH (Shukla et al., 2007; Schreml et al., 2011; Jones et al., 2015; Power et al., 2017), oxygen (Schreml et al., 2010), and exudate composition, with the focus on matrix metalloproteinase analytes (MMPs) (Muller et al., 2008; Liu et al., 2009; Power et al., 2017). Also, temperature (Dini et al., 2015; Chanmugam et al., 2017; Jaspers et al., 2017; Power et al., 2017; Cwajda-Białasik et al., 2020) and moisture (Bishop et al., 2003; Milne et al., 2016) are the physical parameters that are frequently associated with the wound healing process. In particular, the temperature is considered as an informative parameter for all types of wounds, and it can provide information about infections of wounds, even before clinical signs appear (Power et al., 2017).

A common way to measure wound temperature is by means of Infrared (IR) thermography (Ring and Ammer, 2012). Thanks to the development of portable high-resolution affordable thermal cameras, interest in their use for wound assessment has increased. This is mainly due to their non-invasive and easily interpretable results obtained in a very short period. Various studies (Dini et al., 2015; Chanmugam et al., 2017; Jaspers et al., 2017; Martínez-Jiménez et al., 2018; Cwajda-Białasik et al., 2020; Ganon et al., 2020) have used the temperature difference between wound and healthy skin (ΔT), measured by using IR thermography, to characterize the wound status. An increase of ΔT can be related to hyperaemia, inflammation, or infection in venous leg ulcers (Dini et al., 2015; Cwajda-Białasik et al., 2020) or pressure ulcers and surgical wounds (Chanmugam et al., 2017). In burns, the temperature is often correlated to the burn depth and the burn healing time (Jaspers et al., 2017; Martínez-Jiménez et al., 2018; Ganon et al., 2020). Instead of just measuring the temperature locally, these studies demonstrated the importance of measuring the temperature distribution on the wound site and the surrounding healthy skin. Despite these promising results, IR thermography is not yet applied in daily clinical practice for wound assessment, mainly because standard guidelines and protocols have not been established yet (Shterenshis, 2017).

The field of flexible and wearable bioelectronics, capable of monitoring physiological information and assisting in proper treatment is growing exponentially (Rogers et al., 2018; Chen et al., 2020). Researchers and engineers are working on the development of new technologies for smart point-of-care systems (Zhang et al., 2016; Coppola et al., 2021). Therefore, the development of a point-of-care device for wound monitoring is more feasible. Such a device could reduce hospitalization times, the suffering of the patients, and costs (Mehmood et al., 2014; Derakhshandeh et al., 2018). Moreover, it could provide the ability to face emergency surgery, acute trauma, burns, and wounds, in remote and closed environments, such as Space. At present, wearable technology in wound care is limited to laboratory testing, and commercial wearable point-of-care systems are not broadly available. The reason could be found in the complexity of the wound healing process, the broad variety of wounds' types, and the limited understanding of relevant wound biomarkers.

Ideally, a smart sensor for wound monitoring should have specific properties, such as 1) wearability/ability to adapt to the body shape, 2) biocompatibility, 3) high sensitivity, 4) easy-to-use, and 5) no external power. Due to these requirements, sensors based on the colourimetric approach appear as an ideal solution (Ajay et al., 2017; Isapour and Lattuada, 2018). In that respect, liquid crystals (LCs) have emerged as a promising technology for wound management sensing. The most interesting property of LCs is their structural colouration that can be manipulated by changing the external parameters, such as temperature. This makes them ideal candidates for the development of easy-to-use, label-free, and passive sensors, where the output signal is a change in colour detectable by the naked eye. LCs have a long history of being used as responsive materials in different technologies, thanks to their unique properties. Numerous studies have demonstrated the possibility to produce rapid diagnostic optical sensors for temperature (Gao et al., 2014), pH (long Chen et al., 2018), humidity (Saha et al., 2012; Zhao et al., 2019), gas (Esteves et al., 2020) and molecules (ang Niu et al., 2017; Zhang et al., 2018) based on LCs. The most widespread sensors are those for temperature, owing to the diversity and availability of thermotropic LCs.

LCs are a unique state of matter between crystalline solid and isotropic liquid. In thermotropic LCs, phase transitions from crystalline solid to smectic, cholesteric and, finally, isotropic liquid, are caused by temperature changes, and they are mainly composed of rod-like molecules. In cholesteric LCs, also known as chiral LCs, molecules are inherently chiral, and the average molecular orientation is twisted with a certain periodicity, leading to a helical structure. This structure is characterised by a helical pitch that refers to the distance over which the LC molecules undergo a whole 360° twist. The size of this pitch determines the wavelength of the reflected light. The pitch of a cholesteric LC can be of the order of magnitude that corresponds to the wavelength spectra of visible light, allowing structural colouration to occur. An increase in the temperature results in a decrease in the pitch, which causes a shift in the wavelength of the reflected light. This presents the basis of the sensing principle of cholesteric LCs (Mitov, 2012). Thermotropic LCs, intended to be used as colourimetric temperature sensors, are characterized by three parameters: the lower clearing point temperature, optical activation range, and the higher clearing point temperature (Abdullah et al., 2010). The lower clearing point temperature is the temperature at which LCs first reflect

colour in the visible spectrum (red). The optical activation range is the temperature range at which thermotropic LCs actively reflect visible light. When the thermotropic LCs pass through their optical activation range, they reflect visible light from longer wavelengths (red) to shorter wavelengths (blue) as temperature increases until their higher clearing point temperatures are reached. The higher clearing point (further in the text referred as clearing point) temperature is the temperature at which thermotropic LCs stop to reflect colours in the visible spectrum. Beyond the clearing point temperature, thermotropic LCs are transparent again (Abdullah et al., 2010).

A study from 2017 (LeSar et al., 2017) investigated the possibility to use commercial thermotropic LC–coated fabric for the early detection of high-risk foot complications. Using direct visual analysis, they demonstrated that the fabric could accurately map temperatures on the surface of a hand, which supported the hypothesis that this approach can be used to develop a temperature-sensitive system to monitor complications high-risk foot. They used two different types of LC fabrics to expand the active range. The most recent prospective study (Hodorowicz-Zaniewska et al., 2020) used the Brast Tester–LC foil (Braster SA, Ozarów Mazowiecki, Poland) for the early detection of breast cancer. Despite the good performance, this contact LC thermography did not find its way into commercial use. Reasons for this include that the protocol still requires a medical specialist for its implementation, it lacks standardization, and it has been commonly replaced by contactless infrared thermography. Gao et al. (2014) were the first ones to propose a skin-like system that consists of thermotropic LCs patterned into large-scale, pixelated arrays on thin elastomeric substrates, demonstrating that such a system could be used as an epidermal temperature sensor.

This work compares the sensing ability of LCs with respect to IR thermography. To this aim, temperature mappings of surfaces of different topographies obtained using LCs sensing elements are compared against the ones obtained using IR thermography. The IR thermography is chosen as a reference since it is the most widespread technique used in wound temperature studies (Dini et al., 2015; Chanmugam et al., 2017; Jaspers et al., 2017; Martínez-Jiménez et al., 2018; Cwajda-Białasik et al., 2020; Ganon et al., 2020). This comparison will bring new insights into the possibility of using LCs-based systems for wound temperature monitoring. Some challenges in their application in clinical practice will be highlighted. Although, the final aim of the research here presented is temperature wound analysis, this paper is focused on preliminary construction and analysis of the measuring potential of the envisaged device.

2 MATERIALS AND METHODS

2.1 Cholesteric Liquid Crystals

In this work, thermotropic LCs were prepared using Cholesteryl oleyl carbonate, Cholesteryl pelargonate and Cholesteryl benzoate (Sigma-Aldrich). Four thermotropic LCs were prepared, with different pitch values, by varying the concentration of the aforementioned components, as shown in **Table 1**. In order to mix the components appropriately, the powder mixture was heated until 60°C, at which a uniform isotropic liquid is obtained.

2.2 Spectrophotometry of LCs

The first step in the research presented here was the choice of LCs formulation that has an optical activation range in a temperature range that is useful for wound monitoring. For this purpose, transmission spectra of 4 LCs formulations (see **Table 1**) were measured. Spectrophotometric analysis of LCs thin films was carried out to determine the temperature range of the cholesteric liquid crystals in which they exhibit reflection peaks in the visible spectrum (390–700 nm). For this analysis, UV-Vis Spectrometer (UV3600, Shimadzu, Japan) was used. The temperature inside the spectrophotometer was controlled using a water temperature controlling system. A thin film of LCs was uniformly coated on the cell wall. A thermocouple was placed directly inside the cell on the level where the beam is passing, and the temperature was measured in real-time on the screen. During the test, the temperature was increased with steps of 0.5°C. Transmission measurements were carried out once the temperature inside the cell was stabilized. Transmittance spectra of different LCs were collected while changing the temperature from 29.9 to 44.6°C, in steps of 0.5°C.

2.3 Patch Design and Fabrication

The liquid crystal sensing element (also referred as sensing patch) consists of three layers: a bottom polydimethylsiloxane elastomer/carbon black (PDMS/CB) layer, a middle LC sensing layer and a top transparent PDMS elastomer (PDMS) layer. The sensing patch was produced following the steps shown in **Figure 1A**.

The first step in the fabrication process was a production of a thin black bottom elastomer layer. PDMS SYLGARD™ 184 (Dow Inc.) and CB, particle size 4 μm (Nanografi) were used to produce elastomer layers. PDMS components were mixed in a ratio 10:1, with the addition of 1 wt% of carbon black powder to produce the black bottom layer. The mixture was placed under the vacuum for 15 min to remove air bubbles. To make a thin film, PDMS/CB was spin-coated on the glass substrate (50 mm × 50 mm) and cured at 100°C for 30 min. For the glass substrate with 50 mm × 50 mm dimensions, PDMS/CB was spin-coated for 30 s using a speed of 500 rpm. The thickness of the bottom layer was measured using a digital optical microscope (Keyence VHX-6000) and its build in-feature–*Plane measurements between two points*. After the curing sample was peeled from the glass substrate and cut in half and fixed between two acrylic blocks. The fixed sample was placed under the microscope (magnification ×20) in a vertical position to visualise the cross-section. The thickness was then measured ten times across the section, and the average value of approximately 200 μm is calculated. The black bottom layer was chosen for the best visualization of the colours. Thermal conductivity of pure PDMS and PDMS/CB was measured using Hot disk TPS 2500 S. Sample thickness was 5 mm, heating power 20 mW and time 10 s. Thermal conductivity of pure PDMS was 0.19 W/mK, while PDMS/CB had thermal conductivity of 0.18 W/mK. This measurement showed that addition of CB in this small

TABLE 1 | Liquid crystals samples series.

Sample name	Cholesteryl oleyl carbonate (wt%)	Cholesteryl pelargonate (wt%)	Cholesteryl benzoate (wt%)
LC1	35	55	10
LC2	32.5	57.5	10
LC3	30	60	10
LC4	25	65	10

concentration (1 wt%), does not influence the thermal conductivity of PDMS.

The second step was PDMS/CB surface activation with oxygen plasma, to increase the hydrophilicity of PDMS/CB film. Before plasma treatment, PDMS films were cleaned from dust using scotch tape. Films were treated with oxygen plasma using a plasma cleaner (PDC-002 (230 V) Haarrick Plasma) for 15 min, using the maximum power–30 W. The water contact angles before and after the treatment are shown in **Figure 1B**. An oxygen plasma treatment of 15 min using this procedure increased hydrophilicity of PDMS enough for successful printing. The contact angle before surface treatment was 112°, and after treatment, it became less than 10°.

The third step was an LCs dots array fabrication on the PDMS/CB layer, achieved using the blade coating technique. The plane surface was converted to a grid of dots to obtain the uniform colour response, and this was achieved by blade coating over the patterned paper mask. The mask was designed in CorelDraw, with the final dimensions of 50 mm × 50 mm, containing 324 dots with a diameter of 1.5 mm, and a 1 mm distance between them. A laser cutter (EpilogLaser mini) was then used to cut the paper sheet (thickness 100 μm) into the designed pattern. After removing spin-coated PDMS/CB from the plasma cleaner, the mask was glued on top of the sample, and LCs in liquid form (0.5 g for each patch) were blade coated, using a glass slide, moved by a motor with the speed of 50 rpm. The mask was removed once the LC film cooled down. The goal of the process is to cover as much as possible the original shape to maximize the sensing area, keeping at the same time the high sensibility related to homogeneity. With this configuration, the sensing area was covering approximately 23% of the total one, with 324 homogeneous highly sensitive components.

The final step was to cover LC pattern film with the protective transparent PDMS layer, using the spin coating technique (speed 500 rpm, time 30 s). Digital photos of the patch adapting to the different body surface is shown in **Figure 1C**.

2.4 Experimental Setup

In general, an experimental setup for the calibration of thermotropic LCs should consist of a calibration surface with

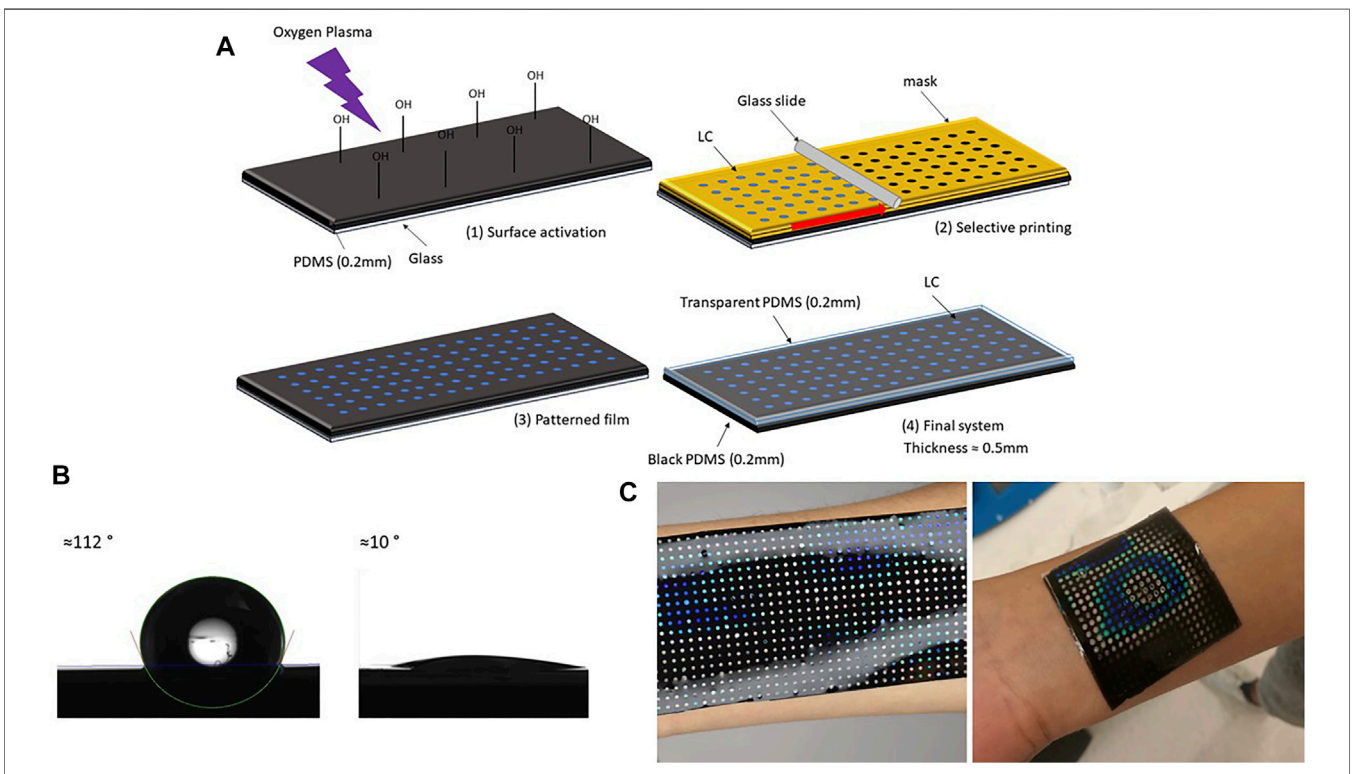

FIGURE 1 | Liquid crystal sensing patch with arrays: production steps. **(A)** Schematic description of LC sensing patch preparation: (1) thin layer (200 μm) of black PDMS (PDMS and CB) was spin-coated on a glass substrate, after curing thin film was transformed into hydrophilic surface using oxygen plasma (2) patterned paper mask (thickness 100 μm) was glued on top of PDMS film, and a drop of LC was poured on top of the mask. A uniform layer was created by blade coating (3) mask was removed when LC cooled down (4) another thin layer of transparent PDMS was spin-coated on top. After curing at the room temperature patch was peeled off from the glass. **(B)** PDMS contact angle before and after oxygen plasma treatment. **(C)** Different forms of LC patches adapting to different body parts.

a temperature sensor, imaging system, heating and cooling system, and illumination source. The experimental setup, shown in **Supplementary Figure S1**, was used to record the LC patch's colour changes with the temperature. Peltier elements with an aluminium plate was used as heating and cooling systems. The aluminium plate was used to obtain uniform plate temperatures. Two temperature sensors were within system. A temperature sensor, TS1, was placed below the aluminium plate and was used to control the temperature of the Peltier element, while another temperature sensor, TS2, was placed on top of the aluminium plate. The imaging system includes hardware [colour camera (Jai GO-5000-USB)] and software for colour extraction. A 5500K LED light was used as an illumination system. There are different types of illumination-viewing arrangements. Here, an on-axis arrangement was used. This arrangement was achieved by placing an LED light ring around the colour camera.

2.5 Assessment of the Liquid Crystal Sensor Functioning

2.5.1 The Relation Between Temperature, Colour and Hue

In order to use thermotropic LCs for quantitative temperature measurements, the determination of the relation between temperature and colour is a necessary step. Several ways to specify colour include the Red, Green and Blue (RGB), and the Hue-Saturation-Value (HSV) model. Researchers have widely used Hue to quantify colour due to its simplicity and independence with respect to illumination intensity. It is considered that the analysis of HSV data represents the simplest and most straightforward analysis approach. Hue (in degrees °) is what people typically refer to when using the term "colour." Saturation describes the degree to which a pure colour is diluted with white. It identifies how pure or intense the colour is. The value (brightness) of colour identifies how light or dark the colour is. In the following graphs, Hue will be used to quantify the colour.

This calibration of the patch should be done in an environment as close as possible to the conditions in which it will be used. The calibration was then performed in a laboratory open space so as to simulate normal utilization conditions, e.g., patients at home. Before each test, temperature and humidity in the laboratory were measured.

Before placing the sample on the aluminium plate, an automatic white balance was carried out on the white background to calibrate the colour temperature. Once the sample was placed, TS2 was in direct contact with the bottom of the patch. During the test, the temperature was increased or decreased in the cooling case, with steps of 0.1°C. When the temperature measured with TS2 was stable, photos were taken. Exposure time was set to 10,200 ms and ten pictures for each temperature were taken. RGB values were collected from dots that were in direct contact with the TS2.

2.5.2 Repeatability Test

In general, the sensor's repeatability is considered to be an important parameter, mainly if the sensor should be used multiple times and be exposed to heating and cooling conditions, like in the case of a sensor that would be used in wound monitoring. To test this property systematically, six different full-range calibrations were performed within 3 days. Each day, the first test was dedicated to heating, and the second test for cooling. Between these calibrations, the patch was stored in ambient conditions. The sample was not moved from its original place during this 3-day period, and hue values were calculated on the same spot.

2.5.3 The Dynamic Test

Response time is another important sensors' characteristic. In this study, response time was measured using a dynamic test. First, the sample patch was placed on the aluminium plate and then the temperature was set to 34°C. The monitoring started after the system was stabilised, i.e., TS2 temperature and Hue were not changing. During the test, temperature from sensor TS2 was constantly monitored (20 values per second), and images were taken automatically (10 frames per second) using a colour camera. When the monitoring started, the set-point temperature was changed from 34 to 37°C.

2.5.4 Application for Colour/Hue Quantification

A C++ application was developed to allow quantitative reading of the temperature using the LCs patch. An example of the temperature reading is shown in **Figure 2**. The size of the selected area can be directly modified in the integrated window. We can choose the number of pixels on each side of the central point, which provides the Hue values for each pixel. From this, the application will then calculate the average hue value converting it to the temperature of the selected area, using the data from the calibration curves (see later in **Figure 5**).

2.5.5 Comparison With IR Thermography

The goal was to directly compare the temperature distribution of the same surface obtained using the LCs patch with the one recorded using an Infrared Thermal Imaging Camera, FLIR T425 with a 320 × 240-pixel resolution. This test was performed on the same setup used for calibration. In this case, instead of an aluminium plate, we used flat surfaces composed of two materials with different thermal conductivities, aluminium (thermal conductivity 237 W/mK) and an acrylic sheet (0.2 W/mK). Three different surfaces were prepared, where the base was an aluminium plate with different 3D shapes engraved using a CNC machine and opposite acrylic masks. The goal of creating these plates is to get a flat surface that will, during heating, have different temperature distributions on the top. This will mimic the temperature distribution at the wound and surroundings. Digital photos of the surfaces are shown in **Supplementary Figure S2**. All surfaces were sprayed with conductive black spray to make IR imaging more accurate. Firstly, an LC patch (50 mm × 50 mm) was placed on the heated surface and photos were taken. Following that, the patch was removed, and a corresponding IR images from the same surface were taken. The IR photos were taken at room temperature, and FLIR was left on for at least 10 min for stabilization before imaging. The distance between the FLIR camera and the mould was 0.2 m, and

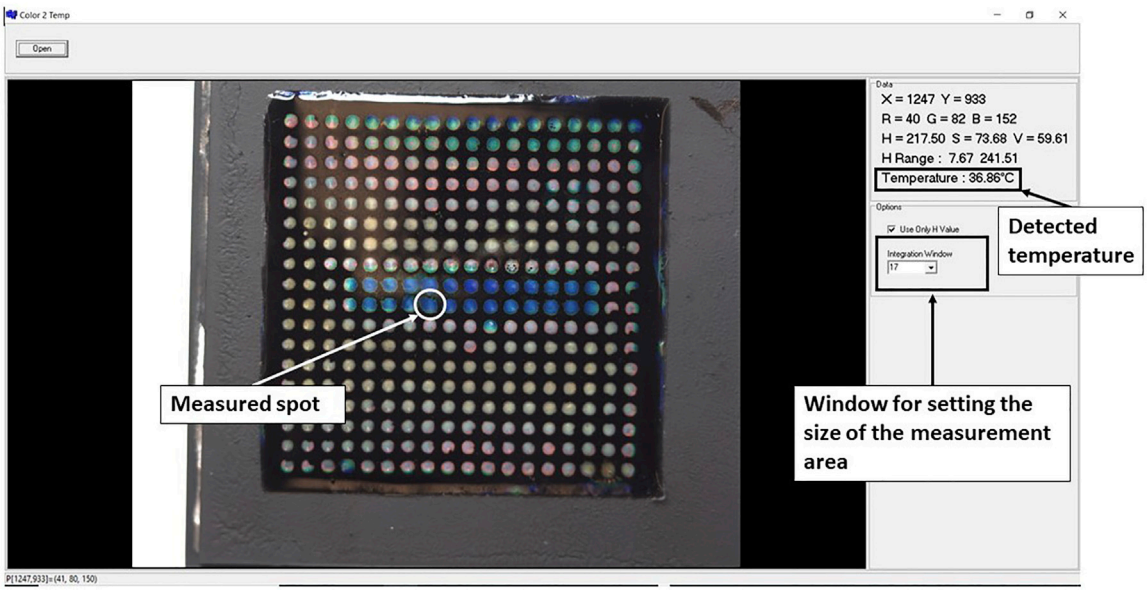

FIGURE 2 | The interface of the application used for quantitative analysis of temperature from the LC patch.

emissivity was set to 0.75. The value for the emissivity was chosen based on the calibration measurements, where two sensors (with accuracy 0.1°C) were placed directly on the surface and the temperature measurements were compared to the IR measurements from the top.

3 RESULTS

3.1 Choice of LC Formulation

Transmittance spectra of LCs are shown in **Figure 3**. For each LC formulation, reflection peaks were shifted towards a lower wavelength, i.e., blue region, with temperature increase. Calibration curves were created by taking the reflections' peak wavelength values for each temperature, presented in **Figure 4**, to establish which liquid crystals combination was the best for the described application. As expected, different LC formulations showed the optical active range in different temperature ranges, shown in **Table 2**. On average, the temperature span was 5°C. All calibration curves showed a second-order polynomial trend. For more straightforward representation, calibration curves were divided into two linear regions, higher and lower sensitivity regions. Sensitivity and range for these regions are shown in **Table 2**. The sensitivity was calculated as the slope of the calibration curve, and it is presented in nm/°C. All formulations showed the same trend, higher sensitivity (78 nm/°C) for lower temperature ranges (red-green colour range) and lower sensitivity (25 nm/°C) for higher temperature ranges (blue colour range). This trend is well-aligned with reports in the literature (Stasiek et al., 2014). Accordingly, for lower temperature ranges, the resolution is 0.013°C. While for higher temperatures the resolution is 0.04°C. Both resolutions are fulfilling requirements for a wound temperature sensor. LC2 system was chosen to produce the sensing patch, since the temperature range (32.5–38.7°C), where pitch corresponds to the wavelength of visible light, is connected to the wound healing temperature range. However, the described procedure can be used to produce patches with any thermotropic LC formulation.

3.2 Relation Between Temperature and Hue

Photos used for calibration are shown in **Figure 5A**. RGB values were taken from two spots, each 50 × 50 pixels, that were in direct contact with sensor TS2. Representative calibration curves, including RGB-temperature dependence and Hue-temperature dependence, are shown in **Figures 5B,C**. The Hue—temperature relationship is strongly non-linear, as can be seen in **Figure 5C**. The non-linear trend of Hue-temperature dependency for thermotropic LCs is expected and previously reported in the literature (Sabatino et al., 2000; Anderson and Baughn, 2004). The hue-temperature curve shown in **Figure 5C** can be well fitted by a 5-order polynomial (R^2 0.991), which is used to convert LCs colour images to the temperature distribution. The part where hue increases monotonically with temperature is known as the effective temperature range or hue bandwidth. From **Figure 5C** it can be seen that the effective temperature range in LC2 is approximately 3.5°C, from 34 to 37.1°C. These types of LCs that have bandwidths within the range of 0.5–4°C are typically referred to as narrowband thermotropic LCs. Although they cover smaller temperature ranges than wideband LCs, the advantage is their higher precision in temperature measurements and are less affected by variations in illumination intensity (Abdullah et al., 2010). Sensitivity in the effective temperature range, corresponding to lower temperatures (34–37.1°C) and red and green hue values, is 73°/°C. However, entering the blue regions (37.1–38.6°C), sensitivity drops to 4.5°/°C.

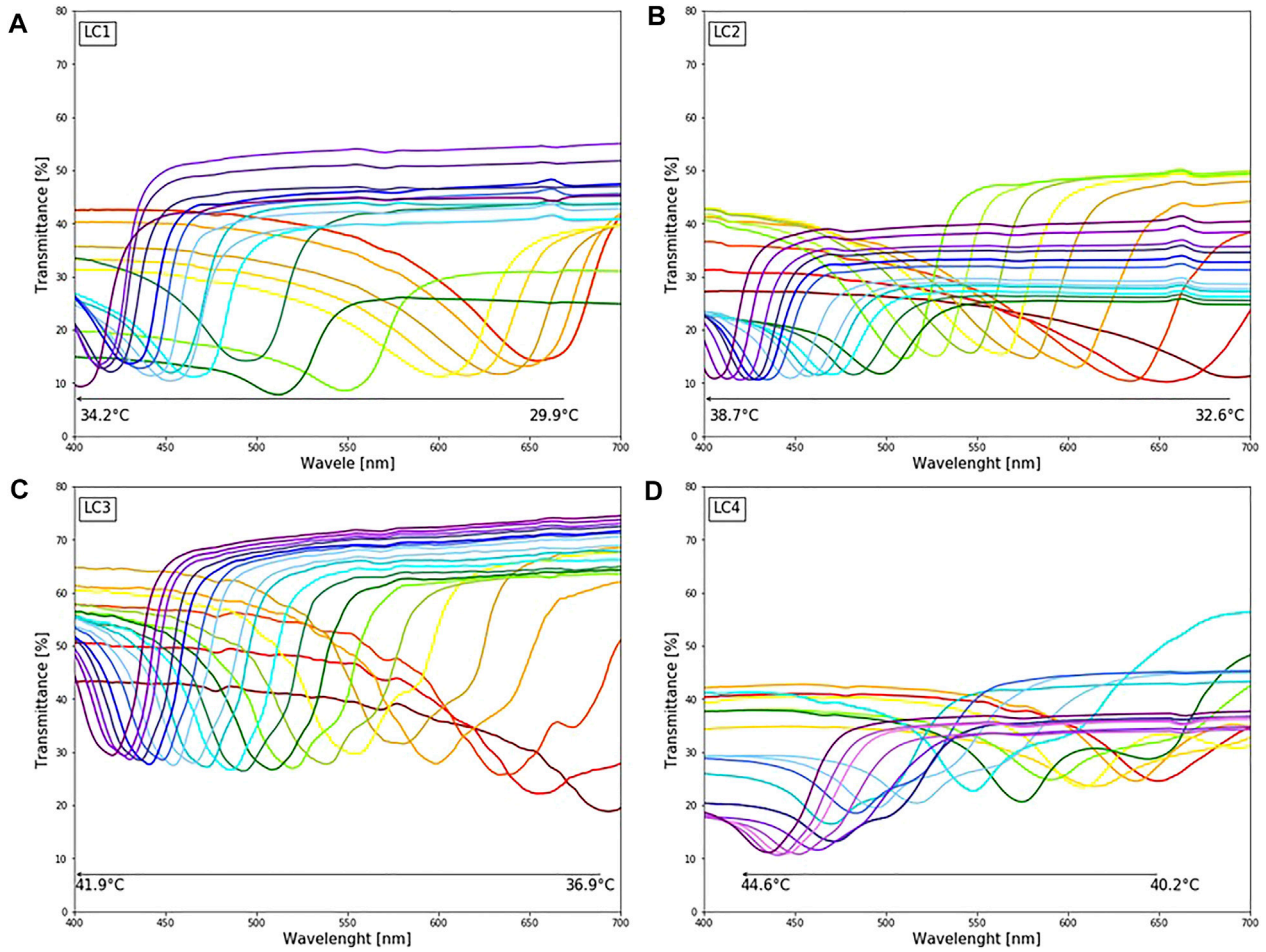

FIGURE 3 | The colorimetric response of different LC upon changing temperature (29.9–44.6°C): **(A)** LC1: reflection peaks in the visible spectrum for temperature range 29.9–35°C **(B)** LC2: reflection peaks in the visible spectrum for temperature range 32.6–38.7°C **(C)** LC3: reflection peaks in the visible spectrum for temperature range 36.9–41.9°C **(D)** LC4: reflection peaks in the visible spectrum for temperature range 40.9–44.7°C.

3.3 Repeatability

3.3.1 Temperature Shift in Time

Hue-saturation values are presented in **Figure 6A**, and Hue-temperature curves on **Figure 6B**. It is observed from **Figure 6A** that colours are less saturated as time increases. The temperature shift in the calibration curves at four points (Hue = 50°, 100°, 150° and 200°) is presented in **Table 3**. Compared to the first calibration curve, the temperature shifts increased with time, and was the highest on the last day during cooling, reaching 0.47°C. The ageing had a maximum effect on the hue-temperature relation for lower hue values. The magnitude of the temperature shift is in agreement with literature, but it shows the opposite trend. Wiberg and Lior (2004) reported a maximum shift of 0.4°C but towards higher temperatures, while shifts towards lower temperatures were observed in our study. In addition, this experiment showed that time is causing the drop in sensitivity, see **Table 4**.

3.3.2 Hysteresis

During the repeatability test, it was observed that the hue temperature relationship depends upon whether the crystals are undergoing a cooling or heating cycle. This difference is known as the hysteresis effect. The hysteresis effect was studied by heating the patch until 40°C and cooling it down until 32.5°C. Hue was calculated during heating and cooling for steps of 0.1°C. Corresponding RGB-temperature and Hue-temperature curves are presented in **Figures 7A,B**. In the cooling cycle, a shift in R, G and B peaks is observed, resulting in a shift to a higher hue for the same temperature. The hysteresis effect was the highest in the temperature range around 35°C, whereas for the same hue value the difference in the temperature was approximately 0.26°C. Several other studies (Dixon and Scala, 1970; Zink and Belyakov, 1997; Sabatino et al., 2000; Anderson and Baughn, 2004; Kakade et al., 2009) reported a hysteresis effect in thermotropic LCs. These studies observed that the hysteresis effect appears if LCs are heated above their clearing point.

3.4 Dynamic Test–Response Time

Changes in temperature and hue are presented in **Figure 8**. The first change in Hue was detected 0.25 s after the first changes in temperature, which indicates an almost immediate response of hue on temperature colour. This response cannot be clearly seen in the

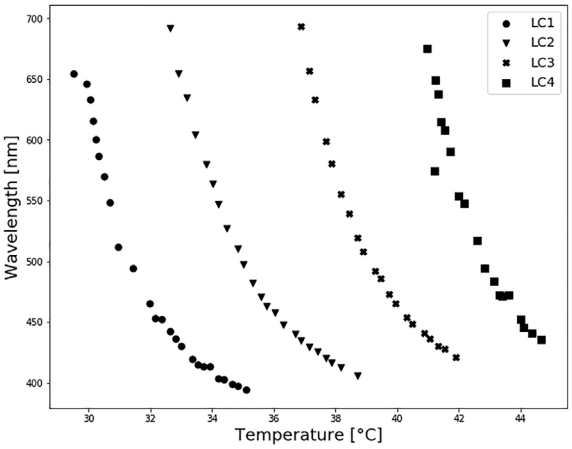

FIGURE 4 | Thermotropic LC spectrophotometry: calibration curves. All calibration curves showed a second-order polynomial trend.

wound studies. IR thermal cameras are sensitive to environmental conditions. Before imaging, IR cameras require input values including room temperature, humidity, distance from the object and its emissivity. It is not always possible to correctly determine these parameters, although they are directly influencing the reading output. The emissivity of human skin is considered to be between 0.97–0.99 (Boylan et al., 1992; Keenan et al., 2017), while different studies have found that the emissivity of wounds can be greater by 0.01–0.03. This could result in an underestimation of the ΔT value by 0.1–0.2°C (Boylan et al., 1992), and could prove significant in clinical evaluations of some studies. Depending on the location and size of the wound, the temperature of the healthy surrounding skin can vary for several degrees (Carriere et al., 2020). Hence directly influencing the ΔT value and the reading outcome. Moreover, the wound bandage should be removed for each temperature measurement using IR thermal cameras. This is a disadvantage because it prevents continuous temperature monitoring.

TABLE 2 | Liquid crystals response range and sensitivity. Measurement done using the spectrophotometer.

Sample Name	Linear response range 1 (°C)	Linear range sensitivity 1 (nm/°C)	R^2	Linear response range 2 (°C)	Linear range sensitivity 2 (nm/°C)	R^2
LC1	29.9–32.0	89.7	0.95	32.1–35.1	25.3	0.98
LC2	32.6–35.9	71.2	0.98	36.0–38.7	18.9	0.98
LC3	36.9–39.5	78.6	0.97	36.6–41.9	23.7	0.98
LC4	40.9–43.1	81.6	0.88	43.2–44.7	31.2	0.94

graph because the first change occurred in the red region, before the effective temperature region. The response time is the same in both the heating and cooling cycles.

3.5 Comparison Between LCs Wearable Patch and IR Imaging

Results are shown in **Figure 9**; **Table 5**. Although a relatively accurate temperature distribution of the surface using the LC paths was achieved, the pattern recognition and sensing quality were higher using the IR camera. ΔT was calculated for IR and digital images for each shape (see **Figure 9**), as the difference between the average temperature of the hotter and colder surface. The difference in ΔT, calculated using IR and LC images varied for different shapes. The highest difference was observed for shape 1, where the difference between the two measurements was 0.7°C. In contrast, there was no difference between IR and LC techniques for shape 3. This phenomenon can be contributed to the quality of the contact between the surface and the bottom of the patch. Although the results are encouraging, some improvements are in order, which will be discussed in the following section.

4 DISCUSSION

As described in the introduction, IR thermography is commonly used to measure temperature distributions in

In general, this study showed that LCs have the potential to fabricate advanced temperature distribution sensors. This is not common to other sensing elements, such as resistance or impedance-based ones, that could measure only point-wise values. Another important advantage of this configuration is its simplicity in reading the output. When compared with infrared imaging, LCs are capable of reproducing the main features of the temperature fields surrounding a wound mimicking shape, even geometrically complex. However, the recognition of the patterns was somewhat less sharp for the LCs with respect to the IR camera. This might be caused by the contact between the patch and the to-be-measured surface.

The thermotropic LCs used in this research can detect even small temperature variations (high sensitivity) and have a good repeatability as well as a fast response time (less than 0.5 s). However, LCs used here have a narrow bandwidth, resulting in high sensitivity but a small effective temperature range. The temperature range can be extended by changing the type of thermotropic LCs or by changing the component ratio. According to (Ochoa et al., 2014), the preferred requirements for temperature wound monitoring sensors depend on the wound's type. However, during the wound healing process, the dynamic temperature range is 25–41°C. The other formulations (see **Figure 4**), showed that it is possible to cover from 29–44°C. In this study only one of them was used

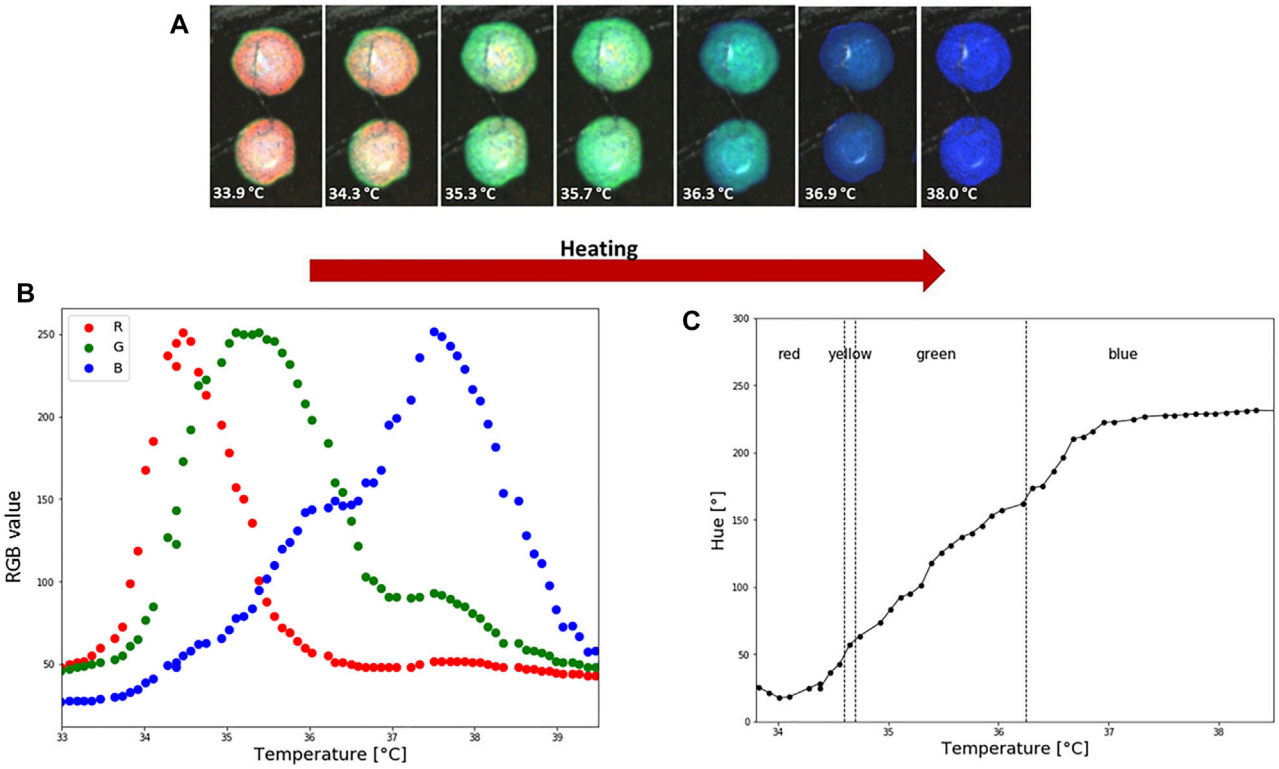

FIGURE 5 | LC2 patch colorimetric response during heating and calibration. **(A)** How the temperature is increasing LC are going through an optical active range, which starts with red and finishes with blue colour. Average RGB and HSV values from two spots were used to create a calibration curve. Each spot is 50 × 50 pixels. **(B)** Temperature and RGB dependence. **(C)** Hue-temperature calibration curve. During the calibration the ambient temperature was 23°C and relative humidity 45%.

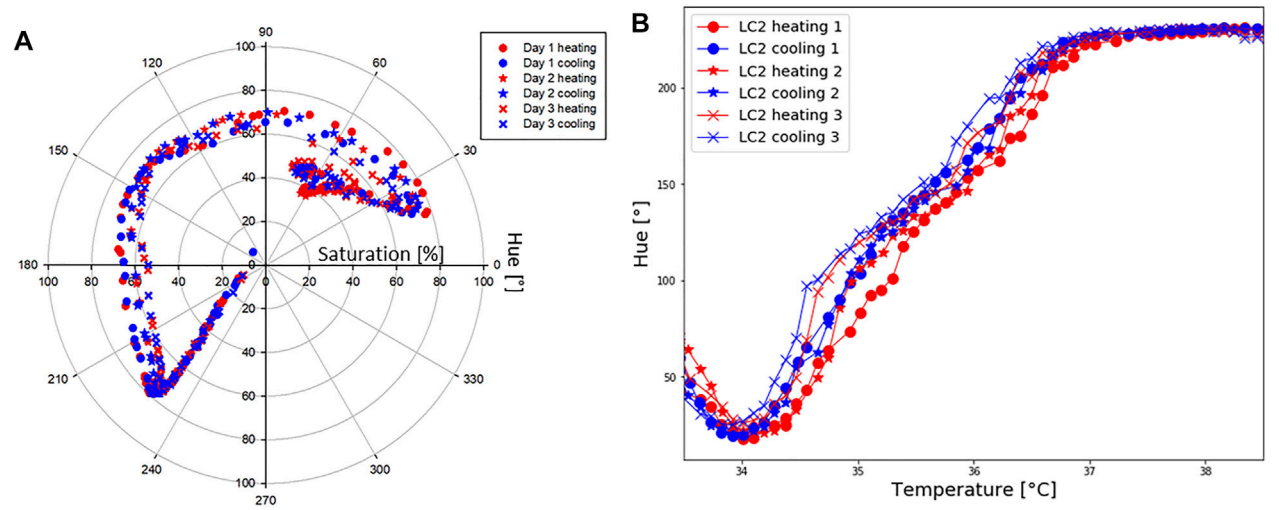

FIGURE 6 | LC2 patch: repeatability. **(A)** Hue [°] and saturation [%] values during six separate tests, done in 3 days. **(B)** Hue [°]-temperature [°C] test performed to study repeatability.

in order to focus on the principle and show the feasibility of such an approach, but the same study can be performed on the other liquid crystals.

The ageing of LCs reduces their shelf life. Proper preparation and storage are necessary to minimize their ageing. It was suggested that pure thermotropic LCs should

TABLE 3 | LC2 patch: Temperature shift (°C) compared to the first heating calibration curve.

Hue (°)	Cooling 1	Heating 2	Cooling 2	Heating 3	Cooling 3
50	0.25	0.09	0.20	0.33	0.47
100	0.24	0.09	0.19	0.29	0.42
150	0.23	0.1	0.18	0.26	0.37
200	0.22	0.1	0.17	0.22	0.32

TABLE 4 | LC2 patch sensitivity in effective temperature region 34–37.1°C.

Measurement	Sensitivity (°/°C)	R^2
Heating day 1	72.8	0.99
Cooling day 1	71.8	0.97
Heating day 2	73.1	0.98
Cooling day 2	71.6	0.98
Heating day 3	69.4	0.97
Cooling day 3	67.74	0.97

not be exposed to fats, greases, organic solvents and dust. They can also be susceptible to UV and IR radiation, and exposure to these sources reduces the shelf life (Abdullah et al., 2010). Another observation is that the hue–temperature dependence is non-linear. The decrease in sensitivity is particularly notable when entering in the blue region, where small changes in temperature do not cause significant changes in hue. The cause of hysteresis in cholesteric LC is complex. Previously it was suggested that hysteresis is strongly associated with chemical composition (Dixon and Scala, 1970) and that hysteresis depends on the sample thickness (Zink and Belyakov, 1997). In the case of a narrowband LC, the thicker sample showed no hysteresis, and the thinner sample showed up to about a 0.5°C bias toward lower temperatures when cooled (Zink and Belyakov, 1997). Moreover, the magnitude of the shift increases with increasing maximum temperature before cooling. For this particular application, hysteresis should be avoided since it can give false information about temperature at the wound during cooling. Hysteresis can be reduced significantly if the operating temperature is regulated. Most importantly, the operating and storage temperature should be kept below the clearing point temperature (Zink and Belyakov, 1997). However, this does not necessarily represent a problem if the calibration is done with small steps (0.1°C) and a computerised way of reading colour is included, such as the application used in this work.

Note that the technological readiness of LCs, although promising, does not directly translate into the possibility to include them in clinical trials. Several issues should be considered in that respect and they will be discussed below.

Wound contamination: the current status of the proposed patch requires that it should be in direct contact with the wound. In clinical practice, medical personnel are obliged to use wound dressings to avoid contamination, protect wounds, and promote wound healing. Nowadays, an ideal wound should fulfil the following characteristics: 1) creating a moist and warm environment around the wound, 2) allowing gas exchange, 3) protecting the wound from bacterial infections, 4) creating a mechanical protection, 5) controlling the exudate level, 6) being biocompatible, non-toxic, non-allergenic, 7) being easily removable, i.e., the material should be non-adhering to avoid removing of a newly formed tissue, 8) being able to stimulate healing, and 9) being costly acceptable (Sood et al., 2014). Applying the proposed LC system on top of the wound dressing could influence some of the aforementioned dressing's characteristics, such as gas exchange, exudate and moist control properties. Moreover, the LCs sensing characteristics could be affected by the micro-environment of the wound and

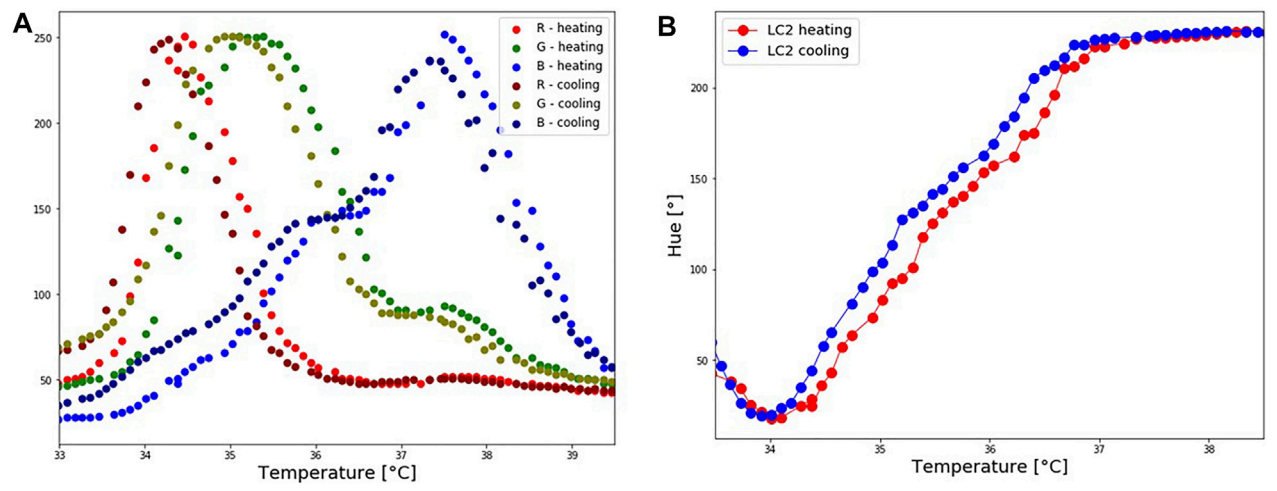

FIGURE 7 | LC2 patch: Hysteresis effect. The patch was first heated until reaching the isotropic phase, and cooled down. **(A)** Shift in RGB values during the cooling process. **(B)** A corresponding shift in Hue values during the cooling process.

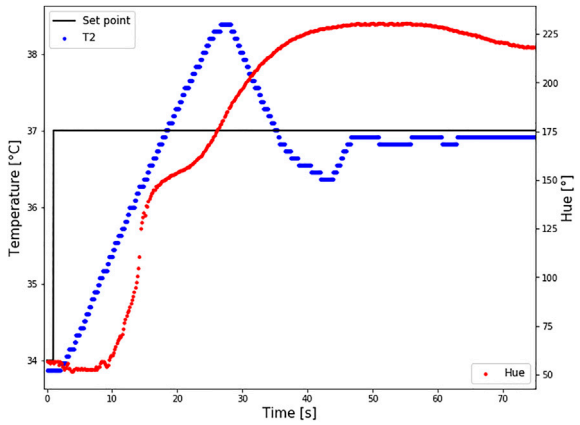

FIGURE 8 | Response time of LC2 patch. The set point temperature was changed from 34 to 37°C (black line), and the temperature sensor TS2 measured the temperature at the bottom of the patch (T2-blue curve). At the same time changes in Hue were calculated (red curve). Intimidate reaction in hue changes how the temperature is changing was reported.

surroundings. Another option would be to use the LC-based sensor just during the bandage removal. In this case, continuous monitoring is not possible.

Size of the wounds: in literature it is possible to find an outstanding number of wound sensors, either based on LCs or not. Everyone who participated to a real clinical study on wound imaging knows that serious wounds, and especially burns, could cover large portions of the body, sometimes even the whole body. It is evident that the development of a contact sensor that would monitor such a wound is currently not possible.

Shape and 3D nature of the wounded tissues distributions: although maybe technically possible, it is very difficult to imagine a contact sensor when the shape and distribution of the wounds is not flat or even worse, changing "volumetrically" during the healing time. It would be possible to think of an intermediate layer hosting the sensing element. If this solution could potentially solve the flatness problem, it will also challenge the capability of this layer to transmit reliably biophysical signals from the wound to the sensing element.

Although it is beyond the scope of this paper to provide a complete overview of the clinical requirements for wound diagnosis and follow-up, it is possible to draw preliminary conclusions. LCs could represent a complementary element to support the medical decision and follow-up in the case of wounds, especially burns. However, the application of such kinds of sensors in clinical practice is far from being straightforward, and the plethora of sensors' concepts presented in the literature are still at a low-TRL stage.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

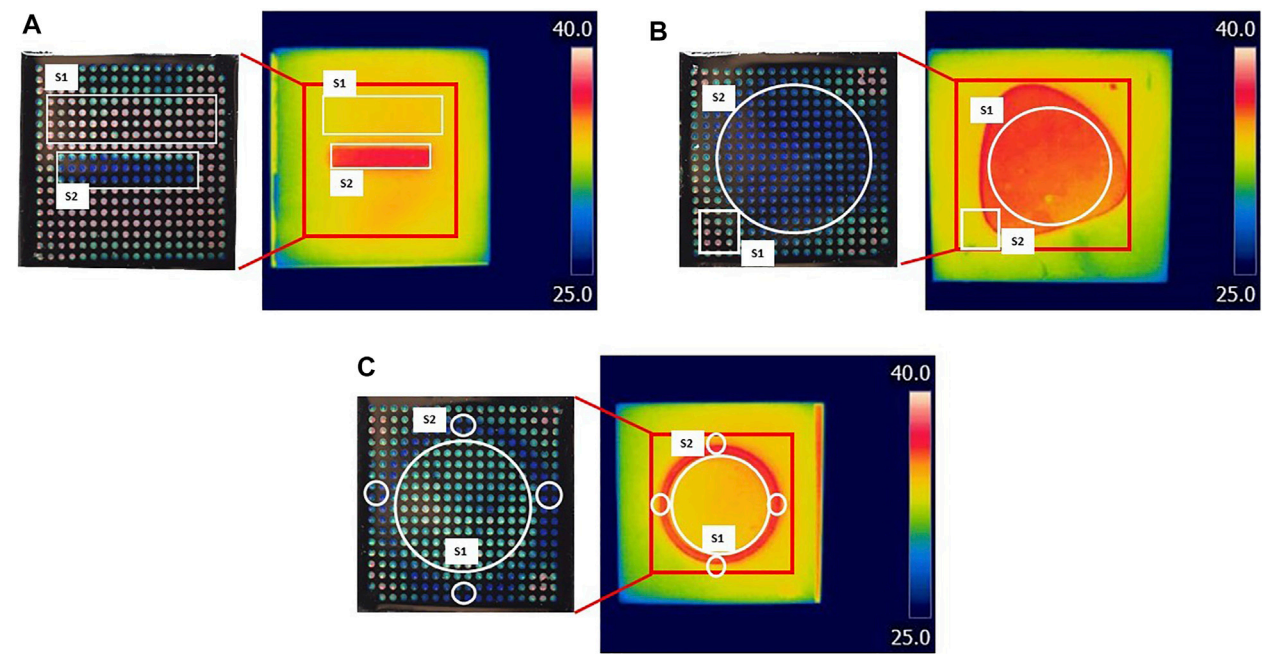

FIGURE 9 | Comparison between LCs patch and IR imaging. Three surfaces **(A–C)** with different temperature distributions were tested. On the left are digital photos of LCs patch response when in contact with the heated surface; on the right are IR images of the same surface. The temperature was calculated from the surfaces marked with white on both digital and IR images. The red square on IR images represents the surface covered by the LC patch.

TABLE 5 | Comparison between temperature measurements using LCs patches and application against the ones measured using IR imaging.

Measured area			Tmax [°C]	Tmin [°C]	Taverage [°C]	ΔT [°C]
Shape1	IR	S1	34.8	32.8	33.9	2.6
		S2	37	35.8	36.5	
	LC	S1	36.8	33.2	35.4	1.9
		S2	38.4	36.5	37.3	
Shape2	IR	S1	34.6	32.1	32.8	3.1
		S2	37.5	31.6	35.9	
	LC	S1	36.5	33.1	34.9	2.5
		S2	38.8	36.3	37.5	
Shape3	IR	S1	35.8	33.6	34.4	1.8
		S2	36.4	35.9	36.2	
	LC	S1	36.5	34.0	36.1	1.8
		S2	38.3	36.6	37.9	

AUTHOR CONTRIBUTIONS

VM and CI contributed to the design and conception of the study. VM, with the help of CM and EM, carried out measurements. VM and CI drafted the article. VM created tables and figures. VM, CI, CV, and HM participated in the revision process of the article and gave final approval of the submitted version.

FUNDING

This work was funded through the WHISKIES, a ESA-sponsored Microgravity Application Promotion. We also thank the Alice and David Van Buuren Fund and Foundation Jaumotte-Demoulin for the provided funds.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.806362/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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NOMENCLATURE

LC liquid crystal

IR Infrared

CB carbon black

TS temperature sensor

frontiers | Frontiers in Bioengineering and Biotechnology

MINI REVIEW
published: 13 May 2022
doi: 10.3389/fbioe.2022.868999

Space Flight-Promoted Insulin Resistance as a Possible Disruptor of Wound Healing

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During space flight, especially when prolonged, exposure to microgravity results in a number of pathophysiological changes such as bone loss, muscle atrophy, cardiovascular and metabolic changes and impaired wound healing, among others. Interestingly, chronic low-grade inflammation and insulin resistance appear to be pivotal events linking many of them. Interestingly, real and experimental microgravity is also associated to altered wound repair, a process that is becoming increasingly important in view of prolonged space flights. The association of insulin resistance and wound healing impairment may be hypothesized from some dysmetabolic conditions, like the metabolic syndrome, type 2 diabetes mellitus and abdominal/visceral obesity, where derangement of glucose and lipid metabolism, greater low-grade inflammation, altered adipokine secretion and adipocyte dysfunction converge to produce systemic effects that also negatively involve wound healing. Indeed, wound healing impairment after traumatic events and surgery in space remains a relevant concern for space agencies. Further studies are required to clarify the molecular connection between insulin resistance and wound healing during space flight, addressing the ability of physical, endocrine/metabolic, and pharmacological countermeasures, as well as nutritional strategies to prevent long-term detrimental effects on tissue repair linked to insulin resistance. Based on these considerations, this paper discusses the pathophysiological links between microgravity-associated insulin resistance and impaired wound healing.

Keywords: insulin resistance, wound healing, microgravity, spaceflight, rehabilitation, diabetes complications

OPEN ACCESS

Edited by:
George Alexander Truskey, Duke University, United States

Reviewed by:
Noha M. Mesbah, Suez Canal University, Egypt

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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *03 February 2022*
Accepted: *14 April 2022*
Published: *13 May 2022*

Citation:
Strollo F, Gentile S, Pipicelli AMV, Mambro A, Monici M and Magni P (2022) Space Flight-Promoted Insulin Resistance as a Possible Disruptor of Wound Healing. Front. Bioeng. Biotechnol. 10:868999. doi: 10.3389/fbioe.2022.868999

INTRODUCTION

Humans engaged in space flight show a number of physiological changes due to a set of known (microgravity, confinement, isolation, space radiation) as well as still unknown stressors related to this specific environmental condition (White and Averner 2001). The opportunity of prolonged or even very prolonged space flights, like long-term stay at the International Space Station (ISS) or the planned travel to the Moon, Mars and possibly to other planets, represents a major health challenge for astronauts, since exposure to the above-mentioned factors may result in relevant and even permanent pathological changes, such as bone loss, muscle atrophy, dysregulation of the immune

system, cardiac and metabolic alterations and impaired wound healing (White and Averner 2001; Frippiat et al., 2016; Grimm et al., 2016; Riwaldt et al., 2017), to mention just some of them. Among these stressors, microgravity has been extensively studied by means of in-flight as well as on-Earth experiments, such as the 6-degrees head down tilt (HDT) (Grimm 2021). Microgravity is known to promote a series of cardio-metabolic changes, including fluid shift, impaired glucose and lipid metabolism, increased oxidative stress and pro-inflammatory cytokine release and chronic low-grade inflammation through muscle unloading and other causes. Interestingly, a central pathophysiological event linking most of these processes is insulin resistance at specific organs, like the liver, the skeletal muscles and the adipose tissue, but also the blood vessels and the skin and epithelia (Tobin et al., 2002; Bergouignan et al., 2011).

In the context of space flight, the relevance of correct wound repair is increasingly important, since, in addition to unwanted injuries, some surgical procedures may also be implemented during prolonged space missions (Drudi et al., 2012). However, several studies indicate that microgravity can induce an impairment of processes specifically related to wound repair (Delp 2008), for example by altering the functionality of cell populations involved in such events (Cialdai et al., 2020). Thus, wound healing impairment after traumatic events and surgery in space remains a relevant concern for space agencies.

According to knowledge obtained from on-Earth biomedicine, it has been clearly observed that chronic wounds, with defective repair processes, are a common complication in patients with type 2 diabetes mellitus (T2DM) and the related insulin resistance, and often lead to amputation. Such non-healing wounds are characterized by a persistent inflammatory state promoted by pro-inflammatory macrophages, pro-inflammatory cytokines and proteases (Salazar et al., 2016).

Based on these considerations and within the specific context of astronaut health during prolonged space flight and exposure to microgravity, the occurrence of insulin resistance may thus contribute to the observed impairment of wound healing (Bacci and Bani 2022). This paper discusses the evidence and knowledge gaps regarding this pathophysiological association. To this aim, the PubMed and Excerpta Medica Database (Embase) were searched from inception until April 2022. Used search terms, with a combination of MeSH terms if applicable in each database, included: (space flight), (microgravity), (insulin resistance), (wound), (healing).

INSULIN RESISTANCE AND METABOLIC SYNDROME PATHOPHYSIOLOGY AND ASSESSMENT

Insulin resistance is the pathophysiological hallmark of the metabolic syndrome (MS), which is currently a global epidemic, accounting for some 15% or more of the overall population worldwide, and is tightly linked to increased obesity prevalence and chronic low-grade inflammation (Saklayen 2018; Neeland et al., 2019; Ross et al., 2020). The MS is defined as a cluster of cardio-metabolic clinical conditions (increased waist circumference, arterial hypertension, hyperglycemia, reduced HDL cholesterol and increased triglyceride), which results in increased individual risk for cardiovascular morbidity and mortality (Alberti et al., 2009). In this context, a pivotal role in the pathophysiology of the MS and the related increased cardiovascular risk is played by the expanded and dysfunctional visceral adipose tissue compartment (Neeland et al., 2019; Ross et al., 2020). Indeed, far from having a mere storage function, adipose tissue can be referred to as a diffuse endocrine organ (Ahima 2006), secreting several intertwined signals which contribute to appetite and energy regulation, immunological and metabolic balance, and wellbeing. These molecules include the adipokines leptin and adiponectin, which modulate a relevant set of physiological functions along with other signals from the gut (ghrelin) and other organs (Magni et al., 2000; Bertoli et al., 2006; Dozio et al., 2009; Magni et al., 2009), and whose changes are associated with important alterations of cardio-metabolic functions (Norata et al., 2007) and bone metabolism in patients (Magni et al., 2010), as well as under microgravity conditions. When growing well above its normal volume or outside its natural boundaries into the abdominal space and other sites, adipose depots soak the liver, the pancreas, and other organs with fatty acids and cause local metabolic changes leading to excess oxygen demands. As a consequence, adipose tissue attracts macrophages bound to get rid of damaged cells and releasing high amounts of pro-inflammatory cytokines, in addition to altered adipokine amounts released by dysfunctional adipocytes (Magni et al., 2005; Cao 2014) (**Figure 1**).

These changes tend to progress over time since hypertrophic fat depots expand spontaneously through various self-maintaining mechanisms related to chronic low-grade inflammation, associated polarization of macrophages to the Th2 phenotype (**Figure 2**) and triggered by adipokines and other factors (Cao 2014), including insulin resistance and muscle dysfunction (Dyck et al., 2006), that are also characteristic of the aging process, and, therefore, can be defined "inflammaging" ((Franceschi and Campisi 2014; Franceschi et al., 2018; Fülöp et al., 2019).

Moreover, nephropathy is frequently observed within the frame of an ill-regulated aging process due to metabolic aberrations negatively affecting acid-base balance, calcium, glucose, and lipid metabolism (Samaras and Campbell 2005). Insulin resistance is characterized by impaired insulin activity, which is initially compensated for by higher circulating insulin levels. Individuals with severe insulin resistance report: 1) altered glucose metabolism including type 2 diabetes mellitus (T2DM) or paradoxical late post-prandial hypoglycemia; 2) acanthosis nigricans, velvety hyperpigmentation of axillary and flexural skin; and 3) hyperandrogenism in women (Savage et al., 2007).

Different approaches have been proposed to identify and monitor insulin resistance in humans. Fasting insulin levels only reflect a specific time point and are influenced by dynamically intertwined metabolic components, including overnight hepatic glucose production and its effects on insulin output, hepatic insulin extraction, spontaneous pancreatic insulin secretion spikes, and the balance between kidney gluconeogenesis and insulin excretion rate (Laakso 1993). Thus, fasting insulin

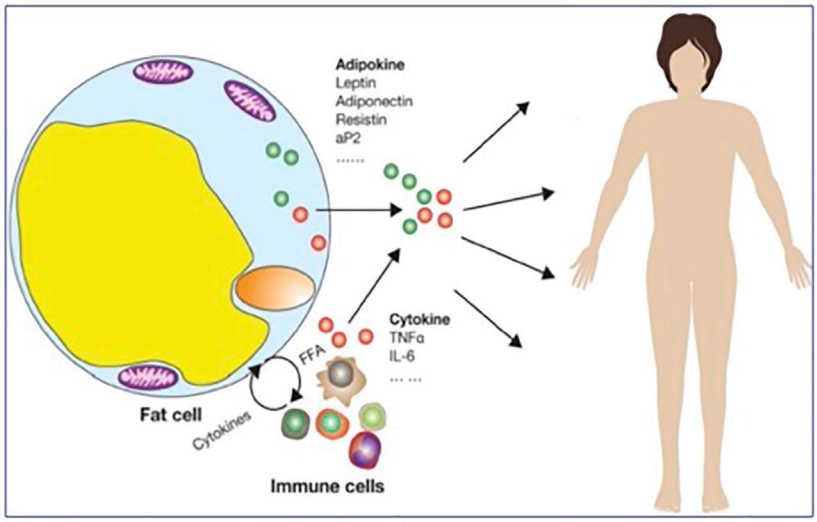

FIGURE 1 | Mechanisms underlying chronic low-grade inflammation after excess/ectopic/dysfunctional adipose tissue accumulation (adapted from (Cao 2014)).

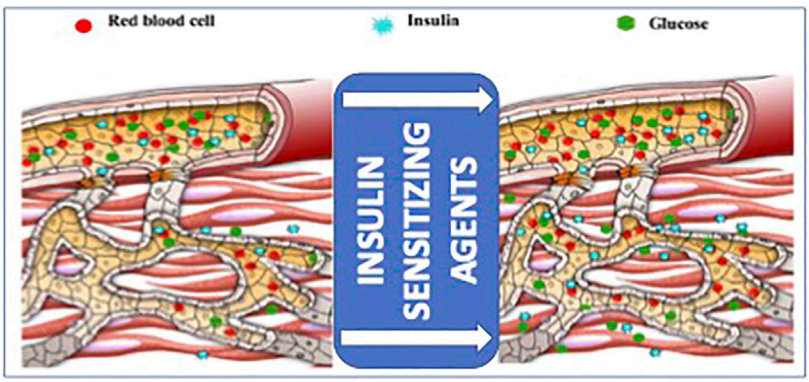

FIGURE 2 | Schematic diagram of the effects of insulin sensitizing agents, including adiponectin, on microvascular recruitment and insulin delivery in the muscle, providing an at least partial explanation of the negative impact of insulin resistance on anabolic signal-related wound healing (adapted from (Zhao et al., 2014)).

levels are quite variable within the same individual and unreliable, when considered alone, for diagnostic approach.

The "Homeostasis Model Assessment (HOMA)-1" (Radziuk 2014) and "HOMA-2" (Levy et al., 1998) index calculations can be easily adapted to large-scale studies (Demir et al., 2020), thanks to their straightforward mathematic approach. Hyper-insulinemic euglycemic clamp (HIEGC) is the reference method for quantifying insulin resistance, but it is a complex, time-consuming, and expensive method, thus less likely to be utilized in specific experiments, like those designed for space flight studies (Johns et al., 2012). In the future, promising results are expected from metabolomics (Milburn and Lawton 2013). Recently, an indirect non-invasive method has been developed based on pulse wave velocity analysis, taking advantage of impaired vascular elasticity as a function of inflammaging, but yet still awaits final validation as for insulin resistance when applied to clinical research (Petrasek et al., 2015). The potential usefulness of measurement of the circulating levels of leptin and adiponectin may also be considered, as the former is known to have pro-inflammatory effects, while the latter acts as an insulin sensitizer, a good marker of insulin sensitivity, counteracts apoptosis and reduces inflammation in various cell types (Lihn et al., 2005; Yadav et al., 2013; Caselli 2014; Achari and Jain 2017). Therefore, the leptin/adiponectin ratio has also become a sensitive marker of insulin resistance by combining two intertwined signals into a single parameter (Norata et al., 2007; Frühbeck et al., 2018; Frühbeck et al., 2019).

MICROGRAVITY AND ITS EFFECTS ON HUMAN HEALTH: FOCUS ON GLUCOSE METABOLISM AND INSULIN RESISTANCE

Space flight is characterized by unloading-dependent muscle atrophy and impaired immune response as well as different

degrees of metabolic dysregulation, which, if persisting long enough, as expected during interplanetary missions with extended duration, might result in increased risk of cardiovascular and metabolic diseases (Lane and Smith 1999; Strollo 1999; Demontis et al., 2017; Strollo et al., 2018a). More specifically, space flight research, ground-based studies (both bed rest and dry immersion), and experimental studies in animal models and on pancreatic islets of Langerhans support the observation that prolonged space flight and experimental exposure to microgravity result in peripheral insulin resistance at different sites (liver, skeletal muscle, adipose tissue and others) (Tobin et al., 2002; Linossier et al., 2017; Wang et al., 2019; Downs et al., 2020; Strollo and Vernikos 2021). Under these environmental conditions, insulin resistance specifically occurs also in other insulin-sensitive tissues, such as the blood vessels (Hughson et al., 2016). Moreover, slight dehydration and inadvertently occurring traumas can cause skin lesions that might suffer healing problems during such missions based on the aforementioned mechanisms. To elaborate a holistic view of the long-term effects of space flight, to understand the resulting health risks, and, possibly, to unravel the mechanisms leading from temporary functional alterations to the onset and progression of chronic diseases, it is needful to shed light on the concurrent outcomes of the multiple alterations concomitantly occurring at those three levels. Indeed, altered metabolism, sarcopenia, and immune deficiency represent models of Earth-bound age-related chronic diseases having oxidative stress and consequent chronic low-grade inflammation as the common underlying mechanisms (Mancuso 2016; Nishida and Otsu 2017).

To this regard, a significant issue is to dissect the confounding effects of multiple space-related stressors, which are direct contributing causes of pathophysiological adaptation processes in the space environment. Stressful tasks and diet changes, confinement-related artificial light, isolation, and reduced motor activity might worsen the effects of weightlessness and radiation by further contributing to muscle and immune system deconditioning and to altered energy metabolism and endocrine balance (Strollo et al., 2014; Strollo et al., 2018a; Strollo et al., 2018b). Indeed, several studies conducted during manned space missions highlighted endocrine alterations in the crew (Strollo 2000; Macho et al., 2001) potentially linked to weightlessness and stress factors including confinement, isolation, demanding tasks, and circadian rhythm disturbances. For instance, rodent research showed that psychosocial stressors and altered gravity could affect immune function and inflammatory mediators known to interact with metabolism (Gaignier et al., 2018). However, we currently know that insulin sensitivity changes occur both in space (Tobin et al., 2002; Hughson et al., 2016) and in Earth-bound simulated microgravity by bed rest esperiments (Downs et al., 2020) and long-term-flight-simulating isolation experiments (Strollo et al., 2014; Strollo et al., 2018b). Indeed, it is well established that prolonged (60–70 days) head-down bed rest studies are associated with significant insulin resistance in healthy volunteers, which is worsened by inactivity and is only partially improved by physical exercise (Downs et al., 2020). A similar finding has also been observed in male subjects undergoing a 3-days dry immersion experiment (Linossier et al., 2017).

Environmental stress, associated to confinement, which is a typical feature of space flight, is another relevant factor disrupting insulin sensitivity, as observed in crew members participating to the Mars-105 and Mars-500 on-ground simulation mission (Strollo et al., 2014; Strollo et al., 2018b).

Moreover, the Mars-500 human volunteers showed significantly lower plasma levels of total and high-molecular weight adiponectin, especially in the first 120 days of mission, which is also supporting the impact of environmental stress upon metabolic adaptations and significant glucose metabolism changes, independently of microgravity contribution (Strollo et al., 2018b).

Vitamin D3 deficiency is also known to negatively affect insulin sensitivity and astronauts are at risk for vitamin D3 deficiency, due to chronic exposure to artificial light conditions. Presumably due to that, vitamin D supplementation has proved beneficial in wound healing of people with T2DM and foot ulcers (Urbaniak and Reid 2016). Another factor potentially contributing to insulin resistance during space flight is the altered gut microbiota environment (Razzaghi et al., 2017; Turroni et al., 2017). Interestingly, urbanization is strongly related to changes (including reduced biodiversity) in stool bacteria composition through intrinsic isolation from the surrounding "bacteria-rich" country. Similarly, due to artificial lighting, closed environment, and confinement, space flight might be considered as an extreme "urban-like" potentially diabetogenic condition (Tasnim et al., 2017; Parajuli et al., 2018; Wang et al., 2018). Indeed, some space-related studies reported a negative impact on gut microbiota composition in experimental models and humans (Ritchie et al., 2015; Cervantes and Hong 2016; Harada et al., 2016; Mahnert et al., 2021; Siddiqui et al., 2021).

Taken together, the available data suggest that strong evidence is present about spaceflight impact on insulin resistance, although future studies are still needed to identify specific exercise, nutritional and nutraceutical countermeasures to mitigate such deleterious pathophysiological event.

Metabolic Alterations and Impaired Wound Healing

Obesity, and specifically abdominal/visceral obesity, is a condition of greater insulin resistance and chronic inflammation (Bashir et al., 2016), as well as markedly impaired cutaneous wound healing. This concept also applies to T2DM, a catabolic disease typically characterized by a severe insulin-resistant condition leading to hyperglycemia and a global metabolic derangement. In most cases, T2DM represents the natural evolution of a long-standing, often obesity-driven insulin resistance state slowly exceeding functional pancreatic reserve and associated with insulin receptor or post-receptor abnormalities. On the other hand, type 1 DM (T1DM) is not only the consequence of severe beta-cell deficiency, but is also burdened, over time, by some degree of insulin resistance due to insulin overtreatment-related receptor downregulation, advanced

glycation endproducts (AGE) accumulation, and more (Kaul et al., 2015). Then, through several pathophysiological mechanisms, including insulin resistance, both DM types undergo macro- and micro-vascular complications over time. These complications are tightly linked to higher cardiovascular risk, renal damage, eventually resulting in chronic kidney disease (Ndisang et al., 2017; Vladu et al., 2017; Wolosowicz et al., 2020). In any case, apart from their extreme post-ischemic expressions, i.e. the diabetic foot and severe leg ulcerations, wound-healing problems often represent an underestimated complication in this context. When the wound is ischemic, the resolution of neutrophils and macrophages appears to be significantly delayed. Excess pro-inflammatory cells release reactive oxygen species (ROS), cytokines, and metalloproteinases, thus generating and maintaining a pro-inflammatory microenvironment that further increases tissue damage, extracellular matrix (ECM) degradation and denaturation of growth factors (Mizuno et al., 2004). An abnormal inflammatory response is also a major feature and the leading cause of impaired diabetic wound healing. Dysfunctional repair is even more apparent in the presence of high levels of local advanced glycation end-products (AGEs), which impair ECM deposition by inducing fibroblast apoptosis and dysfunction of ECM production. The imbalance of deposition and degradation of ECM affects keratinocyte and endothelial cell function, eventually leading to impaired re-epithelialization and angiogenesis (Yang et al., 2016).

Increasing evidence has accumulated on insulin ability to stimulate cell migration and wound recovery, and insulin administration has been proposed to overcome the adverse effects of insulin resistance on wound healing on the ground (Yang et al., 2016). Physical exercise typically decreases insulin resistance and speeds up cutaneous wound healing in aged mice (Goodson and Hunt 1979). The interplay between insulin-mediated glucose supply and wound healing mechanisms is schematically shown in **Figure 2**.

All these pathogenetic events deserve careful consideration in light of upcoming long-duration interplanetary travels (Demontis et al., 2017) and, even more, of Moon colonization missions, potentially placing astronauts at risk for higher cardiovascular disease risk, in addition to altered wound healing and other dysfunctions.

EFFECTS OF MICROGRAVITY ON WOUND HEALING: POTENTIAL ROLE OF INCREASED INSULIN RESISTANCE

An important health problem of astronauts in space is skin deterioration and this may specifically impact on the wound repair process, due to exposure to microgravity (Gössl et al., 2010; Choi et al., 2021; Bacci and Bani 2022), radiation (Hellweg and Baumstark-Khan 2007; Hu et al., 2020) and other factors. Wound healing is a complex series of events including partially overlapping phases (inflammation, tissue formation, and tissue remodeling) (Eming et al., 2014). This process is based upon a series of cell populations playing specific roles at different stages of the repair process (Pasparakis et al., 2014), which should follow a precise sequence of events. Mechanical factors, among different biochemical and physiological factors, play a significant role in wound healing, and include skin tension, mechanical forces at the wound margins, mechanical stress produced by stitches (after suture) and wound contraction due to myofibroblasts (Darby et al., 2014). The coordinated modulation of the expression of a large number of genes in the involved cells is clearly important for the success of the whole repair process. Moreover, a relevant role is played by the apoptotic process in all phases of wound healing, balancing cell growth and elimination of cells that are not necessary anymore (Riwaldt et al., 2017). Several studies show that microgravity can impair repair processes (Delp 2008), altering the behavior of some cell populations involved in wound repair (Morbidelli et al., 2005; Riwaldt et al., 2017). Experimental studies showed alterations in the behavior of cutaneous cell lineages under microgravity, especially regarding the apoptosis process in wound healing (Riwaldt et al., 2021). Moreover, altered modulation of haemostasis is also possible under microgravity condition, since some *in vivo* and *in vitro* studies show that microgravity affects the number and function (i.e., production of platelet-derived growth factor) of platelets, which contributes to impair wound healing (Farahani and DiPietro 2008; Locatelli et al., 2021). Another critical event in wound repair is physiological angiogenesis which occurs in the granulation tissue to allow supply of oxygen and nutrient supply and removal of waste products. Thus, understanding the alterations of angiogenesis during real and experimental microgravity conditions will allow to understand this component of wound repair alterations during space flight (Morbidelli et al., 2021). Currently, no specific studies have addressed the issue of the selected impact of insulin resistance on (experimental) wound healing during real or simulated space flight. In this regards, the Earth-based paradigm of patients with T2DM, with important microvascular complications and relevant wound repair impairment, may suggest at least in part what are the consequences of marked/prolonged insulin resistance on the complex process of wound healing (Fu et al., 2021; Rodríguez-Rodríguez et al., 2022), and suggest some potential experimental approaches to begin to disentangle this pathophysiological problem.

CONCLUSION AND FUTURE DEVELOPMENTS

Among the multiple health issues that are observed during a long-term stay in microgravity conditions, like prolonged space flight, to achieve appropriate wound healing represents an important goal, as this repair process could often be impaired by different factors, including insulin resistance. This goal may be relevant for both minor injuries and even for some (possibly limited) surgical procedures that may need to be conducted during longer space flights. Thus, some countermeasures should be at least envisioned to manage this issue, possibly following a personalized approach, in a context of precision medicine. Starting from the individual health

profiling, it is conceivable, for example, to propose an accurate monitoring of individual insulin resistance and chronic low-grade inflammation by wearable devices, in order to identify the astronauts at greater risk. Moreover, monitoring of the visceral adipose compartment, even by simple waist circumference measurement, may be another way to assess personal susceptibility (Ross et al., 2020). Additionally, a nutritional profile that mitigates insulin resistance may be crucial and could be based upon food with lower glycemic load and possibly rich in antiinflammatory and antioxidant components. Such approach may then be conducted with a plant-based diet, like, for example, the Mediterranean diet (Schwingshackl et al., 2015). Interestingly, engineering progress has recently been done to develop strategies to produce nutritious and palatable food directly on spacecrafts involved in interplanetary missions (Douglas et al., 2020). In particular, a suitable way to produce edible vegetables on spaceship and carbon dioxide absorption and oxygen production to maintain the air circulation system is currently under development (Jiang et al., 2020). Such a choice could also ensure an excellent solution against environment-related oxidative stress (Kyriacou et al., 2017). A further strategy may also include the validation and use of specific food supplements with nutraceutical properties (curcumin, berberine, etc.) and, where necessary, selected drugs targeted to reduce insulin resistance (Rochlani et al., 2017).

The development of effective countermeasures able to mitigate the association of insulin resistance and impaired wound healing during prolonged space flight thus deserves further studies. In this context, our group has been suggesting investigating this topic since a long time and now has finally been selected for a space experiment dealing with it. Next studies will need to combine nutrition, supplements and pharmacological approaches also with any possible physical, endocrine and metabolic countermeasures to prevent long-term detrimental effects on tissue repair potentially related to any long-lasting, unopposed insulin resistant state.

AUTHOR CONTRIBUTIONS

FS and MM conceived the study, FS, SG, MM and PM wrote the paper after deeply discussing its content with AP and AM, who revised the literature and offered their experience in the field of kidney and muscle pathophysiology, respectively.

ACKNOWLEDGMENTS

The preparation of this paper has been supported by the ESA-TT "Tissue Healing in Space: Techniques for promoting and monitoring tissue repair and regeneration".

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frontiers | Frontiers in Bioengineering and Biotechnology

REVIEW
published: 01 July 2022
doi: 10.3389/fbioe.2022.780553

Facing Trauma and Surgical Emergency in Space: Hemorrhagic Shock

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OPEN ACCESS

Edited by:
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Reviewed by:
Xiaodong Xing, Nanjing University of Science and Technology, China
Vasilios E. Papaioannou, Democritus University of Thrace, Greece

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Specialty section:
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *21 September 2021*
Accepted: *22 April 2022*
Published: *01 July 2022*

Citation:
Pantalone D, Chiara O, Henry S, Cimbanassi S, Gupta S and Scalea T (2022) Facing Trauma and Surgical Emergency in Space: Hemorrhagic Shock. Front. Bioeng. Biotechnol. 10:780553. doi: 10.3389/fbioe.2022.780553

Although the risk of trauma in space is low, unpredictable events can occur that may require surgical treatment. Hemorrhage can be a life-threatening condition while traveling to another planet and after landing on it. These exploration missions call for a different approach than rapid return to Earth, which is the policy currently adopted on the International Space Station (ISS) in low Earth orbit (LEO). Consequences are difficult to predict, given the still scarce knowledge of human physiology in such environments. Blood loss in space can deplete the affected astronaut's physiological reserves and all stored crew supplies. In this review, we will describe different aspects of hemorrhage in space, and by comparison with terrestrial conditions, the possible solutions to be adopted, and the current state of the art.

Keywords: hemorrhage, trauma, hemostats, blood substitutes, space missions

INTRODUCTION

Treatment of unforeseen health events in space is a major concern that can jeopardize crew health and mission success (Hamilton et al., 2008; Alexander 2016; Patel et al., 2020). This concern has given rise to several studies on open surgery, laparoscopic surgery, and robot-assisted surgery as well as studies of human physiology in microgravity (for a review of the literature, see Panesar et al., 2018 (Melton et al., 2001; Campbell 2002; Dawson, 2008; Kirkpatrick et al., 2009a; Kirkpatrick et al., 2009b; Kirkpatrick et al., 2009c; Doarn et al., 2009; Pletser et al., 2009; Haidegger et al., 2011; Canga et al., 2016; Ashrafian et al., 2017; Panesar and Ashkan 2018; Baker et al., 2019; Hinkelbein et al., 2020; Kirkpatrick et al., 2020; Robertson et al., 2020). Although in space the risk of trauma is low, objects in movement conserve their mass and still carry a kinetic energy (Komorowski et al., 2018; STEMonstrations 2022) so that damages to the human body are likely to occur. It must be remembered that kinetic energy greatly increases due to velocity rather than mass, consequently a small increase in speed will result in an increased risk of injury (Komorowski et al., 2018; STEMonstrations 2022). Although so far the likelihood of severe

trauma or surgical emergency has been considered low, crushing trauma and penetrating trauma as well as unpredictable events requiring surgical treatment are considered a major concern (Hamilton et al., 2008). Their impact affects an organism that has to find a new balance for the absence of gravity and hence is more fragile even in case of minor trauma than on Earth. Crushing trauma and penetrating trauma can directly harm the astronaut and also damage the protective suit, a potentially catastrophic event as it can cause spacesuit decompression or even ignition (Khorasani-Zavareh et al., 2014; Panesar and Ashkan 2018). Even if to date there have been no accounts of major hemorrhage in space, it can be a life threatening condition during a long term mission or mission to Mars. These exploration missions required a different policy than rapid return to Earth, currently adopted on the International Space Station (ISS) in low Earth orbit (LEO). Although the likelihood of hemorrhage may be prevented through vehicle design adaptation, adequate crew training, and accurate medical crew selection, the occurrence of significant blood loss must be taken into account. Launching, landing and docking or extra vehicular activity (EVA) are dynamic stages at risk of truama. A severe bleeding after landing on another celestial body would have consequences that are difficult to predict given the still poor knowledge of human physiology in such environments, (Roth 1968). On Earth, trauma severity, either blunt or penetrating, can be generally defined by a mechanical damage to the body caused by an external force (Khorasani-Zavareh et al., 2014).

Kirkpatrick had already reported in 2005 (Kirkpatrick et al., 2005) that hemorrhage is the leading cause of potentially preventable deaths on Earth and, in space exploration, considered to be the most significant risk for astronauts, followed by infections (Kirkpatrick et al., 2009c). Blood loss in space can deplete the physiological reserves of the affected astronaut for lack of the standard requirements normally available on earth. In addition, such an occurrence is not only a challenging situation for any individual astronaut, but it can deplete crew resources (Hamilton et al., 2008) as well, as we will explain in this review.

MATERIALS AND METHODS

A literature review was done to identify publications on PubMed and Medline, Embase. The following key words were searched: “hemorragic shock,” “microgravity,” “zero gravity,” “astronauts,” “blood,” “transfusion,” “hemorrhage,” “space missions,” and “mission to Mars.” Collected papers were selected according to the following criteria: English language or, if in another language, be provided from online translation tools or at least come in the form of a structured English abstract. References obtained were crosschecked for additional relevant publications.

IN MISSIONS

The Space Environment

Presently, in spaceflight, the most critical moments are launch and landing due to the stressful conditions crew members are under in the spacecraft. In fact, human losses in space missions have occurred in those phases (Barratt 2019). Inside the spacecraft, the Environmental Control and Life Support System (ECLSS) maintains the atmosphere steady, normoxic, and normobaric while at neutral temperatures. However, the carbon dioxide concentration inside the cabin is on average 10 times higher than on the ground (0.3%–0.5%). The environment outside the spacecraft is the most hostile ever experienced before, nil barometric pressure, high levels of radiation, and extreme temperatures (−150°C to +120°C) (Komorowski et al., 2016; Barratt 2019). An immediate hazard is caused by a loss of cabin pressure (e.g., in case meteorite or satellite debris were to hit the station), fire, release of toxic substances, or malfunction of the ECLSS (Environmental Control and Life support System) (Kirkpatrick et al., 2005; Kirkpatrick et al., 2009c). In space, any of these events could potentially cause and aggravate a potential injury.

Hints on Changes in Human Body Physiology in Space Related to Hemorrhagic Shock

In space, numerous changes occur in human body physiology (**Table 1**). Some of these changes may particularly affect the body’s response to bleeding, mainly the cardiovascular system. The cardiovascular system is involved in response to a hemorrhagic event. The redistribution of body fluids toward the head is a well-known phenomenon in microgravity (Komorowski et al., 2016; Barratt 2019; Nowak et al., 2019). The fluid shift initiates at the launch position with the lower limbs raised above the thoracoabdominal coronal plane, a condition that continues during orbit, producing a displacement of blood and other fluids from the lower limbs to the torso and head (Williams et al., 2009; Nowak et al., 2019). When astronauts arrive in space, the gravitational pull that drives the circulation toward the feet stops working and fluids shift toward the head and torso (Alexander 2016). This shift and the compensation by the cardiovascular system can make the human body more subject to potentially harmful cardiovascular effects. The compensation for volume redistribution includes the activation of central baroreceptors (Convertino 2003; Di Rienzo et al., 2008; Panesar and Ashkan 2018) and suppression of the renin–angiotensin–aldosterone axis with the release of the atrial natriuretic peptide. Salt and water are excreted, with a reduction in plasma volume and a transient increase in hematocrit levels. A decrease in both erythropoietin secretion and red cell mass is also present, with a reduction in blood volume (Panesar and Ashkan 2018) (see Panesar and Ashkan (2018)) for the review of the literature). The decrease in cardiac workload in prolonged spaceflight may reduce the overall myocardial mass (Convertino 2009; Demontis et al., 2017; Panesar and Ashkan 2018), but despite the loss of contractile mass, ejection fraction and arterial pulse wave velocity are preserved. The human body is able to compensate the fluid shift through diuresis, with a

reduction of extracellular fluid and plasma volume, an event that produces a decrease in body mass during the first 30 days (Panesar and Ashkan 2018).

Cardiac Function

Cardiac function adapts to the fluid shift and to the alterations by increasing cardiac output (Demontis et al., 2017; Panesar and Ashkan 2018). In spaceflight, the cardiovascular system is affected by one of the major alterations in human physiology. Over time, the shift of fluids from the lower body to head and torso produces loss of ventricular mass (cardiac atrophy) (Perhonen et al., 2001; Demontis et al., 2017; Evans et al., 2018), decreased sensitivity of the carotid-cardiac (vagal) baroreflex (Williams et al., 2009; Norsk 2014), and a greater responsiveness of sympathetic neural activity to inflight simulations of standing (Williams et al., 2009; Demontis et al., 2017). The effect is a decreased blood pressure and elevation of cardiac output throughout flight (Perhonen et al., 2001; Ertl et al., 2002a; Ertl et al., 2002b; Norsk 2014; Evans et al., 2018).

Vasodilation, present in space permanence, may reduce (Norsk et al., 2015; Norsk 2020) plasma volume and associated cardiovascular effects. This scenario is called cardiac deconditioning (Demontis et al., 2017), and decreased compensatory responses, exacerbated by relative hypovolemia and anemia manifesting during spaceflights (Nowak et al., 2019). However, increased levels of red blood cell platelets and higher hemoglobin concentration are reported in long duration flights, but these effects are probably linked to the plasma volume decrease occurring in space (Smith 2002; Kunz et al., 2017). The altered physiologic conditions of the cardiovascular system may result in a decreased ability to respond to blood loss in weightlessness. Hence, in case of hemorrhage, the time to intervene effectively is probably shorter (Kirkpatrick et al., 2009b; Williams et al., 2009) and rescue must be rapid, making fluid resuscitation a priority.

Autonomic Nervous System and Hypothalamic–Pituitary–Adrenal System in Space Conditions—Hints on Their Possible Role in Hemorrhage

The hypothalamic–pituitary–adrenal HPA (axis) plays an important role in the adaptation to stress (Buckey and Homick 2002; Smith 2006; Welt et al., 2021). It is the most important interconnection between the nervous system and the endocrine system. The activation of the HPA axis leads to the secretion of glucocorticoids, which act on multiple systems and organs to redirect the energy resources necessary to meet a real need or even a possible need that could occur. The HPA axis response to stress is driven primarily by neural mechanisms, with responses that may be inhibited by feedback (e.g., production of glucocorticoid hormones). In a stressful situation, the axis mediates the effect of stress factors by regulating numerous physiological processes, such as metabolism, immune responses, and activation of the autonomous nervous system (ANS) (Chouker 2020; Tobaldini et al., 2020). In the presence of hypovolemic shock, there is an activation and release of adrenaline and noradrenaline by the adrenal medulla and glucocorticoid hormones by the adrenal cortex, in addition to glucagon from the pancreas (Smith 2006; Mandsanger et al., 2015; Herman et al., 2016; Crucian et al., 2018; Chouker 2020; Tobaldini et al., 2020; Kageyama et al., 2021).

ANS regulates the cardiovascular system and controls visceral functions in order to maintain homeostasis and the homeodynamic state of the body. It is also an interface between the body, the central nervous system (CNS), and external stimuli (Mandsanger et al., 2015; Chouker 2020; Tobaldini et al., 2020). Its sympathetic branch plays a role in the control of many activities, for example, cardiovascular, gastrointestinal, pulmonary, cutaneous, genitourinary, and immune. Despite being defined as an "autonomic" system, there are complex control mechanisms that act both centrally and peripherally (Mandsanger et al., 2015; Herman et al., 2016; Chouker 2020; Tobaldini et al., 2020) in pathological conditions such as hypertension, heart failure, and myocardial infarction stress (Malliani 2000; Wallin and Charkoudian 2007; Herman et al., 2016; Tobaldini et al., 2020). ANS plays an important role in the regulation of the vegetative state and also in the modulation of the responses of the immune system (Kirkpatrick et al., 2009b; Mandsanger et al., 2015; Chouker 2020; Tobaldini et al., 2020), metabolism, and inflammation (Kirkpatrick et al., 2009b; Chouker 2020; Tobaldini et al., 2020; Welt et al., 2021), suggesting an integration at different levels of control (Kirkpatrick et al., 2009b; Mandsanger et al., 2015; Chouker, 2020; Tobaldini et al., 2020).

When we talk about modifications present in space, we refer mainly to the studies conducted on parabolic flights. In them, there are short phases in which there is a change in severity. In this way, the effects of the different phases of flight on hemodynamics and the cardiovascular system were studied: 1) 1 g (before and after each parabola), 2) hypergravity during the ascending part, 3) microgravity phase at the apex of parabola, 4) hypergravity during the descending part of parabola, and 5) 1 g at the end of the parabola (Iwase et al., 2020).

In fact, these experiments simulate the hypergravity and microgravity characteristics of space missions (Criscuolo et al., 2020). These studies were born with the intent to explore the effects of different gravity levels (zero, lunar, and Martian gravity) on cardiovascular and autonomous control for missions to Mars in the near future. During the parabolic flight, the correlations between the level of gravity and the cardiovascular autonomic modulation have been at the center of many studies (Widjaja et al., 2015). Despite this, to the best of the authors' knowledge, these effects have not yet been sufficiently studied to predict what might happen in the event of a severe hemorrhagic shock.

CARDIOVASCULAR AUTONOMIC CONTROL DURING SPACE FLIGHTS

The cardiovascular function is profoundly influenced by microgravity and thus also by autonomous cardiovascular control. In the case of space flights, each component of this

system can be affected by the new conditions to which it is subjected. It is known that in microgravity there is a reduction in cardiac mass and vascular function is worsened by presenting stiffened arteries and affected by endothelial dysfunctions (Kirkpatrick et al., 2009b; Widjaja et al., 2015; Alexander 2016; Hughson et al., 2018; Criscuolo et al., 2020; Iwase et al., 2020). In other words, the cardiovascular system shows a reduced ability to respond to stressful situations. Consequently, it is presumable that in the event of a hemorrhagic shock, the response capacity is compromised in space. There is currently no evidence on this topic, but it is presumable that all the aforementioned modifications would force the cardiovascular system to a non-optimal performance. Furthermore, on Earth, there are neither cosmic radiations nor microgravity, which instead act synergistically in space (Jones et al., 2019). It is important, in fact, that adequate shielding for deep space flights is implemented because even low doses of radiation are able to increase the risk of cardiovascular mortality (Jones et al., 2019).

Hints on Inotropes and Other Medications for Space Missions

On the ground, the main indication in massive bleeding shock is surgery, i.e., damage control surgery (Ball 2017), which must be applied as soon as possible (Ertl et al., 2002a; Ertl et al., 2002b; Kirkpatrick et al., 2005; Wallin and Charkoudian 2007). Persistent hypotension after fluids administration (ATLS protocol) (American Committee for Trauma-American College of Surgeons 2018) is treated with vasopressors (epinephrine and norepinephrine) to improve systolic pressure. However, drugs (Standl et al., 2018) with different targets are available in case of failure of inotropic infusion. For example, dobutamine can be used in cardiogenic shock and in any type of shock with insufficient ventricular pump function. Other drugs (Standl et al., 2018) such as milrinone, levosimedan, vasopressin, glyceryl trinitrate, and sodium nitroprusside have found applications in different types of shock, mainly cardiogenic, with the exception of cafedrine hydrochloride and theoadrenaline hydrochloride that are used for neurogenic shock. However, a specific review for this topic should be the best option to discuss it in depth.

Hemorrhage Control in Space

The ability to control hemorrhage after a traumatic injury in space is crucial in astronaut's health care (**Table 2**). The crew must continue its mission autonomously, and any medical care is performed in a setting where resupply, evacuation, and communication are difficult (Hamilton et al., 2008). In space, medical systems as well as supplies, equipment, and crew training are limited.

It must be underlined that estimates of traumatic injuries are mainly based on terrestrial populations and do not include spaceflight data. They are extrapolated to the astronauts and referred to the environment of the International Space Station (ISS). In addition, estimates do not account for injury risks due to long duration surface operations under the influence of gravity, and for increased risks of acute radiation sickness (ARS) (Jones et al., 2019), both conditions are expected in Moon or Mars missions (Nowak et al., 2019).

In case of medical issues, stabilization and expeditious evacuation back to Earth are the present policy (Hamilton et al., 2008; Kirkpatrick et al., 2009c; Hodkinson et al., 2017) on the ISS, although standard advanced trauma life-support (ATLS) (American Committee for Trauma-American College of Surgeons 2018), intravenous insertions for infusion, endotracheal intubation, and chest tube placement are practices that the crew medical officer (CMO) must know (Hamilton et al., 2008; Kirkpatrick et al., 2009c; Hodkinson et al., 2017). A major traumatic hemorrhage in space would be catastrophic. Hemorrhage can occur either externally, from open wounds, or internally, into closed anatomic spaces. It can be categorized as compressible and non-compressible, depending on the location (Kirkpatrick et al., 2005; Ball 2017; American Committee for trauma-American College of Surgeons 2018). Non-compressible torso hemorrhage (NCTH), coming from the torso vessels, the pulmonary parenchyma, solid abdominal organs, or disruption of the bony pelvis, is occult and not treatable by simple compression and therefore be fatal (Ball 2017). On Earth, a blunt, polytrauma patient can be a challenge due to difficulty in detecting internal bleeding and could require advanced surgical skills and more dedicated devices than with external bleeding (Brenner et al., 2018; Cannon et al., 2018). Vice versa, in weightlessness, this type of injury can be devastating and seemingly impossible to treat, despite the help of ultrasound in diagnosis (Hamilton et al., 2008; Kirkpatrick et al., 2009c; Pletser et al., 2009; Alexander, 2016; Garrigue et al., 2018; Mashburn et al., 2019) It can absolutely drain a mission's resources with no chance of resupply. The need for blood supplies in resuscitation is another major issue in space. It is known that morbidity and mortality resulting from hemorrhage decrease with the use of blood products and the derivatives of blood (Nowak et al., 2019). Therefore, blood transfusion, already a life-saving procedure on Earth, should be considered all the more important in space.

In an article, Nowak et al. (2019) reported on the need for research on alternative blood products in hostile environments. For example, lyophilized blood components like plasma that undoubtedly have advantages over liquid storage for mass, volume, and limited shelf life (Pusateri et al., 2016; Garrigue et al., 2018; Nowak et al., 2019).

Another possibility is the use of hemoglobin-based oxygen carriers (HBOCs) (Moore et al., 2009; Weiskopf et al., 2017; Nowak et al., 2019), which are artificial red blood cell substitutes able to deliver oxygen and provide volume expansion. They require little preparation; however, mass and volume are similar to red packed cells, occupying room in the space craft. Additionally, they are still experimental (Nowak et al., 2019).

In space, limitations due to mass, volume, and power (Nowak et al., 2019) affect blood storage capability which requires a significant use of refrigeration power. Refrigeration is also associated with a limited shelf life, 35 days at 1–6°C, while a trip to Mars lasts at least 6 months. However, to date, the most practical application for transfusion in space is fresh whole blood despite the limits set by circulatory physiology, resupply issues,

and personal restraints in the spacecraft (Nowak et al., 2019). Other issues have to be taken into account in space (Nowak et al., 2019) such as the following:

-The need to restrain CMO and patients during spaceflights.
-The risk of venous thromboembolism after a venous line insertion due to loss of stratification of liquids and gases present in microgravity.
-Froth formation in agitated solutions makes it difficult to measure their volume.

Additionally, in space, a severe hemorrhage may demand an immediate surgical intervention before diagnostics may have localized the source of bleeding (Kirkpatrick et al., 2001; Doarn 2007; Kirkpatrick et al., 2017a). A group of flight surgeons, trauma surgeons, and biomedical engineers emphasized that laparotomy could be required to stabilize a patient prior to further procedures (Doarn 2007; Ball 2014; Lamb et al., 2014; Kirkpatrick et al., 2017a).

Damage control surgery (DCS) (Doarn 2007; Ball 2014; Lamb et al., 2014) should be used to provide surgical control of hemorrhage. However, DCS should be performed in association with Damage Control Resuscitation (DCR) (Doarn 2007; Ball 2014; Lamb et al., 2014; Kirkpatrick et al., 2017b; Chang et al., 2017). This paradigm states that essential surgery is needed to preserve the physiological reserves of patients implementing only the necessary tasks by means of a few, selected procedures. Besides limiting the procedures to the essential, this method does not require large equipment outlays. A special protocol for austere environment is the Remote Damage Control Resuscitation (RDCR) (Kirkpatrick et al., 2017b; Chang et al., 2017), a treatment strategy for the severely injured trauma patient, designed to limit hemorrhage and produce or preserve adequate levels of physiological reserves for the DCS in the prehospital phase (Kirkpatrick et al., 2017b; Chang et al., 2017). For RDCR on Earth, the doctrine of permissive hypotension has been adopted (Lamb et al., 2014). This practice aims at limiting ongoing hemorrhage by reducing pressure while maintaining a "critical level" of vital organ perfusion. In this approach, few signs are necessary to evaluate the patient status such as the presence of a palpable radial pulse, mental status, and a systolic blood pressure (SBP) of 80–100 mmHg. Traumatic brain injury (TBI) needs a higher SBP to preserve cerebral perfusion pressure and avoid a secondary ischemic injury to the brain (Kirkpatrick et al., 2017b; Chang et al., 2017). However, applying this strategy during spaceflight would be difficult, especially in missions beyond low Earth orbit (LEO) due to the challenging issue of blood transfusion storage.

The early use of blood products, fresh warm whole blood, and other blood components, as well as other devices (hemostatic dressing, extremity tourniquets, junctional tourniquets, abdominal aortic and junctional tourniquets (AAJT), non-absorbable expandable, injectable hemostatic sponge (XSTAT), resuscitative endovascular balloon occlusion of the aorta (REBOA), intra-abdominal self-expanding foam, tranexamic acid administration, and expandable hemostatic sponges (Gordy et al., 2011; Jenkins et al., 2014; Bjerkvig et al., 2016; Rappold and Bochicchio 2016) is representative of DCR and RDCR. These protocols should be particularly useful in space missions where conditions are extreme rather than only austere. In fact, despite the numerous experiments on the feasibility of emergency procedures in microgravity (Campbell 2002; Dawson 2008; Kirkpatrick et al., 2009c; Alexander 2016; Panesar and Ashkan 2018; Robertson et al., 2020), the complex pathophysiology of hemorrhagic shock is mostly still unknown.

Space missions, in any case, may not allow all the procedures we are accustomed to on Earth, for example, the use of a "massive transfusion" protocol with fresh frozen plasma in conjunction with blood and platelets for a severe ongoing bleeding.

ATLS (Ball 2017) protocols have been adapted to the unique pathophysiological mechanisms (Panesar and Ashkan 2018; Nowak et al., 2019) present in space (Kirkpatrick et al., 2009c), but it is still not known how a severe bleed or cardiac failure might affect the hemodynamic state secondary to microgravitational fluid shifts (Kirkpatrick et al., 2009b). Plus, prolonged fluid infusion may have the effect of draining most of the limited supplies of the crew, adversely affecting the clotting profile and/or induce hypothermia (Kirkpatrick et al., 2009b; Panesar and Ashkan 2018). For limb and extremity trauma, tourniquets or hemostatic dressings may be adequate (Rappold and Bochicchio 2016; Panesar and Ashkan 2018). On the contrary, the region of the trunk cannot be treated by external pressure to control hemorrhage and, so far, hemorrhagic shock. Hemodynamic deterioration may prove difficult to address, owing to homeostatic decompensation, the fact that there is no access to facilities and equipment, or due to the lack of trained staff. As a result, DCS has been introduced also in space (Doarn 2007; Ball 2014; Lamb et al., 2014; Kirkpatrick et al., 2017a). In the exploratory phase, hemostasis and/or control of endogenous bacterial contamination must be achieved.

On the ISS, the only resuscitation fluids available are 4 L of normal saline (Nowak et al., 2019), and methods to generate crystalloids in flight are under investigation (Kirkpatrick et al., 2001; McQuillen et al., 2011). On the other hand, response to reduced gravity on other planets such as Mars, or on the Moon is largely unknown, and no appropriate protocols are available to date.

Missions to Mars and Other Long-Term Mission Peculiarities for Hemorrhagic Shock Treatment

As already stated, blood transfusion in space depends on, among many conditions, limited vehicle dimensions (Summers et al., 2005; Hamilton et al., 2008; Alexander 2016; Nowak et al., 2019). In fact, during a spaceflight, mass, volume, and the necessary power to preserve stored items are known constraints. In addition, blood transfusion in the microgravity environment presents numerous difficulties. For example, although intravenous cannula infusions, phlebotomy and catheterization are possible, in weightlessness once the CMO and the patient are restrained, liquids have a different behavior than on Earth. Blood collection in microgravity should be possible with the use of a

TABLE 1 | Effects of microgravity that could affect physiologic response to hemorrhage.

Fluid redistribution	Fluid redistribution is followed by decrease in blood volume, cardiac size, and aerobic capacity with a post-flight orthostatic intolerance known as "cardiovascular deconditioning." The redistribution is caused by fluid shifts from the intravascular to the interstitial spaces due to lower transmural pressure for reduced compression of all tissues by gravitational forces, by fluid shifts from intravascular to muscle interstitial spaces due to lower muscular tone required to maintain the body posture. Decreased diuresis in the initial phases of space flight is due to the increased retention after stress-mediated sympathetic activation Antonutto and di Prampero (2003), Demontis et al. (2017), Tanaka et al. (2017), Iwase et al. (2020), Gallo et al. (2020)
Blood	-Reduction in circulating blood volume (a loss of 10–23% of circulating blood volume) resulting in an earth hypovolemic state Kirkpatrick et al. (2001), Kirkpatrick et al. (2005), Diedrich et al. (2007), Nowak et al. (2019) -Reduction in red cell mass (10–20% with respect to the preflight baseline) although this effect diminishes with the increase in mission duration Nowak et al. (2019) -Missions that last more than 6 months cause an increase in red blood cells, platelets, and hemoglobin concentration, probably related to reduction in plasma volume Nowak et al. (2019), consequent blunting of the baroreceptor response, and vasodilatation with a decrease in heart rate and blood pressure -The cephalad fluid shift causes an increase in venous return with increased stroke volume that produces alterations in the autonomic and endocrine systems designed to control the cardiovascular functions Iwase et al. (2020)
Heart	-Cardiac atrophy and reduced cardiac output Nowak et al. (2019), Demontis et al. (2017), Iwase et al. (2020)
Neuroumoral and cardiovascular system	-Reset of the working parameters of the neuroumoral and cardiovascular system -Possible global resetting of the centronomic nervous system with either a beta receptor bias or impaired receptor sensitivity resulting in an overall attenuation of the cardiac chrono tropic response Baker et al. (2019) -Attenuation of the aortic cardiopulmonary and carotid baroreflex responses to hypotension would presumably decrease the ability to respond appropriately to hypovolemic stress Baker et al. (2019).
Vessels	Muscle sympathetic nerve activity (MSNA) is designed to control the vasomotor function of the muscular bed Iwase et al. (2020), and its response to blood pressure changes against gravitational stress. MSNA responds also to the loading or unloading of the cardiopulmonary receptors when stimulated by the cephalad fluid shift Iwase et al. (2020).

vacuum, syringe, or pump. Infusion could be accomplished with a pressure bag or a syringe (Hamilton et al., 2008; Nowak et al., 2019).

It must be remembered that on deep space exploration missions, communication with mission control may be delayed or impossible and the possibility of evacuation will be largely dependent on distance and trajectory from Earth. Therefore, the crew should be more able to function autonomously (Hamilton et al., 2008; Alexander 2016; Nowak et al., 2019) because even on the ISS in low Earth orbit, emergent evacuation could take more than 24 h (Summers et al., 2005; Hamilton et al., 2008; Alexander 2016).

Topical Hemostatics in Space

The improved understanding of the coagulation process has produced a growing number of hemostatic agents that can be topically applied (Alexander, 2016; Chiara et al., 2018; Huang et al., 2020; Tompeck et al., 2020). In a systematic review in 2018, Chiara et al. (2018) selected four categories: 1) adhesives (liquid fibrin adhesives and fibrin patch), 2) mechanical hemostats, 3) sealants, and 4) hemostatic dressings (mineral and polysaccharides). Each one of them is described according to their employment and scientific foundation.

Despite their different utilization and activation modality, it must be generally underlined that the first concern of the surgeon is the state of the patient's endogenous coagulation system. A mechanical agent after surgical hemostasis or packing is the right choice in case of normal coagulation. If the patient's coagulation cascade is not reliable, the hemostat of choice should be an agent that may be effective even when coagulation factors are not, for example, adhesive products (Chiara et al., 2018). In the case of ongoing arterial or high flow bleeding, a patch-supplemented adhesive agent is indicated, as it is directly applicable under pressure on the site of the bleeding. A sealant agent is useful when the bleeding source is an organ like the liver, pancreas, and kidney, or to close a lung wound. Finally, hemostatic dressings should be considered in junctional and non-compressible hemorrhages, for example, in the neck, groin, or axilla (Chiara et al., 2018).

All these hemostats should be available in hospital service because of their different usages and indications. On the ground, tourniquets, conventional bandages, and advanced hemostatic dressings should be available to stop the bleeding in complex situations (Chiara et al., 2018).

New hemostats (nanotechnology) defined as self-assembling peptide nanofibers and chitosan nanofibers have also been used in clinical applications (Corwin et al., 2015; Chaturvedi et al., 2017; Chiara et al., 2018; Estep 2019; Huang et al., 2020; Tompeck et al., 2020).

Hemoglobin-Based Oxygen Carriers

Artificial red blood cell substitutes (hemoglobin-based oxygen carriers (HBOCs)) are under study to find a way of providing oxygen delivery and volume expansion in such extreme environmental conditions as deep space missions (Kirkpatrick et al., 2009c; Nowak et al., 2019). Originally, their application was studied to prevent transfusion reactions and/or bloodborne disease transmission. Other issues related to HBOCs need to be mentioned. For example, religious motives

TABLE 2 | Approach to hemorrhage control in space and on Earth. Research studies conducted on the ISS (Neurolab Missions).

	Space	Earth
Initial treatment	Applications of ATLS American Committee for Trauma-American College of Surgeons (2018) protocols for airway protection, drainage of hemopneumothoraces, and initial resuscitation. Needs to rapidly localize and address major hemorrhage Kirkpatrick et al (2009c)	Applications of ATLS American Committee for Trauma-American College of Surgeons (2018) protocols for airway protection, drainage of hemopneumothoraces, and initial resuscitation. Needs to rapidly localize and address major hemorrhage
Diagnostics	Ultrasounds (FAST) Kirkpatrick et al. (2007) (possible detection of an alarming rate of bleeding (on earth bleeding rates over 25 ml/min: estimated window before death –2 h) In space, intracavitary, thoracic, and abdominal hemorrhage will be more difficult to detect than on the earth and will require higher levels of skills and resources for treatment Kirkpatrick (2017a), Kirkpatrick (2017b)	Ultrasounds (FAST) Kirkpatrick et al. (2009c), Kirkpatrick et al. (2007) CT scan (hemodynamically stable patient) ATLS American Committee for Trauma-American College of Surgeons (2018)
Blood administration	Lyophilized blood products, hemoglobin-based oxygen carriers (HBOCs) Nowak et al. (2019). The use of human blood is to be excluded for now Astronauts have an estimated 15% decrease in circulating red blood cells and plasma on-orbit, the equivalent of Class I hemorrhage Alexander (2016)	Early use of fresh warm whole blood transfusions and of blood products Nowak et al. (2019)
Cardiac output	(Animal studies): changes in cardiac output and blood pressure when subjected to + G centrifugation (Antonutto and di Prampero, 2003; Komorowski et al., 2016; Demontis et al., 2017; Tanaka et al., 2017; Barratt 2019; Nowak et al., 2019; Iwase et al., 2020; Gallo et al., 2020)	
Intravenous fluid administration	Gravity absence no longer pulls fluids out of fluid bags into the body without an external force (risks for bubble formation). It is difficult to control the rate of fluid administration using such techniques (Antonutto and di Prampero, 2003; Komorowski et al., 2016; Demontis et al., 2017; Tanaka et al., 2017; Barratt 2019; Nowak et al., 2019; Iwase et al., 2020; Gallo et al., 2020)	
Surgery	To date, no information on possible major surgery for severe trauma, use of damage control surgery Jenkins et al. (2014), Lamb et al. (2014), Kirkpatrick et al. (2017a), Kirkpatrick et al. (2017b), and damage control resuscitation No information on the use of other techniques as the role of the resuscitative endovascular balloon for occlusion of the aorta (REBOA) Brenner et al. (2018), Cannon et al. (2018) or angioembolization techniques Also the use of hemostatics Gordy et al. (2011), Rappold and Bochicchio (2016), Chiara et al. (2018), Huang et al. (2020), Tompeck et al. (2020) possible on Earth, lacks strong evidence relatively to space missions	Damage control surgery Jenkins et al. (2014); Lamb et al. (2014), Kirkpatrick et al. (2017a), Kirkpatrick et al. (2017b), damage control resuscitation, staged surgery, and multidisciplinary team activation Junctional and compressive device Gordy et al. (2011), Rappold and Bochicchio (2016); Chiara et al. (2018); Huang et al. (2020); Tompeck et al. (2020) Balloon occlusive device for the aorta (REBOA) Brenner et al. (2018), Cannon et al. (2018) Angioembolization techniques Hemostats Gordy et al. (2011), Rappold and Bochicchio (2016), Chiara et al., 2018, Huang et al. (2020), Tompeck et al. (2020) Intracavitary foam Rago et al. (2016) Expandable hemostatic sponge and other devices Huang et al. (2020), Tompeck et al. (2020)

against transfusions or the need for a rare blood type. In addition, HBOCs may be used as an alternative to blood products in areas where the usual form of blood donation is not available, such as in space missions far from the Earth's orbit (Moore et al., 2009; Corwin et al., 2015; Weiskopf et al., 2017; Estep 2019; Nowak et al., 2019). Although HBOCs have many advantages as they can be stored at room temperature and require relatively little preparation, they are liquid and have a mass and volume similar to packed red blood cells. Although Moore et al. (2009) published a systematic review showing there is no statistical difference in the mortality rates of shock patients with placebo and HBOC-treated patients, adverse events have been present, and these substitutes are still under review (Nowak et al., 2019).

Lyophilized Blood Products

As reported by Nowak et al. in 2019, plasma is the only lyophilized blood component in clinical use (Pusateri et al., 2016; Garrigue et al., 2018; Nowak et al., 2019). It has the advantage to be stored in a powder form at ambient temperatures for up to 2 years, and at the moment of transfusion, it can be reconstituted for infusion within a few minutes. In this way, it is advantageous with respect to the limited shelf life of other products and also with respect to the reduced mass and volume of the lyophilized component. In addition, lyophilization may provide storage of autologous blood products with lower risks for infection and transfusion reaction. However, to date, lyophilization of red blood cells and platelets is still experimental.

Some of the Hemorrhagic Shock Issues With Unknown Effects in Space

Bacterial translocation: To date, the theory suggesting that the gut, when suffering from oxygen debt, starts to leak endotoxin and bacteria systemically which then initiates an inflammatory reaction, has not been studied in space (Lord et al., 2014). On Earth, the severity of organ damage that starts in this way depends on bleeding severity and shock duration. The mesenteric lymphatics seem to be the major conduit for the transport of gut-derived bioactive factors into the systemic circulation (Diebel et al., 2012). Organ damage starts depending on bleeding severity and shock duration. Vital organ hypoperfusion usually begins in the gut and progresses to the kidney, liver, and lungs. The emission of large amounts of damage-associated molecular patterns (DAMPs) in polytrauma, systemically circulating, affects the patient's whole body as initiators of systemic inflammation that is an exaggerated defense response with consequent organ failure (Wutzler et al., 2013; Huber-Lang et al., 2018; Matheson et al., 2018; Relja et al., 2018; Relja and Land 2020).

The endothelium while the linkage between oxygen debt and traditional organ failure (renal, hepatic, lung, etc.) has been long recognized; two additional highly dynamic tissues should be considered: the endothelium and the blood. These can be thought of as an integrated organ system, and are strongly related to oxygen delivery in the body (Lord et al., 2014). Microcirculation approximately represents an area of 4,000–7,000 m^2 with endothelium being a major target for trauma induced hemorrhage and hypoperfusion damages. When endothelial damage is present, hemorrhage and hypoperfusion therapy can produce damages (reperfusion damages) in the epithelium. In this case, the primary goal in trauma care should be the fast mitigation of oxygen debt (Jenkins et al., 2014).

DISCUSSION AND CONCLUSION

The current ever renewed interest for space missions, in deep space or to another planet, has brought novel conclusions on astronaut health. Although the risk of hemorrhage is relatively low, the next missions to Mars pose a new set of health challenges. Mars is farther than any planet to which humans have traveled before. From or to it, astronauts will not be able to return in case of a health emergency. Also, repairing any injured area of our body would be impossible and even what we consider a "common condition" could turn out to be a devastating event (Nowak et al., 2019). Any traumatic injury or medical condition could possibly lead to life threatening hemorrhage and may be fatal. Although to date there have been no accounts of major hemorrhage on any space missions, protocols similar to those prepared for austere environments on Earth are needed, given that space is considered the most extreme environment ever experienced. Even if the likelihood of blood loss may be reduced through preventive measures, the occurrence of significant blood loss cannot be completely excluded. Even if blood loss from traumatic injury is more likely to occur during the dynamic stages of flight (launch, landing, and docking, and extravehicular activities (EVA)) (Alexander 2016; Nowak et al., 2019), there are additional risks on Lunar, Martian, or other planetary where gravity creates the possibility of fall and crush injuries. Other causes of nontraumatic blood loss, such as gastrointestinal bleeding from ulceration or sequelae from high-dose radiation exposure, may also be possible.

If the blood transfusion process is challenging in austere environments, where access to stored blood, equipment, and personnel may be limited or nonexistent, to date in space such an event can cause the entire mission to be aborted, or the exhaustion of mission supplies. On Earth, protocols used in mass casualties (SALT: Sort. Assess, Life-saving intervention, Treatment or transport) are helpful tools to decide the strategy of cure and treatment in case of multiple injured people. In space, the bulk of risk mitigation for health issues in low Earth orbit (LEO) is placed more on preventive medicine rather than treatment. The medical protocol (International Space Station ISS Astronaut Medical Treatment Algorithms/Protocols ed US NASA, 2015 (U.S. NASA 2015)) for the ISS is designed to "stabilize and transport" an ill or injured crewmember to reach a definitive medical care facility (DMCF) on Earth (Hamilton et al., 2008). Exploration class missions to the moon have a similar plan, and 4–5 days are needed to transport an ill or injured crewmember to DMCF on Earth (Hamilton et al., 2008).

In case of bleeding, in deep space missions or on Mars, the major issue to face is the absence of a storage possibility for blood and blood derivatives. Blood and its derivates as stated before are the main treatment for hemorrhage. Until today, no resource coming from Earth will last enough to reach the red planet, storage on the spacecraft has limitations, and even in case of a prompt resolution of these problems, a severe trauma requiring transfusion could drain the entire resources of the crew, resources that, at this moment, are not possible to reinstate. This is probably the main problem that needs to be tackled for cure, either surgical or non-operative. Concerns are many: Should the CM O be only a surgeon or at least a physician? What kind of aid could he/she need? A surgical robot, humanoid, or other computerized devices? What degree of autonomy should they have? The delay in communication could be a significant problem, or even the lack of them, just to mention some of them. A great deal of work needs to be done to study and prepare protocols for hemorrhage treatment.

While programs need to be based on proven methods and further studies on hemorrhage control are required before they can be applied in a peculiar environment as space research, hemorrhage control innovations in austere and extreme environments will probably provide the best scenarios to prepare a strategy for missions in deep space.

AUTHOR CONTRIBUTIONS

DP made the design and wrote the manuscript. OC, TS, and SH carried out a check for the current terrestrial treatment in case of trauma and supervised the manuscript. SC and SG performed the

literature search based on keywords and cross-checked for further relevant publications.

ACKNOWLEDGMENTS

This review was written within the activities of the ESA-Topical Team on "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration"; the ESA-MAP Project "WHISPER—Wound Healing In Space: problems and PErspectives for tissue Regeneration and engineering," SciSpacE Microgravity Application Promotion Programme, ESA Contract Number 4000130928/20/NL/PG/pt—the SUTURE in SPACE experiment, selected by ESA (ESA-AO-ILSRA-2014) and supported by ASI (ASI Contract N. 2018-14-U.O).

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Conflict of Interest: The handling editor AC declared a shared research group, WHISPER, with the author DP at the time of review.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Opinion
PUBLISHED 29 August 2022
DOI 10.3389/fbioe.2022.958515

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

RECEIVED 31 May 2022
ACCEPTED 01 August 2022
PUBLISHED 29 August 2022

CITATION
Puhl C, Caplin N, Fogtman A and Van Ombergen A (2022), Wound management and healing in space. *Front. Bioeng. Biotechnol.* 10:958515. doi: 10.3389/fbioe.2022.958515

Wound management and healing in space

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KEYWORDS

wound healing, tissue engineering, wound management, 3D bioprinting, space exploration

Wound healing allows the body to repair damage by regenerating the skin barrier, protecting the skin from the external environment, and allowing it to maintain its homeostasis. It is a complex and multi-step process involving a set of biological mechanisms that should follow a specific sequence, modality, and timeline. Any alterations to this, endogenous or exogenous, can lead to impaired healing which could result in the formation of scar tissue or other pathological conditions (Wilkinson and Hardman, 2020).

The space environment comes with environmental conditions (such as microgravity and ionising radiation) and several constraints in terms of available medical care. Previous studies (Locatelli et al., 2021; Morbidelli et al., 2021; Riwaldt et al., 2021; Marvasi et al., 2022) have already shown that wound healing is slowed down and impaired in space due to the unloading related to the microgravity environment.

Additionally, the combined effects of different stressors of spaceflight, including isolation, a hostile environment or ionising radiation (IR) can further impact an increased susceptibility to infection and delayed wound healing (Chancellor et al., 2018). IR can be a single showstopper to deep space exploration, and it was previously demonstrated that repetitive irradiation impairs cellular replication, causes chronic inflammatory responses, and disturbs proliferation. This leads in consequence to many acute and long-term effects and delayed wound healing is one of them. Skin atrophy, soft tissue fibrosis and microvascular damage has been observed in patients undergoing radiotherapy, and those effects increase the likelihood of impaired tissue regeneration (Jacobson et al., 2017).

As humans venture deeper into space, with longer missions and limited medical capabilities, as well as the higher risk of acute radiation effects due to Solar Particle Events (SPE), investigating wound healing mechanisms adds to the list of knowledge gaps in space research.

Furthermore, a serious risk of long-term space travel and exploration is the potential for the space crew to sustain injuries. The inability to plan for every possible situation, and the lack of a hospital or specialised medical care, means that even a relatively small injury can become serious and potentially threaten the life of the crew and the long-term viability of a given mission. It is critical to address this concern to be prepared for longer-term and deep space missions.

The know-how and applications stemming from this research will have a significant impact on terrestrial medicine, as the problem of insufficient medical resources has been presented in the frame of an exponentially growing human population, more often populating remote areas with access difficulties. Lastly, relocation of large human populations due to climate change will call for novel medical applications for treatment in remote locations and understanding the mechanism of wound healing is a crucial step in this process.

There are several important considerations (non-exhaustive list):

1. Space exploration requires careful planning for payloads. Limited space and other constrained resources make it difficult (and at times impossible) to give medical care in accordance with terrestrial standards.
2. Transporting medical grade materials from Earth into space potentially requires temperature control and the fragility of these materials also needs to be considered. The extreme environmental factors in space will require developing new ways of storing medical supplies and pharmaceuticals to ensure their survival. Potential new materials with adjusted properties need to be developed to ensure they are robust enough for application in space.
3. The physical properties of matter are altered in microgravity, therefore standard ways of drug storage and administration are often not sufficient in spaceflight.
4. The number of possible medical problems that might arise during long-term space exploration is vast. Future mission scenarios involve crew travelling deeper into space and therefore further from help if required. As the temporal aspect of wound management can be critical, novel ways of conducting this in space will be a necessity.
5. The understanding of the mechanisms underlying wound healing in space, and how these are altered remains to be accurately identified.

Consequently, the topic of wound healing in the context of spaceflight has been approached by ESA in a similar fashion to other potentially mission critical problems. ESA has set up Topical Teams—think tanks of experts and leaders in a particular field with the goal of identifying potential concerns and developing recommendations to address these using practical solutions.

The culmination of the Topical Team "*Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration*" is the series of articles in this special issue, as well as several other ongoing and planned activities. In June 2022, ESA's *Suture in Space* experiment is scheduled for flight on the International Space Station (ISS). This experiment aims to investigate the behavior of sutured wounds and the repair process in microgravity, using two models of sutured wound healing based on tissue cultures from human skin and blood vessels, respectively. The *Suture in Space* experiment will lead to improved knowledge of the molecular, cellular and tissue mechanisms involved in the healing of sutured wounds, as well as yield important information to develop strategies to manage wounds in space and promote healing, both in space and on Earth (Monici et al., 2021). ESA also has on-going activities in this area in its Microgravity Application Programme (MAP), i.e., WHISKIES and WHISPER. Both projects grew out of the Topical Team and focus on space environment induced changes to biomedical materials for wound treatment and the fundamental biological wound healing mechanisms.

ESA is also building a 3D bioprinting and 3D cell maturation capability in Low Earth Orbit which will provide support for research and preparation activities to enable long-term human deep space exploration. This will enhance our fundamental understanding and characterisation of the effects of space stressors. Looking further into the future, 3D bioprinting offers the potential to generate personalised grafts or implants for repair of tissue injuries for crew members during long-term deep space exploration missions, where a rapid return to Earth is not possible (Cubo-Mateo and Gelinsky, 2021).

There are still many unknowns, but we are convinced that the contributions to this special issue provides the current state of the art, as well as important next steps and fascinating foresights for wound healing in the context of space exploration. Without question will the knowledge gained from these activities not only advance space research and human exploration, but also deliver answers to fundamental questions in the area of wound healing and yield translational applications to terrestrial medicine related to trauma care and emergency surgery amongst other fields.

Lastly, we would like to thank the editors and the authors for their efforts in compiling this vital work, as well as the many dedicated peer reviewers for reinforcing the excellence in the quality of the papers. A special thanks goes out to Prof. dr. Monica Monici, whose dedication and contributions have been pivotal in these efforts and activities.

Author contributions

CP and AVO wrote the first draft of the manuscript. CP, NC, AF, and AVO wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

Author CP was employed by the company Telespazio Belgium S.R.L.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Perspective
PUBLISHED 30 August 2022
DOI 10.3389/fbioe.2022.937709

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

RECEIVED 06 May 2022
ACCEPTED 08 August 2022
PUBLISHED 30 August 2022

CITATION
Aliberti F, Paolin E, Benedetti L, Cusella G and Ceccarelli G (2022), 3D bioprinting and Rigenera® micrografting technology: A possible countermeasure for wound healing in spaceflight. *Front. Bioeng. Biotechnol.* 10:937709. doi: 10.3389/fbioe.2022.937709

3D bioprinting and Rigenera® micrografting technology: A possible countermeasure for wound healing in spaceflight

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Plant and animal life forms have progressively developed mechanisms for perceiving and responding to gravity on Earth, where homeostatic mechanisms require feedback. Lack of gravity, as in the International Space Station (ISS), induces acute intra-generational changes in the quality of life. These include reduced bone calcium levels and muscle tone, provoking skin deterioration. All these problems reduce the work efficiency and quality of life of humans not only during exposure to microgravity (μG) but also after returning to Earth. This article discusses forthcoming experiments required under gravity and μG conditions to ensure effective and successful medical treatments for astronauts during long-term space missions, where healthcare is difficult and not guaranteed.

KEYWORDS

microgravity, 3D bioprinting, micrografting, skin, regenerative medicine, wound healing, space, tissue engineering

1 Introduction

Wound healing (WH) is a dynamic and complex biological process consisting of tightly coordinated interactions between growth factors, cytokines, chemokines, different cell types, extracellular matrix (ECM), and proteases (Nourian Dehkordi et al., 2019; Morbidelli et al., 2021). WH is conventionally separated into several events: coagulation, inflammation, granulation tissue formation, proliferation, and remodeling (Jimi et al., 2017; Cialdai et al., 2020). Alterations or blocking of one or more stages of the repair process can lead to the formation of chronic or intractable wounds, issues that may arise in astronauts during long-term space explorations (Riwaldt et al., 2021). Indeed, astronauts' complaint of skin deterioration during space missions has been reported by the National Aeronautics and Space Administration (NASA) (Riwaldt et al., 2021; Garcia, 2022). Long-term exposure to μG induces mechanical stress upon mammalian tissue, leading to rapid alterations that increase the risk of physiological degeneration of the bone, muscles, cardiovascular capacity, and WH. High fibrinogen concentrations, the presence of thrombin, and endothelial damage markers were detected in the blood of

astronauts in spaceflight missions in addition to the reduction of blood flow (Kim et al., 2021). Prolonged exposure to μG may also result in the development of anemia and the alteration of cardiac physiology (Trudel et al., 2020).

As the interest in space travel and identifying other habitable planets increases, it becomes more important to deepen our understanding of how a low-gravity environment may impair and affect the human body. In particular, the impacts of the absence of gravity, the absence of atmosphere, and the lack of Earth's magnetic field on organ systems are under-researched.

Initial research by Blaber et al. (2014) reported experiments conducted in collaboration between NASA and the Russian Space Agency over the course of the Foton M2 and M3 missions, where various tests on tissue regeneration were conducted. Specifically, it was observed that μG is responsible for the inhibition of the transition from progenitor cells to differentiated adult cells, suggesting that prolonged exposure to μG may result in a decrease in the tissue regeneration capacity.

Furthermore, NASA designed an automated Bioculture System meant for use on the International Space Station (ISS) to carry out bioscience research exploiting ten independent cell culture cassettes, each with a standalone cell culture bioreactor to allow multiple experiments simultaneously (Blaber et al., 2014).

Among several approaches which have been developed to fully characterize the biological skin model, tissue engineering (TE) acquired a pivotal role due to its capability to produce three-dimensional (3D) biological *in vitro* models that mimic the skin's physiological environment (Singer and Boyce, 2017). Indeed, Zhou et al. (2020) have designed and fabricated a functional living skin formed by the human skin fibroblast and biomimetic bioink, using the 3D bioprinting technology, which is one of the most innovative and promising techniques of tissue engineering. They obtained a 3D structure model capable of widely promoting cell viability, migration, and proliferation compared to 2D *in vitro* models. Furthermore, the *in vivo* application of the investigated model demonstrated very high performance in tissue regeneration and, above all, an acceleration in wound repair.

Moreover, another main goal of TE is to recreate biological *in vitro* substitutes with the aim of providing tissue models to be used in the replacement or regeneration of damaged tissues (Berthiaume et al., 2011). In this regard, Cao et al. (2020) have engineered a scaffold based on human-like collagen and carboxymethylated chitosan, whose geometry and composition allow to mimic the extracellular matrix intrinsic characteristics (transportation of materials, cell adhesion, and proliferation), making it an advantageous candidate as a project strategy for skin defect repair as it has been demonstrated by promising *in vivo* collected data. Hence, in the last decades, new materials, methodologies, and technologies have emerged for the creation of skin substitutes, including the use of arising methodologies (Tresoldi et al., 2019; Cubo-Mateo and Gelinsky, 2021; Tottoli et al., 2022). Considering the challenges that WH research encounters under normal gravity conditions, it is ambitious to identify a process to address WH under μG conditions. However, on board the ISS, it is necessary to consider the additional limitations that a medical treatment on board or an *in vitro* experiment would encounter, e.g., the number of devices and limited transportable materials, time constraints, changes in mass, chemical–physical properties, and the levels of containment to protect the crew, ferry vehicle, and ISS itself (Ferranti et al., 2021). The aim of this work is to discuss the forthcoming experiments that need to be carried out to study and characterize potential countermeasures to guarantee new, effective, and successful medical treatments for astronauts.

Rigenera® (Human Brain Wave, Turin, Italy) is a new autologous micrografting technology (AMG), already successfully employed in WH (Uehara and Shimizu, 2019). It is also under investigation to assess its potential to favorably influence WH in a low-gravity environment. Furthermore, AMG technology, together with 3D bioprinting, is acquiring a pivotal role in the field of tissue engineering, removing limitations associated with autograft transplants (e.g., risk of infections, secondary diseases, and low compliance for the patient).

2 Biological design of a novel approach for wound healing in space

The skin is the largest single organ of the body. It is the main barrier to the external environment and, therefore, has a protective function (Gollnick et al., 2019).

Aging can lead to the development of skin fragility due to the defective migration of WH cells (Afshinnekoo et al., 2020). In space flights, environmental factors such as μG and radiation can have a severe impact on the skin, resulting in impaired WH mechanisms in case serious traumatic injuries occur (Tronnier et al., 2008; Braun et al., 2018; Cubo-Mateo and Gelinsky, 2021).

Among several re-epithelialization technologies developed to establish a physiological WH process, it has been demonstrated that the AMG technology is able to respond to the principal limitations to the current gold standard approach of autologous grafting. This includes the need to use large quantities of tissues, long sample preparation time, and long-term hospitalization (Niimi et al., 2022). It has been shown by Balli et al. (2020) that the Rigenera® technology plays a key role in the re-epithelialization stage by modulating the genes responsible for angiogenesis, cell migration, and MAPK/ERK activation, and inducing the migration of fibroblasts. They observed quicker wound closure in cell scratch assays in the presence of AMGs. These findings could be tested under simulated μG conditions on Earth using a random positioning machine (RPM) to rapidly establish if AMGs could be employed as a potential solution for in-space medical care to investigate further in conjunction with a tissue-engineered scaffold (Cialdai et al., 2020). Subsequently, a

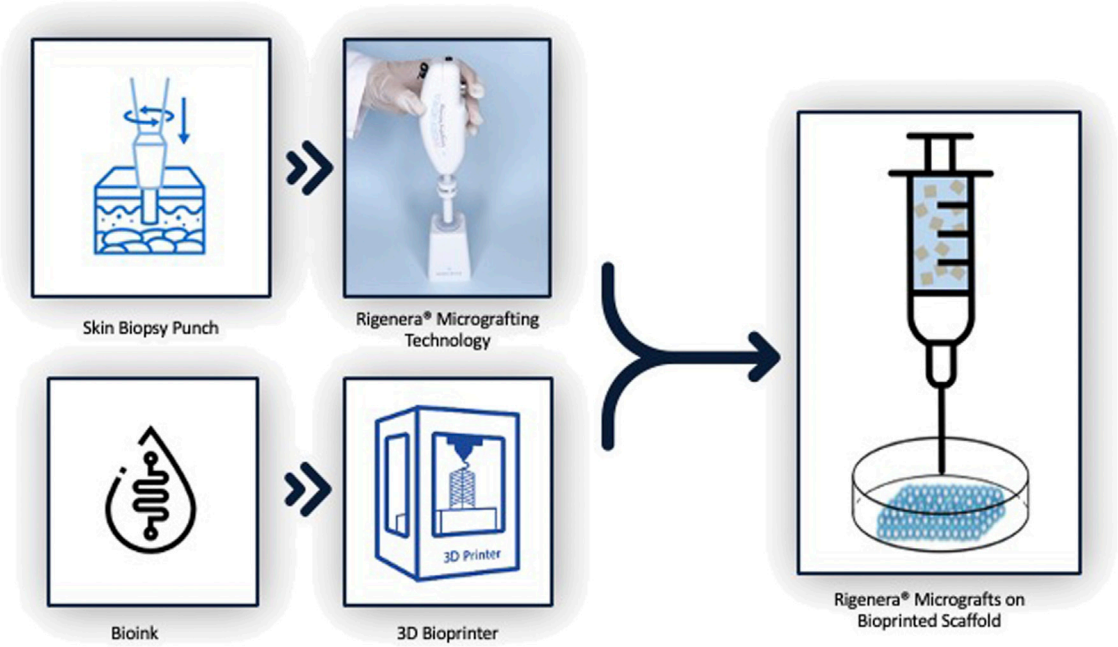

FIGURE 1
Process to obtain Rigenera ® micrografts on the bioprinted scaffold.

gene expression analysis must be conducted, like RNAseq or microRNA analysis, to comprehensively evaluate the AMG technology.

Balli et al. (2020) suggested the investigation of all genes related to the matrix metalloproteinases (MMPs) and whose expressions increase during AMG treatment, leading to an expansion of chemokine regulators, remodeling due to myofibroblasts, and a faster wound contraction. Moreover, in the study by Cialdai et al. (2020), it was observed that when α-smooth muscle actin (α-SMA—gene related with fibroblast–myofibroblast transdifferentiation) expression decreased on fibroblasts exposed to μG, vascular endothelial growth factor (VEGF—gene active in the processes of angiogenesis and vasculogenesis and promotes cell migration) expression significantly increased.

Moving toward a higher level of complexity, along with the use of a solution of micrografts, it is also useful to exploit dermal substitutes designed to mimic the physiological architecture of the skin. For full-thickness wounds, the use of scaffolds would not only be useful but would also be necessary to provide a 3D structure to the solution of micrografts and ensure, therefore, a faster and more efficient WH (Negut et al., 2020).

The production of a suitable skin substitute has been a long-sought goal for modern medicine; in fact, in the last decades, significant advances have been made in the field of skin tissue engineering, both in the development of engineered substitutes useful for the replacement of skin lost due to trauma, wounds, or burns and as a realistic model to be used for *in vitro* tests. The design implementation of skin tissue is realized by combining materials, cells, biochemical mediators, and innovative culture systems (Berthiaume et al., 2011).

Pellegrini et al. developed a new life-saving therapeutic strategy to replace and repair severely damaged tissues using new substrates and new culture technologies. After isolation and characterization of keratinocytes (holoclones, meroclones, and paraclones), a fibrin scaffold was designed, and cells were seeded. The results show that cell percentage is maintained when cultured on fibrin, demonstrating that the fibrin scaffold allows the maintenance of the epidermal phenotype.

Moreover, *in vivo* experiments have been carried out using the bioconstruct (scaffold and seeded keratinocytes), which has been shown to support permanent cell proliferation and ensure high reproducibility if it is applied to injured skin regions (Graziella and Luca, 1999).

Based on this research, it would be advantageous to fully exploit the 3D bioprinting technology together with the Rigenera® technology with the aim of obtaining and engineering biological and biocompatible skin substitutes with an enhanced regeneration efficiency that could be applied to astronauts' wounds during future interplanetary space missions, as shown in Figure 1.

In recent years, one of the major challenges has been the realization of a device that can be considered a novel effective countermeasure for severe skin injuries. The implementation of

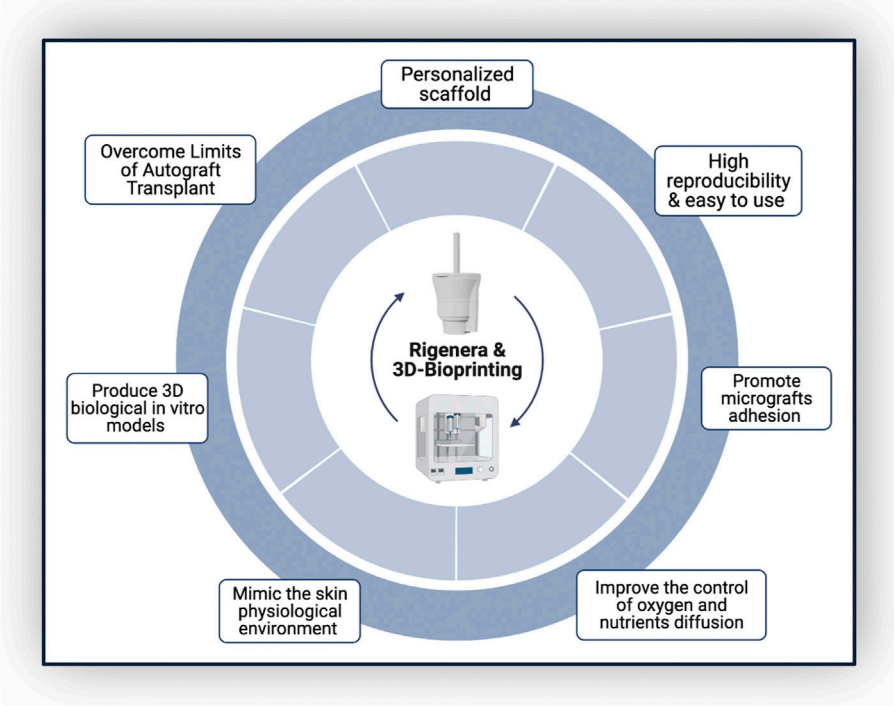

FIGURE 2
Applicability and suitability of the Rigenera® technology with 3D bioprinting.

such a device could overcome the common treatment disadvantages, such as telemedicine that, despite its effective use on the ISS, it would have an increased communication delay when the astronauts get further from Earth, the high amount of tissue required, and the long sample preparation times (Farkas and Farkas, 2021; Niimi et al., 2022).

Obtaining an effective skin substitute that can be readily used, even in space missions, requires easy-to-use equipment and materials. Indeed, the characterization of a proper formula for an appropriate 3D skin substitute bioink is crucial. The term bioink refers to biomaterials that possess good printability characteristics and allow cells to survive when mixed with the bioink itself (Weng et al., 2021). Indeed biomaterials suitable for skin bioprinting, such as collagen, hyaluronic acid (HA), agarose, and alginate, possess good biocompatibility, promote cell adhesion, proliferation, and migration, do not cause inflammation, and can be enzymatically degraded. Moreover, they can be printed at low temperatures, significantly decreasing cellular stress (Murphy et al., 2013; Li et al., 2016; Weng et al., 2021).

Combining Rigenera® technology and 3D bioprinting would take advantage of both their high reproducibility and ease of use, allowing astronauts to produce personalized scaffolds in space, avoiding prior skin substitute production on Earth. This approach would allow mechanical scaffold features to be modulated to mimic biological tissues and, parallelly, improve the control of oxygen and nutrient diffusion, as well as promote cell adhesion and proliferation (Figure 2). In fact, AMGs could be applied directly over the wound, enhancing healing time and preventing fibrotic scar formation (Giaccone et al., 2014; Baglioni et al., 2016).

The experimental part is divided into a three-step cascade: in the first two steps, the scaffold will be designed (engineered in Computer Aided Design—CAD) and then bioprinted, layer-by-layer, into a fibrin hydrogel, and finally, AMGs will be seeded on it. To analyze the efficiency of hereby proposed mixed technology, AMGs will be applied on scratch-damaged bioprinted constructs, under the presence or absence of μG. Furthermore, to mimic the condition whereby an injured astronaut would come back to Earth to receive healthcare, the scratch-damaged bioprinted bioconstruct will be manufactured in μG conditions and subsequently, AMGs will be applied in a standard atmosphere environment. The goal of the above-mentioned experimental setup is to analyze a possible change in cell migration capability, in the presence and absence of AMGs and μG. The aim of this prospective research is to perform studies that can demonstrate, during μG tests, any alterations in pathways involved in skin regeneration confirmed through gene expression and microRNA analysis (Banerjee et al., 2011; Balli et al., 2020; Cialdai et al., 2020). Hence, the novel

bioconstruct hereby proposed could *in vitro* predict the skin-related alterations in astronauts during μG exposure and be used as a reference model for the in-space construction of personalized patches to ameliorate the regeneration of damaged tissues. Prior to performing an *in vivo* study, it is advisable to proceed with *ex vivo* experiments, for instance, Botchkareva et al. devised an *ex vivo* model for testing WH-promoting compounds. Similarly, an experimental setup could be adopted using the *ex vivo* tissue alone under μG conditions as the negative control, the *ex vivo* tissue under μG conditions treated with AMGs as the test group, and the *ex vivo* tissue treated with AMGs in Earth gravity conditions as the positive control (Botchkareva and Westgate Editors, 2022).

3 Discussion

Effective WH is a challenging task to be achieved under normal gravity, and it becomes even more complex in μG conditions. Wounds that could arise in incidents on routine spaceflights may potentially affect astronauts' health. Based on medical data collected in past spaceflights, skin alterations cause significant trauma to crew members (Singer and Boyce, 2017). It, therefore, becomes crucial to better understand how to minimize and treat skin deterioration in spaceflight (Riwaldt et al., 2021).

Currently, many different techniques are being used to treat WH, resulting in different outcomes. Certainly, the split-thickness autologous skin grafting treatment is deemed to be the gold standard for full-thickness wounds, although it exhibits disadvantages such as the need for a secondary surgical site, limited availability, and low cost-effectiveness.

For the xenograft technique, where a graft from an animal is collected, the main limitations concern graft rejection and the risk of transmitting viruses or zoonotic disease (Oryan et al., 2017).

It is, therefore, necessary to identify an easy, quick, and clinical-effective technique to improve WH outcomes. Furthermore, considering a μG environment, such as the one in the ISS, or during a long-term space exploration mission, it is essential to find an application that is easy to learn by the staff present in order to minimize infections, human errors, and rejection. It is proposed that the Rigenera® AMG technology, described here, could address these limitations. Therefore, with the aim to investigate the cellular and molecular mechanisms behind AMG technology under μG conditions, as a first approach, an *in vitro* WH model (fibroblasts scratch assay) could be analyzed, as Balli et al. (2020) and Cialdai et al. (2020) analyzed (Monici et al., 2011). Considering that both migrations of fibroblasts and extracellular matrix (ECM) production are essential to guarantee proper WH, it is interesting that fibroblast migration decreases in a μG, and ECM production is significantly altered (Monici et al., 2011). In this respect, we propose to investigate how AMGs could influence the above-mentioned WH model; in particular, taking into consideration that AMGs consist of progenitor cells, extracellular matrix, and growth factors, and that in normal g condition, a scratch assay with them was already performed by Balli et al. with awesome outcomes, a relation between the Rigenera® technology and its capability to affect fibroblast migration under μG could be speculated and deserves to be further investigated (Trovato and Failla, 2016).

Once the macroscopic feasibility has been settled through a first-level assessment, a second-tier investigation should be followed. Several genes and microRNAs are involved in the process of WH, and evaluating their expression in conditions of normal gravity and μG should help the scientific community to better understand the biochemical reaction behind skin repair and WH. This could be more easily achieved by recreating 3D skin *in vitro* models. The development of a three-dimensional construct would mimic a suitable microenvironment so that each type of cell (associated with the scaffold and with the micrografts) can express its own phenotype and perform its own functions (Yannas, 2018; Negut et al., 2020; Pavez Loriè et al., 2021; Botchkareva and Westgate Editors, 2022). Furthermore, the bioprinting technology provides the design and the manufacturing of bioconstructs that are personalized based on the shape and size of the astronauts' wounds and are biocompatible, biodegradable, and induce angiogenesis (Yu et al., 2019).

Nevertheless, considering the difficulty of being able to perform *in vivo* studies under μG conditions, an *ex vivo* experiment could help in collecting a larger amount of evidence. Data on changes in inflammation, tissue formation, and tissue remodeling would be better observed through an *ex vivo* study, creating the possibility to confirm the preliminary data from the *in vitro* tests.

It is also interesting to be able to exploit what is already present on the International Space Station (ISS) so as to consider not only the parameters such as the difference in gravity but also the presence of different radiations, oxygen levels, and bacteria in the microenvironment that is created inside the station. In fact, astronauts are exposed to different types of radiation, namely, the ionizing radiation (IR) particles outside the Earth's magnetic field, particles derived by solar flares, and galactic cosmic rays. All these factors can influence the outcome of healing of a wound, and considering them would bring all the experiments as close as possible to the environment that a human being affected by a skin injury could actually find onboard the ISS (Pavez Loriè et al., 2021).

For these reasons, our future research will be focused on the development of *in vitro* and *ex vivo* experimental studies that can confirm the expected results depicted in this article and that can provide solid results that afford to perform further studies which can overcome the presented limitations.

4 Conclusion

In this perspective study, a deepened analysis of the current situation of WH in conditions of μG was

performed. Considering the strong desire of humans to push the boundaries and reach other planets and the necessity of finding new resources outside the Earth's space, it is now necessary to understand how to prevent wound degeneration and re-establish a balanced physiological condition even in the absence of injuries. The scientific community is only at the beginning of this long path, and many facets of this research deserve to be addressed.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

FA, EP, and GCe contributed to the conception and design of the perspective study. FA and EP wrote the first draft of the manuscript. GCe, LB, and GCu revised the manuscript. GCe supervised this project. All authors approved the original and revised versions of the manuscript.

Funding

This work was funded by the SciSpacE Microgravity Application Promotion Programme of ESA called: "Wound Healing in Space: problems and Perspectives for tissue Regeneration and engineering (WHISPER)."

Acknowledgments

The authors thank Siobhan Moyes from the University of Plymouth, Peninsula Medical School (United Kingdom), for revising the manuscript.

Conflict of interest

EP belong to the R&D department of HBW srl, the company owner of the Rigenera Micrografting Technology.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 04 October 2022
DOI 10.3389/fbioe.2022.958381

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

RECEIVED 31 May 2022
ACCEPTED 05 September 2022
PUBLISHED 04 October 2022

CITATION
Cialdai F, Risaliti C and Monici M (2022), Role of fibroblasts in wound healing and tissue remodeling on Earth and in space. *Front. Bioeng. Biotechnol.* 10:958381. doi: 10.3389/fbioe.2022.958381

Role of fibroblasts in wound healing and tissue remodeling on Earth and in space

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Wound healing (WH) and the role fibroblasts play in the process, as well as healing impairment and fibroblast dysfunction, have been thoroughly reviewed by other authors. We treat these topics briefly, with the only aim of contextualizing the true focus of this review, namely, the microgravity-induced changes in fibroblast functions involved in WH. Microgravity is a condition typical of spaceflight. Studying its possible effects on fibroblasts and WH is useful not only for the safety of astronauts who will face future interplanetary space missions, but also to help improve the management of WH impairment on Earth. The interesting similarity between microgravity-induced alterations of fibroblast behavior and fibroblast dysfunction in WH impairment on Earth is highlighted. The possibility of using microgravity-exposed fibroblasts and WH in space as models of healing impairment on Earth is suggested. The gaps in knowledge on fibroblast functions in WH are analyzed. The contribution that studies on fibroblast behavior in weightlessness can make to fill these gaps and, consequently, improve therapeutic strategies is considered.

KEYWORDS

fibroblasts, wound healing, tissue regeneration, microgravity (μg), spaceflight, chronic ulcers, scarring, wound healing dysfunction

Wound healing and tissue regeneration

The wound healing (WH) process is the response of the organism to an injury. By stopping bleeding and restoring the protective barrier that counteracts the onset of infections and maintains internal homeostasis, WH allows the organism to survive. The healing process is classically described as a sequence of partially overlapped phases, which, after hemostasis, includes inflammation, proliferation, and, finally, remodeling (Figure 1). In reality, each phase includes a series of events or subphases (e.g., early and late inflammation), which are temporally, quantitatively, and qualitatively regulated by a plethora of biochemical and physical factors (Monaco and Lawrence 2003).

The final goal of the healing process is to repair damaged tissues and restore their function. While in invertebrates and non-mammalian vertebrates, the ability to regenerate functional native tissues without scars is quite common, at least in some periods of the life cycle (Arenas Gómez et al., 2020), in adult mammals, with very few exceptions, the progression of healing leads to wound closure with the formation of scar tissue. It is morphologically and functionally different from the native tissue as regards

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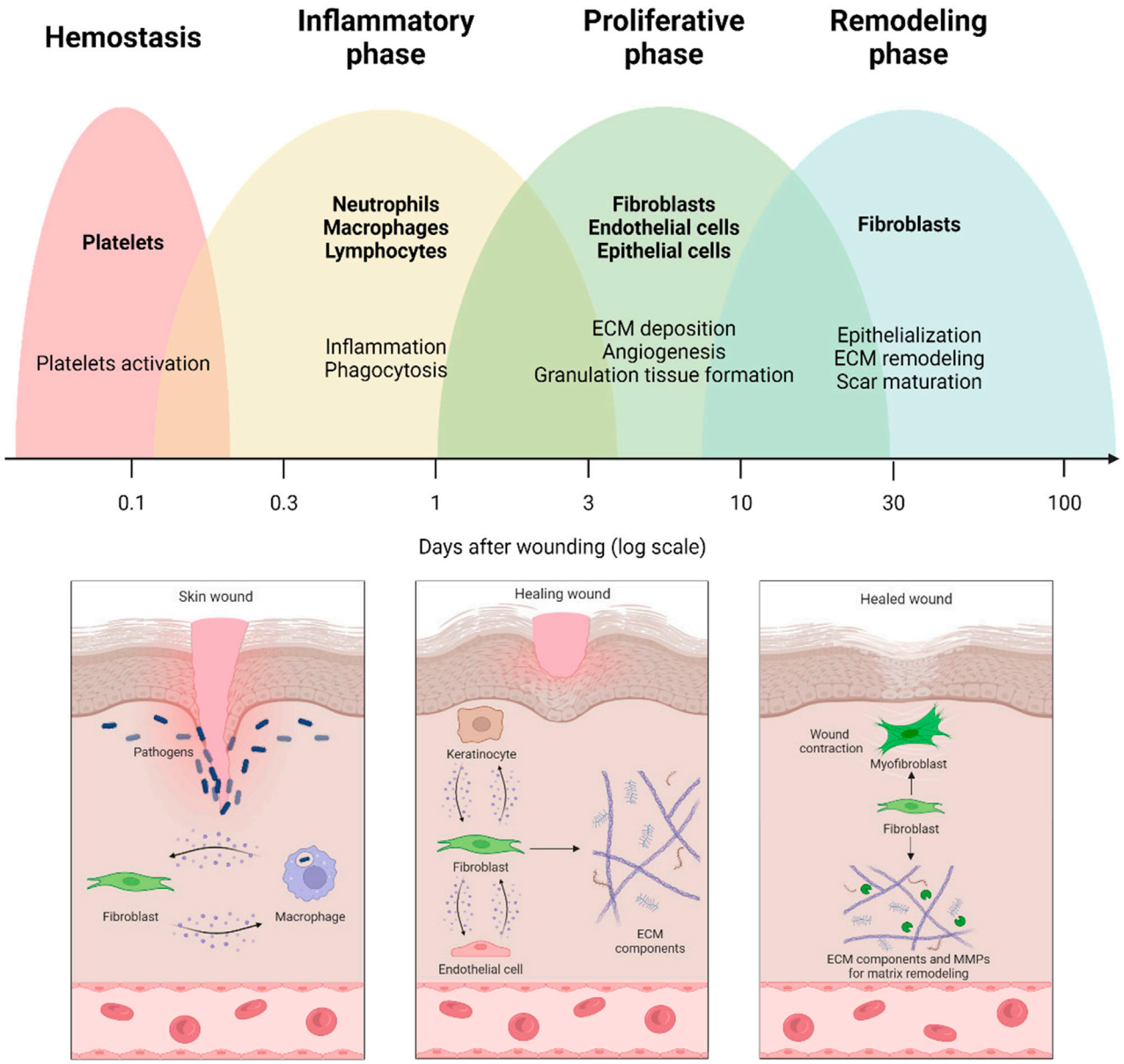

FIGURE 1
Wound healing process and the involvement of fibroblasts in the different healing phases. The early stages of wound healing are characterized by crosstalk between fibroblasts and immune cells. Thereafter, fibroblasts are responsible for producing extracellular matrix (ECM) components and establishing crosstalk with endothelial cells and keratinocytes. Finally, fibroblasts are involved in remodeling the extracellular matrix by secreting matrix metalloproteinases (MMPs) and matrix components.

extracellular matrix (ECM) organization (in particular, collagen quality and assembly) and other features: for example, skin wounds heal with scars lacking hairs and glands (Thulabandu et al., 2017). Therefore, the repair is considered successful when it ends with the formation of a limited amount of scar tissue that restores the integrity of the protective barrier and preserves organ function and tissue integrity (Sawant et al., 2021). However, mammalian embryos (including human ones) can regenerate in the early stages of development, and oral mucosa wounds can heal nearly scarless also in adults (Peake et al., 2014).

Despite numerous in-depth studies, how and why adult mammals have lost the ability to regenerate remain unanswered questions. The most accredited hypothesis suggests that, in the course of evolution, given the complexity reached by the mammalian organism, an imperfect but faster healing modality (with scars) has proved to be more

advantageous than a perfect but slower regenerative process (without scars) (Cohen, 2007). Intense research is underway to answer this scientific problem because it is a common opinion that bridging this gap of knowledge would allow enormous progress to be made in the management of healing dysfunctions, which heavily affect patient's quality of life and drain the health system of an enormous amount of resources (Desjardins-Park et al., 2018). A better understanding of the mechanisms for switching from imperfect healing into full regeneration could also be extremely helpful in space medicine from the perspective of future deep space exploration missions.

In case the normal progression of the healing mechanisms fails, chronic conditions arise that result in non-healing ulcers or fibrotic scars. Of the latter, the most common example is skin hypertrophic and keloid scarring. However, internal organs show analogous scarring, in which lesions or pathological conditions induce fibrosis that may or may not resolve over time (Darby and Hewitson, 2007).

Many factors affect tissue repair and its complications: age, gender (due to the different hormone profiles), overweight, systemic diseases (e.g., diabetes), suturing materials and techniques, wound contamination, mechanical factors, emergency care and wound care, and non-physiological environment. Some of these factors cannot be modified, such as age, overweight, and systemic diseases. In contrast, others, such as suturing techniques and wound care, can be managed to facilitate the proper evolution of the healing process. The most common features of healing dysfunctions are persistent inflammation, persistent stromal activation, altered myofibroblast function, uneven areas in the wound bed (i.e., wound areas that are in different healing phases at the same time) with an uncoordinated transition from one phase to another (Darby and Hewitson, 2007).

Despite the progress made in the last 20 years in understanding the origins of healing dysfunctions, their causes are far from clear. Consequently, current therapeutic strategies are poorly effective (DesJardins-Park et al., 2018; Zou et al., 2021).

The role of fibroblasts in wound healing

Fibroblasts play a crucial role in all three phases of WH. They orchestrate the whole repair process by producing a number of regulatory molecules and crosstalk with the other cell populations involved in the healing mechanisms (Figure 1).

Any injury triggers an inflammatory reaction *via* cytokines deriving from platelet degranulation. Immune cells further increase the level of pro-inflammatory mediators, such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and inducible nitric oxide synthase (iNOS) (Koh and DiPietro, 2011), thus fueling the inflammatory process and stimulating the recruitment and activation of fibroblasts.

During the inflammatory phase, activated fibroblasts engage a crosstalk that strengthens the local immune response and the activation of immune cells in several ways: 1) producing proinflammatory cytokines, such as TNF-α, interferon gamma (IFN-γ), IL-6, and IL-12 (Correa-Gallegos et al., 2021), and releasing a wide range of C–C and C–X–C chemokines, including CXCL1, CX3CL1, and CCL2, to further recruit immune cells to injury sites (Bautista-Hernández et al., 2017); 2) juxtacrine interactions, *via* ICAM1 and CD40 expression, which also activate dendritic cells (Saalbach et al., 2007); 3) remodeling the wound stroma *via* the secretion of matrix metalloproteinases (MMPs) to allow immune cell infiltration; 4) sensing the changing interstitial flow and fluid pressure caused by the inflammatory edema and responding with a modulation of the physical properties of the microenvironment, including rigidity, porosity, elasticity, and viscosity, which make it more immunologically active (Langevin et al., 2013; Correa-Gallegos et al., 2021); 5) migrating collectively from fascia into wounds, thus translocating ECM with embedded immune cells, as recently demonstrated in response to deep injuries (Correa-Gallegos et al., 2019). In summary, fibroblasts can modulate the recruitment of immune cells and regulate their behavior, retention, and survival in damaged tissue. Furthermore, the crosstalk between fibroblasts and macrophages is particularly important in regulating the transition from the inflammatory phase to the subsequent proliferation phase, determining the correct progression of the healing process (Mescher, 2017).

Fibroblast activity becomes even more important during the proliferation phase. After migrating in the provisional fibrin clot, regulated by inflammatory mediators, such as C5a, fibronectin, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-β (TGF-β) (Thulabandu et al., 2017), fibroblasts proliferate and contribute to angiogenesis and the formation of granulation tissue by secreting pro-angiogenic molecules, including vascular endothelial growth factor (VEGF), FGF, angiopoietin 1 (Ang-1), and thrombospondin (TSP) (Tonnesen et al., 2000; Li et al., 2003). Stimulated by growth factors produced by macrophages and other immune cells, fibroblasts produce MMPs, which degrade the fibrin clot favoring cell migration (Mirastschijski et al., 2010), and ECM molecules, including fibronectin, hyaluronic acid, proteoglycans, and collagen (mostly type III), which replace the fibrin clot with a new provisional matrix supporting keratinocyte migration needed for re-epithelialization (Mirastschijski et al., 2012). Although the proliferative phase can last up to 2 or 3 weeks, starting about 4 days after injury, fibroblasts are the prevailing cell population in the wound, and, stimulated by TGF-β and CXCL8, they begin to differentiate into myofibroblasts (Desmoulière et al., 2005), the protagonists of the remodeling phase.

The final phase of the process can last over a year (Gurtner, 2006) and consists of wound contraction, vascularization and cellularity decline, ECM turnover leading to tissue remodeling, and tensile strength recovery. Myofibroblasts regulate wound contraction and tissue remodeling by combining the ability to synthesize ECM proteins and assume a contractile phenotype (Tomasek et al., 2002; Darby et al., 2014). The fibroblast-myofibroblast transdifferentiation is mainly regulated by TFG-β1 and ECM stiffness. Myofibroblasts assume contractile properties by incorporating α-smooth muscle actin (α-SMA) into stress fibers (Sawant et al., 2021). The contractile activity not only causes wound contraction but also increases ECM stiffness, which in turn induces myofibroblast differentiation and persistence (Grinnell and Petroll, 2010). In the meantime, myofibroblasts remodel ECM through a balanced production of MMPs and ECM proteins, including collagens (Bernardo & Fibbe, 2013). In this phase, collagen III is typically replaced by collagen I, and ECM undergoes a sequential remodeling toward increasing complexity, order, and tensile strength. However, the tensile strength of wounded skin after healing reaches, at best, approximately 80% of that of the unwounded skin (Gurtner and Wong, 2013).

As healing progresses, cellularization decreases through apoptosis (or programmed cell death), which firstly affects the cells of the immune system, then endothelial cells, and finally myofibroblasts. It has been shown that myofibroblast apoptosis occurs when the wound closes and the tissue recovers, at least in part, its tensile strength, suggesting that apoptosis triggers the decrease in vascularization and the evolution from the granulation tissue to scar tissue (Bainbridge, 2013).

Fibroblast dysfunction in wound healing

Recent research on WH impairment has strongly focused on fibroblasts and their functions involved in the different WH phases. Fibroblast dysfunction can lead to opposite healing problems, namely, fibrosis and healing delay.

Proper timing for resolution of the inflammation is very important for successful healing progression: a persistent macrophage-fibroblast activation state, with excessive production of pro-inflammatory mediators by fibroblasts and further recruitment of immune cells, generates a feed-forward loop leading to altered repair processes from chronic wounds to fibrosis and scarring (Wynn, 2008; Grinnell and Petroll, 2010; Wynn and Ramalingam, 2012). For example, the excessive fibroblast activity, often occurring in large burns and severe injuries, results in hypertrophic scarring and keloid formation (i.e., dysfunctional and disfiguring scar tissue) (Hinz, 2016; Arif et al., 2021). The persistence of myofibroblast activity can also be caused by altered signaling pathways (Leask, 2021), apoptosis failure (Hinz and Lagares, 2020), and excessive mechanical stress, as in the case of high strains at the wound edges (Wong et al., 2011a) or ECM stiffness (Sawant et al., 2021).

Conversely, myofibroblast dysfunctions can also cause delayed wound closure up to chronic ulcers, which fail to heal due to failure in ECM reconstitution. Chronic ulcers are often associated with cardiovascular and/or metabolic diseases, chronic infection, and inflammation (Wan et al., 2021). A study on diabetic mouse fibroblasts, compared to normal ones, found a 75% reduction in migration, altered MMP9 production, and a tremendous decrease in VEGF production (Lerman et al., 2003). Recently, it has been shown that high glucose impairs the proliferation and migration of human gingival fibroblasts (HGFs), explaining the delayed gingival WH in diabetic patients (Buranasin et al., 2018). The outcomes of these studies unequivocally indicate the inability of fibroblasts to promptly migrate to the wound site, properly remodel ECM, and adequately support neoangiogenesis in conditions of high glucose levels (Table 1). Moreover, prolonged inflammation and wound infection can strongly affect ECM production. Persistent inflammation leads to a wound environment characterized by an excess of pro-inflammatory molecules, proteolytic enzymes, and reactive oxygen species (ROS). Both proteolytic enzymes and ROS directly damage ECM molecules, altering the balance between ECM production and degradation, with the prevalence of degradation. In these conditions, the cells involved in the healing process, including fibroblasts, in particular senescent ones, are stimulated to further produce proteolytic enzymes and ROS, generating a vicious circle that hinders the normal evolution of WH. Wound infection further worsens the scenario as it fuels inflammation while its microbial components interfere with cell-ECM interaction (Eming et al., 2007).

Wound healing in unloading conditions

The studies on WH in space, namely, in microgravity (μg) conditions, are relatively few. To the best of our knowledge, no studies concerning the effect of spaceflight on WH in humans are available. This knowledge gap is largely motivated by the fact that, in space missions within the low Earth orbit (LEO), the likelihood of surgical emergencies or traumatic events causing serious wounds is very low. In any case, a medical evacuation to Earth is always feasible. However, Space Agencies are aware that future interplanetary missions open completely different scenarios. Realistically, medical emergencies will be more likely and will have to be managed aboard spacecraft and space stations or in space bases. Due to the great distance from Earth, medical evacuation would not be feasible, nor would it be easy to guide crew actions remotely due to communication lag.

Therefore, Space Agencies have for some time been conducting studies on the feasibility of surgery in space and the implementation of dedicated techniques (Panesar and

TABLE 1 Impairment of fibroblast functions involved in wound healing in conditions of high glucose level.

	Normal wound healing	Diabetic wound healing impairment
Migration	↑	↓
VEGF production	↑	↓
Inflammation	↓	↑
ROS	↓	↑
Proteolytic enzymes	↓	↑
ECM degradation	↓	↑

↑ higher; ↓lower; extracellular matrix (ECM); reactive oxygen species (ROS); vascular endothelial growth factor (VEGF).

Ashkan, 2018; Pantalone et al., 2021; Rajput, 2021). Already 10 years ago, in a report on the "state of the art" in space surgery, wound management and healing in space was indicated as a critical topic that needed to be studied in depth (Drudi et al., 2012). Hence, Space Agencies have begun to support studies on tissue repair and regeneration mechanisms in the space environment. The ESA-TT on "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration" and the research projects originating from it are clear examples.

Although there are no studies on WH in humans during space missions, the well-known pathophysiological changes induced by spaceflight could affect the ability of the organism to respond effectively to injuries (Kirkpatrick et al., 2009). For example, the deficient immune function (Crucian et al., 2018), chronic low-grade inflammation (LGI) and metabolic alterations (Strollo et al., 2018), changes in hemorrhage evolution (Kirkpatrick et al., 2009), and skin microbiota (Marvasi et al., 2022) could be contributing causes of healing impairment in space.

Recent studies on models simulating *μg* conditions demonstrated an elevated neutrophil-to-lymphocyte ratio (NLR), confirming previous data collected from astronauts and animal models exposed to real *μg*. Investigating the mechanisms involved, the authors found alterations in redox processes and oxidative stress responses leading to the production of pro-inflammatory mediators (Paul et al., 2020a). These changes could significantly affect the inflammatory phase of WH, compromising its normal evolution and sustaining a deleterious persistence of inflammation. Moreover, the *μg*-induced inhibition of T lymphocyte activation (Hauschild et al., 2014) and dendritic cell maturation (Monici et al., 2007) could interfere with the crosstalk between wound fibroblasts and immune system cells.

Research on the hindlimb unloaded mouse model, alone or in combination with acute simulated galactic cosmic rays or solar particle events irradiation, showed that each condition resulted in distinct circulating immune responses (Paul et al., 2020b). These results demonstrate that, in deep space conditions, immune system dysregulation could be aggravated by stressors other than unloading.

Some *in vivo* and *in vitro* studies demonstrated that μg affects platelets' number and function, thus increasing the risk of hemorrhages and contributing to delay WH (Locatelli et al., 2021). Moreover, the reduction in circulating blood volume observed in astronauts during spaceflight might impact the body's ability to withstand blood loss. Finally, changes in skin microbiota during spaceflight might affect WH by predisposing wounds to infections and through interaction with the immune system.

The relatively few studies performed in real and modeled *μg* using animal models did not provide definite results (Riley et al., 1990; Kirchen et al., 1995; Davidson et al., 1999; Campbell et al., 2005; Midura et al., 2006; Delp, 2008; Heinemeier et al., 2009), but most of them showed that the healing of wounds and fractures is delayed and impaired in weightlessness, and alterations have been documented in the three different healing phases (Delp, 2008). Studies performed on rats revealed bone healing impairment, with reduced callus formation and angiogenesis (Kirchen et al., 1995; Midura et al., 2006). Defective microvasculature was found in injured muscles, suggesting impaired neoangiogenesis and delayed muscle repair (Heinemeier et al., 2009). Reduced growth factor responses and ECM deposition in tendons and ligaments, resulting in decreased strength, have been hypothesized to jeopardize repair mechanisms in connective tissues (Provenzano et al., 2003; Martinez et al., 2007). Recent studies, carried out in simulated *μg* using an *in vivo* WH model in *Hirudo medicinalis*, showed delayed healing and alterations in the newly formed connective and epithelial tissues, which appeared less organized than *1xg* controls and showed a lower collagen fiber density. The addition of platelet-rich plasma (PRP) to the culture medium during exposure of injured leeches to *μg* prevented, at least in part, both healing delay and alterations in tissue structure. These results suggest considering PRP among the possible countermeasures for *μg*-induced WH impairment (Cialdai et al., 2020).

Although *in vitro* experiments specifically focused on WH in weightlessness are relatively few, the literature offers several studies concerning the effects of *μg* on the behavior of cell types that, together with fibroblasts, play key roles in WH. About immune cells, it is well known that lymphocyte activation is impaired in *μg*, whereas granulocytes seem to be

overactivated, although their behavior in weightlessness has been little investigated (ElGindi et al., 2021). Studies concerning macrophages, recently reviewed by Ludtka et al. (2021), showed *μg*-induced alterations in cell metabolism, signal transduction, proliferation, cytokine secretion, differentiation, cytoskeletal structure and morphology, migration, gene expression, and inflammatory response (Thiel et al., 2019; Vogel et al., 2019; Shi et al., 2021). Furthermore, endothelial cells, responsible for angiogenesis, are very sensitive to mechanical and gravitational stresses. It has been demonstrated that, when exposed to *μg*, endothelial cells show cytoskeleton remodeling, mitophagy, changes in proliferation, apoptosis, adhesion molecules, migration in response to chemoattractants, production of ECM components, and vasoactive and inflammatory mediators (Morbidelli et al., 2005; Maier et al., 2015; Kruger et al., 2019; Locatelli et al., 2020). Research on keratinocytes demonstrated that exposure to *μg* initially induces changes in gene expression profile, rearrangement of cytoskeleton, and cell–cell and cell–matrix interactions, leading to enhanced epithelial–mesenchymal transition. Later, these changes are reversed and followed by adaptive modifications through which the cells miss the acquired mesenchymal phenotype (Clement et al., 2008; Ranieri et al., 2017; Ricci et al., 2021). Recent studies focused on mechanisms involved in WH have shown that fibroblast behavior is strongly affected by *μg*. The results of these studies will be described in detail in a dedicated paragraph.

In summary, *in vitro* studies show that lymphocytes, granulocytes, macrophages, fibroblasts, endothelial cells, and keratinocytes are sensitive to *μg*. It induces significant alterations in the cell functions involved in WH, such as migration, proliferation, differentiation, apoptosis, production of cytokines, growth factors, and ECM molecules. In agreement with what has been observed *in vitro*, research on animal models shows delayed healing, angiogenesis impairment, ECM disorganization, and, consequently, morphological alterations in tissues. These results and the possibility that pathophysiological changes induced in the human organism by spaceflight (in particular, immune dysfunction, increased low-grade inflammation, and metabolic alterations) interfere with repair mechanisms suggest that WH could be delayed and impaired in space. Therefore, studies on WH in spaceflight conditions and the development of strategies to manage wounds and burns on board spacecraft or space stations are needed in view of future interplanetary missions.

Alteration of fibroblast function in space and possible consequences on wound healing

Literature offers a still limited number of studies on the behavior of fibroblasts in *μg*, mostly published in the last 2 decades (Table 2). The early studies aimed to understand whether fibroblasts, the most representative cells of connective tissue proper, were sensitive to *μg* conditions, as had already been ascertained for bone and immune system cells, belonging to specialized connective tissues. Since it was an almost entirely unknown field, the objective of these early studies was to obtain a general overview of the alterations fibroblasts underwent in *μg*.

Mainly based on the analysis of genomic profiling, these studies provided a broad picture of *μg*-induced changes affecting multiple aspects of fibroblast behavior, such as growth, signaling, adhesion, transcription, and apoptosis. In detail, a study published by Arase et al. (2002) examined the effect of a 24 h exposure to simulated *μg* (3D-clinorotation) on the gene expression level in human fibroblasts. Among the 588 genes examined, the most significant results were as follows: 1) upregulation of *XRCC1,* a repair-related gene that the authors hypothesized to be involved in the response to *μg*; 2) downregulation of *ERB-B2*, a proto-oncogene not directly involved in gravity-dependent signaling cascade but associated with integrins, which have been shown to be important in the transduction of mechanical stress; and 3) reduced expression of p21, a strong inhibitor of the cell cycle. In the same year, other authors identified 10 genes whose expression levels were altered in human WI-38 fibroblasts after 5 days aboard the Space Shuttle. These genes belong to the TNF or IL-related gene families and are thought to be involved in either the regulation of bone density, connected with the development of spaceflight osteopenia, or the development of the pro-inflammatory status (Semov et al., 2002).

In a subsequent Space Shuttle mission, a similar experiment was carried out by Liu and Wang (2008). In human WI-38 fibroblasts analyzed after 5 days of spaceflight, they found an increase in the expression of transcripts encoding oxidative stress response and DNA repair pathways, which could impact cell apoptosis and senescence. The downregulation of genes involved in energy metabolism was also observed. In contrast, Zhang et al. (2016) reported that exposure to the space environment for 3 or 14 days on board the ISS induced minimal changes in the gene and miRNA expression profile of human fibroblasts (AG1522). It is important to point out that, in this experiment, confluent fibroblast cultures were used. In fact, the purpose of the study was to investigate if and how non-proliferating cells sense *μg*, in contrast to previous studies that focused on the effect of *μg* on cell growth and differentiation. Although minimal, the changes were dependent on the number of days of exposure to *μg* and culture conditions. Although the activation of growth-related pathways and downregulation of the Let-7 miRNA family (developmental regulator) were found after 3 days (slowly proliferating cells), after 14 days (not proliferating cells), the gene and miRNA expression profiles of ground controls and flight samples were indistinguishable.

Li et al. (2015) studied the effect of simulated *μg* (3D-clinostat) on DNA damage using mouse embryonic fibroblasts deleted for *Mdc-1*, an important component of DNA damage

TABLE 2 Summary of selected articles addressing research on fibroblast behavior under real and simulated microgravity.

Experimental model	Device and exposure duration	Findings in microgravity (μg)	References
Human fibroblasts	3-D clinostat (24 h)	Upregulation of XRCC1, TNF, ICAM-1. Downregulation of ERB-B2, p21	Arase et al. (2002)
Human fibroblasts (WI38)	Space Shuttle during the STS-93 mission (4 d and 23 h)	Upregulation of genes from TNF superfamily, TNF-inducible genes, TNF-α, IL-1 receptor antagonist and downregulation of IL-15 receptor α chain	Semov et al. (2002)
Human fibroblasts (WI38) quiescent cells	Space Shuttle during the STS-93 mission (5d)	Changes in gene expression associated with oxidative stress and DNA repair pathways. Downregulation of genes involved in energy metabolism	Liu and Wang (2008)
Normal human foreskin fibroblasts (AG1522) confluent culture	International Space Station (3d and 14d)	At 3d (slowly proliferating cells): activation of growth-related pathways and down-regulation of the Let-7 miRNA family At 14d (not proliferating cells): minimal changes in gene and miRNA expression profile	Zhang et al. (2016)
Mouse embryonic fibroblasts (MEF) and Mdc-1-deleted MEF	RCCS (from 1d to 5d)	Increased SMG-induced DNA double strand breaks (DSBs) in Mdc-1-deleted cells but not in wild type cells. Partial adaptation (reduction of DNA damage) at 5d. ROS only partially responsible for SMG-induced DNA damage	Li et al. (2015)
Limbal fibroblasts (LFs)	RCCS (3d)	Lower proliferation rates and higher proportion of cells expressing CD90, CD105 and SSEEA44	Pao et al. (2017)
Human dermal fibroblasts	Space lab D2-mission 1993 (4,7,10 and 20 h)	Increased collagen synthesis. No effect on relative proportions of synthesized collagens I, III, and V	Seitzer et al. (1995)
Juvenile normal human dermal fibroblasts (NHDF)	RPM (3 d)	Changes in cytoskeleton organization and focal adhesion molecules. Presence of two phenotypes (part of the cells grew as 3D aggregates (spheroids), while the remaining part continued to grow as a monolayer adhering to the plate. Overexpression and intracellular increase in collagen type IV, in parallel with a decrease in collagen type I. Increase in MMP-1 and MMP-3 expression	Buken et al. (2019)
Cardiac fibroblasts (CF) derived from porcine hearts	RPM (24 h)	Increased apoptosis. Increased synthesis of ECM proteins. VEGF and bFGF can revert these effects	Ulbrich et al. (2010)
Rat dermal fibroblasts	RPM (4h or 24h)	Decreased adhesion. Reduced expression of proteins involved in cell-surface interaction	Loesberg et al. (2007)
Fetal fibroblasts	RPM (3d)	Decreased collagen I expression. Increased expression of fibronectin, actin and membrane integrins, reorganization of actin filaments, redistribution of membrane integrins and dysregulation in cell adhesion properties	Monici et al. (2011)
Fisher 344 rats	Space Shuttle Endeavour (STS-57) (9d)	Decrease in collagen amount. Delayed wound healing	Davidson et al. (1999)
NIH-3T3 murine fibroblasts	RCCS (3d) w or w/o Platelet Rich Plasma	Rearrangement of cytoskeleton, impaired adhesion ability and inhibition of migration. Decreased expression of alpha-SMA and E-CAD PRP is effective in partly restoring fibroblast chemokinetic properties compromised by SMG	Cialdai et al. (2017)
NIH-3T3 murine fibroblasts and leech model	RPM w or w/o Platelet Rich Plasma [fibroblasts (3 d); leech (6 h, 2 d, 4 d)]	Fibroblasts: decrease in migrating ability and alpha-SMA expression Leech: delayed healing and structural alterations in the repair tissue. PRP partially counteract these effects both in vitro and in vivo models	Cialdai et al. (2020)
Human dermal fibroblasts	RPM (24h)	Increased apoptosis and reduced proliferation. Oxidative damage. Compromised migrating properties. Downregulation of fascin, α-SMA, cofilin actin and vinculin. Impaired fibroblast-keratinocyte cross-talk	Fedeli et al. (2022)
Human dermal fibroblasts cultured within collagen matrix	RPM (3d)	Decrease in alpha-SMA and Smad 2/3 expression. Reduction of matrix remodeling	Sapudom et al. (2021a)
Human fibroblasts (1BR-hTERT cells)	3D-clinostat (24h) + radiation (heavy-ion beam and x-ray)	Increased number of chromosome aberrations (CA) respect to radiation alone	Hada et al. (2018)
Human fibroblasts (1BR-hTERT cells)	3D-clinostat (24h) + radiation (heavy-ion beam and x-ray)	Downregulation of cell cycle-suppressing genes, such as p21, and upregulation of genes promoting cell cycle progression	Ikeda et al. (2019)
STO mouse fetal skin fibroblasts		Decrease in apoptosis (lower level of caspase-3 activity)	Beck et al. (2012)

(Continued on following page)

TABLE 2 (*Continued*) Summary of selected articles addressing research on fibroblast behavior under real and simulated microgravity.

Experimental model	Device and exposure duration	Findings in microgravity (μg)	References
	Irradiation with increasing doses of X-ray then RPM (24h)		
STO mouse fetal skin fibroblasts	RPM (65h) or ionizing radiation (55mSv) (65h) or RPM + ionizing radiation	Microgravity induced upregulation of oxidative stress responsive genes, such as targets of the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). Radiation decreased expression of genes involved in cytoskeleton remodeling. The expression of these genes changed in the combined treatment, indicating that the interaction between effects induced by *μg* and radiation is complex to be understood	Beck et al. (2014)

α-SMA, alpha-smooth muscle actin; bFGF, basic fibroblast growth factor; CF, cardiac fibroblasts; CA, chromosome aberrations; CD105, cluster differentiation 105; CD90, cluster differentiation 90; p21, cyclin-dependent kinase inhibitor 1; DSBs, double strand breaks; E-CAD, E-cadherin; ERB-B2, Erb-B2 Receptor Tyrosine Kinase 2; ECM, extracellular matrix; ICAM-1, intercellular adhesion molecule 1; IL-1, interleukin-1; IL-15, interleukin-15; Let-7 miRNA, Let-7 microRNA; LFs, limbal fibroblasts; MMP-1, matrix metalloproteinase 1; MMP-3, matrix metalloproteinase 3; Mdc-1, mediator of DNA, damage checkpoint 1; MEF, mouse embryonic fibroblasts; NIH, national institute health; NHDF, normal human dermal fibroblast; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; RPM, random positioning machine; ROS, reactive oxygen species; RCCS, rotating cell culture system; SIM, sandos inbred mice; STO, thioguanine and ouabain-resistant; SMG, simulated microgravity; STS-93, Space Transportation System 93; SSEA4, stage-specific embryonic antigen-4; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; XRCC1, X-ray repair cross-complementing protein 1.

response, and wild-type cells. They found that 24 h exposure to *μg* induced significant ROS production and DNA double-strand breaks (DSBs) in Mdc-1-deleted cells but not in wild-type cells. As exposure increased, ROS levels returned to control and DNA lesions gradually decreased, though not to control levels, indicating a partial adaptation of fibroblasts to *μg*. The authors concluded that *μg* increased genomic DNA instability in DNA damage response-deficient cells and that ROS were only partially responsible for *μg*-induced DNA lesions.

More recent research studied the effects induced by simulated *μg* (RCCS) in limbal fibroblasts (Pao et al., 2017). These cells possess mesenchymal stem cell characteristics and multilineage differentiation potential (Katikireddy et al., 2014). After 3 days of exposure to simulated *μg* (RCCS), limbal fibroblasts showed lower proliferation rates and higher proportions of cells expressing MSC markers as CD90, CD105, and SSEEA44. Hence, the authors inferred that the differentiation potential of limbal fibroblasts was enhanced by simulated *μg*, opening interesting perspectives for reconstructive therapy in patients with corneal disease.

These studies on the behavior of fibroblasts in μg, despite being conducted with different types of fibroblasts and in different experimental conditions (true or simulated μg, proliferating or non-proliferating cells), showed alterations concerning signaling pathways related to inflammation, repair, metabolism regulation, and oxidative stress response.

As extensively described in the previous part of this review, fibroblasts play a key role in tissue homeostasis because they can act as transducers of biophysical and biochemical cues. They actively participate in WH and orchestrate all the phases of tissue repair/regeneration through the crosstalk with other cell populations involved in the process. Therefore, some studies have been devoted to understanding whether and how *μg* affects fibroblast functions related to WH.

Seitzer et al. (1995) were the first to evaluate collagen biosynthesis by human dermal fibroblasts exposed to unloading and loading conditions. In detail, qualitative and quantitative data on collagen synthesis under altered gravity conditions were investigated by incubating fibroblasts with [^{3}H]-proline during space lab D2-mission in 1993. The authors found that *μg* induced an increase in collagen synthesis compared to *1xg* conditions. On the contrary, hypergravity, obtained by means of a hyperfuge, induced a decrease in collagen synthesis, which depended on the *g* value. The relative proportions of synthesized collagens I, III, and V seemed to remain unaffected under any applied conditions.

In a more recent study, overexpression and intracellular increase in collagen type IV, in parallel with a decrease in collagen type I, was observed in juvenile normal dermal fibroblasts cultured for 72 h in simulated *μg* (RPM). A significant increase in MMP-1 and MMP-3 expression was also found. In addition, changes in cytoskeleton organization, focal adhesion molecules, and growth behavior were found. Interestingly, in RPM-exposed fibroblasts, two phenotypes were observed. Part of the cells grew as 3D aggregates (spheroids). In contrast, the remaining part continued to grow as a monolayer adhering to the plate (Buken et al., 2019), suggesting the presence of two different fibroblast subpopulations. The formation of multicellular spheroids in porcine cardiac fibroblast cultures exposed for 24 h to simulated *μg* (RPM) had previously been reported by Ulbrich et al. (2010). They also observed a significant increase in apoptosis, which reverted by adding VEGF and bFGF.

In a study aimed to understand whether cell morphology, orientation, and adhesion properties are more dependent on *μg* or substrate micro-topographical features, rat dermal fibroblasts cultured in simulated *μg* (RPM) on smooth and microgrooved surfaces, respectively, were compared. The authors found that

fibroblasts adjusted their shape and orientation according to micro-topographical features, even if simulated *μg* decreased cell adhesion, as proved by the reduced expression of proteins involved in cell-surface interaction (Loesberg et al., 2007). Further confirmation that *μg* significantly affects the production of ECM molecules and cell-surface interaction was obtained in a study on fibroblasts and endothelial cells, protagonists of tissue remodeling and angiogenesis, respectively. After 72 h exposure to simulated *μg* (RPM), a significant decrease in collagen I expression, coupled with increased fibronectin expression, was found in both cell types. In correlation with the increased fibronectin fibrillogenesis, increased expression of actin and membrane integrins, reorganization of actin filaments, redistribution of membrane integrins, and dysregulation in cell adhesion properties were also observed. Interestingly, this study showed that near-infrared (NIR) emission of a Nd:YAG laser source (high power laser, 1,064 nm wavelength) is able to modulate the formation of ordered arrays of fibronectin fibrils and favor endothelial cell monolayer formation. Therefore, it could be investigated as a countermeasure against *μg*-induced effects on tissue remodeling (Monici et al., 2011).

A significant reduction in collagen amount was also demonstrated in an *in vivo* experiment aimed at investigating, for the first time, cutaneous WH in *μg*. In that study, sponge discs were subcutaneously implanted in the ventral panniculus carnosus of Fisher 344 rats sent in the orbiting Space Shuttle for 10 days. Sponges contained growth factors known to accelerate granulation tissue formation. As mentioned above, the authors observed that the spaceflight environment significantly decreased collagen concentration at the injury site compared to ground controls, regardless of the presence or absence of growth factors. Therefore, the authors hypothesized delayed WH in rats exposed to *μg*, although in this experiment, they could not distinguish the effects of *μg* from those of other space stressors (Davidson et al., 1999).

In a study concerning the effect of *μg* on fibroblast functions involved in WH, NIH-3T3 fibroblasts exposed for 72 h to simulated *μg* (RCCS) showed an important rearrangement of cytoskeleton together with an impaired adhesion ability and inhibition of migration in response to chemoattractants. Consistent with alterations in cytoskeleton and adhesion/migration, a decrease in E-CAD and α-SMA expression was found. As α-SMA is the major marker of fibroblast-myofibroblast transdifferentiation, its downregulation suggests that *μg* could affect the differentiation toward the myofibroblastic phenotype, which is a key event in the healing process. Moreover, a decrease in VEGF expression, which could compromise the crosstalk between fibroblasts and endothelial cells, and an increase in COX-2 expression, indicative of induction of an inflammatory phenotype, were also reported. In the same study, PRP, known to promote WH on Earth, proved to be effective in partly restoring fibroblast chemokinetic properties compromised by exposure to modeled *μg*. This finding strengthens the possible use of PRP as a countermeasure to promote WH on Earth and in *μg* conditions (Cialdai et al., 2017).

PRP effectiveness as a countermeasure was the subject of a second study carried out by the same group. *In vitro* and *in vivo* WH models, developed by performing the scratch assay in NIH-3T3 fibroblasts and sutured wounds in the leech, respectively, were exposed to simulated *μg* (RPM). Consistent with the results of the previous study, the scratch assay showed a significant decrease in migrating ability and α-SMA expression in *μg*-exposed fibroblasts compared to *1xg* controls. In the animal model, delayed healing and structural alterations of the repair tissue were observed. The effectiveness of PRP in partially counteracting the alterations induced by *μg* was confirmed in both models. In the scratch assay, it partially restored the ability of fibroblasts to migrate, whereas in the *in vivo* model, it promoted and speeded up wound closure (Cialdai et al., 2020).

A very recent study, carried out with dermal fibroblasts exposed for 72 h to simulated *μg* (RPM), showed a similar panel of results: simulated *μg* induced downregulation of molecular markers involved in WH, such as α-SMA, and alteration of cytoskeleton components, such as the modification of the actin-vinculin apparatus, compromising cell contractility and mechanotransduction, and leading to inhibition of fibroblast-myofibroblast transdifferentiation, with consequent alteration of the fibroblast-keratinocytes crosstalk. Moreover, the authors found signs of oxidative damage and advanced the hypothesis that the *μg*–induced oxidative stress may trigger the mechanisms producing the observed phenotypic changes (Fedeli et al., 2022). Fibroblast differentiation in *μg* was also investigated in the study of Sapudom et al. (2021a) using collagen-based 3D models. After 72 h of exposure to simulated *μg* (RPM), the authors found a significant reduction in α-SMA and Smad 2/3 expression and reduced matrix remodeling, evaluated through the expression of collagen and other ECM components. Once more, these results confirm impairment in fibroblasts-myofibroblast transdifferentiation and altered ECM production in *μg*, in agreement with previous studies.

Although the literature on the behavior of fibroblasts in *μg* conditions is still relatively small and studies have been carried out using different models and exposure conditions, the general overview indicates that unloading conditions strongly affect fibroblast functions, thus jeopardizing the evolution of the WH process. In particular, the results of the most recent studies are very consistent and demonstrate alterations in cytoskeleton and membrane proteins mediating the interaction with the ECM, decreased migration ability, impaired fibroblast-myofibroblast transdifferentiation, alterations in the production of ECM components, signs of oxidative stress, and increase in the levels of inflammation markers (Figure 2). Interestingly, some of these alterations resemble those observed on Earth in the fibroblast dysfunction that characterizes healing impairment. Further

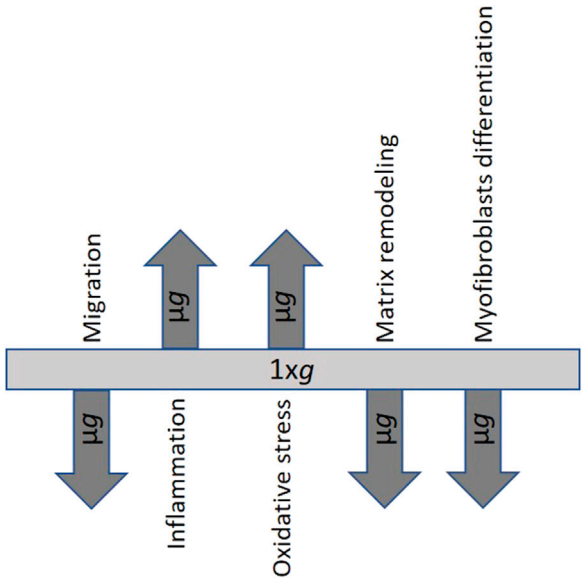

FIGURE 2
Processes and aspects of fibroblast behavior affected by microgravity. The arrows pointing up and down indicate an increase or decrease following microgravity exposure. Inflammation is intended as an increase in inflammatory markers produced by fibroblasts; oxidative stress is intended as an increase in reactive oxygen species and oxidative processes in fibroblasts.

studies can help better understand the mechanisms underlying the morphological and functional changes observed in fibroblasts exposed to *μg* conditions. Knowing how and to what extent *μg*-induced fibroblast dysfunction affects tissue healing and regeneration can lay the groundwork for the development of new strategies for counteracting delayed or dysregulated WH in both weightlessness and normal gravity conditions (*1xg*).

Over the years, some studies investigated the combined effect of *μg* and ionizing radiation to better simulate spaceflight conditions in future exploration missions beyond the LEO. In particular, studies were intended to understand whether *μg*, which affects important biological processes, can also modify cell sensitivity to radiation and subsequent repair of radiation damage. By using a 3D clinostat synchronized to a carbon-ion or X-ray irradiation system, research carried out on human fibroblasts simultaneously exposed to simulated *μg* and radiation [heavy-ion beam (0.5 Gy) and X-ray (0.5 and 1.5 Gy)] investigated chromosome aberrations (CA) as a biomarker of cancer risk associated with radiation exposure. In fibroblasts exposed for 24 h to simulated *μg* and radiation, the number of CA was higher than that in cells exposed to radiation alone. The authors inferred that simulated *μg* increased cell sensitivity to radiation and/or reduced cells' ability to repair radiation damage (Hada et al., 2018). Another study carried out using the same cell model and experimental setup demonstrated that the combined effect of simulated *μg* and radiations (1 Gy of dose both for X-rays and C-ions) induced downregulation of cell cycle-suppressing genes, such as p21, and upregulation of genes promoting cell cycle progression, thereby increasing the risk for events of genomic instability (Ikeda et al., 2019). In addition, Beck et al. (2012) reported that X-rays irradiation (from 0.5 to 4 Gy) followed by 24 h RPM exposure induced in mouse fetal fibroblasts a decrease in apoptosis compared to *1xg* control. This effect could lead to an accumulation of cells with DNA damage and/or mutations.

In a later study, the same authors exposed fetal skin mouse fibroblasts to simulated *μg* (RPM) for 65 h, in combination or not with a low dose of ionizing radiation (55 mSv). The two sample groups were compared to irradiated/non-irradiated *1xg* controls. The authors found that simulated *μg* induced oxidative stress-responsive genes, such as targets of the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), which is supposed to play a role in cell response to *μg*. Furthermore, the decreased expression of genes involved in cytoskeleton remodeling was observed, possibly mediated by Rho signaling pathway. Radiation alone decreased the expression of genes involved in cytoskeleton remodeling in cell cycle regulation and DNA damage response pathways. Interestingly, *μg* showed a dominant impact on single gene expression, whereas ionizing radiation had a dominant effect on gene sets. Moreover, some genes showing altered expression when exposed to the single treatments (simulated *μg* or irradiation) were not altered in the combined treatment

TABLE 3 Differences between wound scarring and tissue regeneration.

	Wound scarring	Regeneration
Inflammatory infiltrate	↑	↓
CXCL cytokine level	↑	↓
TGF-β1 level	↑	↓
TGF-β1/ TGF-β3 ratio	↑	↓
Collagen III/Collagen I ratio	↓	↑
MMP catabolizing collagen level	↓	↑
Cell migration	↓	↑
ECM remodeling	↓	↑
Myofibroblasts	↑	↓

↑ higher; ↓lower; C-X-C motif ligand (CXCL); tumor growth factor beta 1 (TGF-β1); tumor growth factor beta 3 (TGF-β3); matrix metalloproteinase (MMP); extracellular matrix (ECM).

(Beck et al., 2014), indicating that the interaction between the effects induced by *μg* and those induced by ionizing radiation is complex and quite completely unknown.

Although many more studies are needed to shed light on the effects that simultaneous exposure to *μg* and radiation can induce in fibroblasts, the above-mentioned results suggest that the combined effects could further affect fibroblast function, whereas *μg* appears to weaken the response to radiation damage. This result deserves attention because the radiation doses to which crews will be exposed in future missions beyond LEO will be significantly higher.

Gaps to be filled in research on fibroblast function and wound healing

Although much is known about the role of fibroblast in WH, there are still unanswered questions. The large number of patients suffering from chronic ulcers and fibrosis irrefutably attests that there are gaps in knowledge to be filled in order to implement effective strategies in countering WH impairment.

In comparison to scar models, scar-free WH and skin regeneration models, such as WH in embryos, healing of some wounds in adult oral mucosa, and *Acomys* (the African spiny mouse which can perfectly regenerate skin in wounds as large as 60% of its body surface) show less inflammatory infiltrate, lower CXCL cytokines levels, lower TGF-β1 and TGF-β1/TGF-β3 ratio, higher collagen type III/type I ratio, and higher production of MMPs catabolizing collagen, thus enhancing cell migration and speeding up ECM remodeling, as well as low or absent α-SMA expressing myofibroblasts, which are implicated in scarring (Table 3). The origin of these different characteristics has not yet been understood. However, based on recent research, it has been hypothesized that modifying the behavior of adult fibroblasts and bringing it closer to that of the embryonic ones could be a keystone for tissue regeneration (Thulabandu et al., 2017).

Adult human wounds show a spectrum of healing outcomes ranging from nearly scarless healing (close to perfect tissue regeneration) to scarring (including keloids and fibrosis) and delayed/non-healing wounds (including chronic ulcers). Studies on the gene expression profile of fibroblasts derived from these different healing conditions aim to find the corresponding "signatures" and, in particular, identify a signature for enhanced, nearly scarless healing (Peake et al., 2014).

Indeed, for a long time, fibroblasts have been considered a single and fully differentiated cell type. More recent studies demonstrated that fibroblasts are a collection of heterogeneous subpopulations with great morphofunctional plasticity, which can play distinct roles in WH, producing different outcomes of the repair process (Peake et al., 2014; Zou et al., 2021). To date, it is not yet possible to unambiguously identify the different subpopulations of fibroblasts through a combination of surface markers (Jiang and Rinkevich, 2018), and further research is needed to understand the function and behavior of the different subpopulations and distinguish one from the other. It is a common opinion that filling the gaps in knowledge on the specific functions of the various fibroblast subpopulations and how they determine scar quality could allow more effective therapeutic strategies to be developed to prevent scarring and promote tissue regeneration (Jiang and Rinkevich 2020).

Interestingly, a lineage relationship between fibroblasts and adipocytes has been demonstrated during WH. It has been observed that the phenotypic plasticity of fibroblasts toward adipocytes is associated with lower fibrosis (Plikus et al., 2017). Furthermore, in the lung, the reversible myofibroblast-adipocyte transition seems to be associated with reduced fibrosis, whereas the transition of fibroblasts toward a myogenic and contractile phenotype increases fibrosis (El Agha et al., 2017). In the above-mentioned studies, carried out *in vitro* and in animal models, the authors found that the conversion of myofibroblasts to adipocytes (a completely different lineage) is BMP and PPARγ dependent in WH (Plikus et al., 2017) and lung fibrosis (El Agha et al., 2017), respectively. These results pave the way for treatments that, by modulating the plasticity of fibroblasts, direct the healing process toward regeneration rather than scarring.

More than 20 years ago, fibrocytes were described and a leukocyte-fibroblast transition was hypothesized (Bucala et al., 1994). More recent studies indicated that more than half of myofibroblasts in healing wounds derive from myeloid cells and the hypothesis has been advanced of a macrophage-fibroblast transition mediated by extracellular vesicles (EV) released by keratinocytes (Sinha et al., 2018; Guerrero-Juarez et al., 2019). Further research is needed to understand the roots of fibroblast plasticity, if and how it depends on the wound microenvironment, and to what extent it can dictate or shape healing outcomes.

By considering fibroblast plasticity and the variety of subpopulations, an important issue is the crosstalk with the other cell types: it is well known that, in the different healing phases, fibroblasts engage in crosstalk with immune cells, especially macrophages, endothelial cells, and keratinocytes, but it is less clear if, when, and how each different fibroblast subpopulation interacts with the other cell types.

By coinciding with their intense activity at the wound site, fibroblasts undergo transdifferentiation into highly contractile myofibroblasts by developing muscle-like features, including the formation of contractile actin-myosin bundles. It has been widely demonstrated that the phenotype and function of fibroblasts and myofibroblasts are mechanically regulated by matrix stiffness, using a feedback control system integrated with the progress of tissue remodeling (Hinz et al., 2019). However, the interplay between mechanical and biochemical factors in regulating fibroblast subpopulations and its consequences on the healing outcomes are far from clear.

The fibroblast-myofibroblast transdifferentiation is a reversible process controlled by tensile forces (Kollmannsberger et al., 2018), which can sustain the myofibroblast phenotype also in the absence of TGFβ (Jiang and Rinkevich, 2020). The response of fibroblasts to mechanical stress, including that induced by changes in cell–cell and cell–ECM adhesions, involves the Hippo signaling pathway and the nuclear translocation of the Yes-associated protein (YAP) (Dupont et al., 2011). Considerable progress has been made in understanding the molecular mechanisms mediating the fibroblast-myofibroblast transition induced by mechanical stress. However, many aspects remain to be clarified. It would be useful to understand if the various fibroblast subpopulations respond to mechanical stress differently and know if, how, and to what extent the mechanical forces shape fibroblast plasticity.

Studies on 3D fibroblast cultures have shown that wound contraction and closure are driven by fibroblast migration controlled by mechanical forces at the wound edge rather than fibroblast proliferation (Sakar et al., 2016). Both cell–ECM and cell–cell interactions are needed for wound contraction and closure. It has been demonstrated that the actomyosin contraction mechanism and cell–ECM adhesion receptors are crucial for fibroblast mechanosensing and converting mechanical signals of the microenvironment into biological signals (Hinz et al., 2019), which in turn can regulate the fibrotic response. The activation of focal adhesion kinase (FAK) in fibroblasts results in increased scar formation (Wong et al., 2011b), whereas tensile stress reduction results in reduced scarring (Sakar et al., 2016). Furthermore, integrin complexes at cell–ECM focal adhesion sites activate the Hippo pathway and downstream YAP/TAZ, reshaping the actomyosin cytoskeleton as a consequence of changes in ECM (Kim and Gumbiner, 2015). Persistent nuclear expression of YAP/TAZ in fibroblasts supports their mechanoactivation, creating a vicious cycle that increases fibrosis (Liu et al., 2015).

In the wound, tensile forces occurring at fibroblast-fibroblast adhesion sites are as important as those at fibroblast-ECM adhesion sites in modulating wound contraction and scar development. Cadherins, which form the adherens junctions, are integral membrane proteins mediating calcium-dependent cell–cell adhesion (Alimperti and Andreadis, 2015). It has been observed that the expression of cadherin-11 (CDH-11) is higher than normal in the skin of patients with systemic sclerosis or scleroderma, which are autoimmune diseases clinically manifesting as progressive fibrosis of the skin and internal organs (Wu et al., 2014). The growing evidence of the importance of tension forces at the levels of cell–cell and cell–ECM adhesions has suggested various hypotheses on wound contraction mechanisms, and various models of wound contraction have been proposed recently. However, the interplay between tension forces at cell–cell and cell–ECM adhesion sites and their different roles in regulating wound contraction and the development of scars and fibrosis is far from clear (Jiang and Rinkevich 2020).

As *μg* induces alterations in fibroblast function that resemble those observed on Earth in WH dysfunctions, studies in the space environment could represent an interesting model for gaining insights into the molecular and cellular mechanisms leading to healing impairment. Moreover, the space environment (*μg* conditions) offers a unique opportunity to investigate the role of gravity and mechanical forces in WH and mechanotransduction mechanisms in fibroblasts.

While, on ground, the role of fibroblasts in WH has been extensively studied, and the knowledge gaps are quite well defined, in *μg*, the knowledge gaps overwhelm the limited information deriving from the few studies conducted so far. However, this information allows researchers to identify some "main topics" that will certainly need to be studied in depth.

Fibroblast migration

In vitro studies report that *μg* inhibits the ability of fibroblasts to migrate, and this alteration seems to be related to cytoskeletal changes (Cialdai et al., 2017). It has also been reported that *μg* affects the production of ECM components and MMPs (Monici et al., 2011; Buken et al., 2019; Sapudom et al., 2021a). Changes in ECM properties can, in turn, interfere with the migration of fibroblasts and other cell populations involved in WH, such as immune cells, endothelial cells, and keratinocytes. In addition, the production of chemoattractants and the response of fibroblasts to them could change. Therefore, the regulation of fibroblast migration in *μg* needs to be studied in depth.

Fibroblast regulation by biochemical factors

Fibroblast functions involved in WH are regulated by biochemical factors. The studies conducted so far show that,

in *μg*, the levels of growth factors and cytokines produced not only by fibroblasts but also by other cells, such as those of the immune system, can change. Furthermore, in *μg*, fibroblasts seem to respond to biochemical stimuli differently than on Earth (Cialdai et al., 2020). Therefore, the mechanisms of biochemical regulation of fibroblast functions in conditions of altered gravity must be elucidated.

Fibroblast-ECM dynamics and mechanical cues

The dynamic interaction between fibroblasts and ECM in conditions of altered gravity is quite completely unknown. In *μg*, the levels of ECM molecules and MMPs produced by fibroblasts change, and ECM properties (relative percentage of components, content of fluids and biochemical factors, mechanical properties, topographical features, *etc*.) are expected to change accordingly. While fibroblasts undergo cytoskeleton reorganization and redistribution of membrane integrins, focal adhesions and the mechanical cues transmitted by ECM to fibroblasts change as well, modulating cell activity. Therefore, the fibroblast-ECM interaction in *μg* might differ significantly from what has been observed on Earth and needs to be investigated. In particular, altered fibroblast-ECM dynamics might strongly affect wound contraction and the remodeling phase of WH.

Fibroblast crosstalk with other cell populations

It is well known that the crosstalk between fibroblasts and the other cell types involved in WH is extremely important for the proper evolution of the process. Through the production of biochemical factors, cell–cell interactions, and the exchange of materials (e.g., EV), the various cell populations regulate each other and perform their functions concertedly. Particularly important are the interactions of fibroblasts with endothelial cells, keratinocytes, and macrophages, mediating neoangiogenesis, re-epithelialization, the transition from inflammation to proliferation phase, and fibroblast-myofibroblast transdifferentiation (Sapudom et al., 2021b), respectively. In *μg*, these interactions are expected to be altered due to changes in the behavior of the different cell types involved. Moreover, alterations of the microenvironment (e.g., alterations in ECM properties) might interfere with the crosstalk between fibroblasts and the other cell types, including the different immune cell populations.

Fibroblast plasticity

Last but not least, the fact that, in samples exposed to simulated *μg*, two different fibroblast phenotypes have been observed, one growing as a monolayer adhering to the plate and the other forming spheroids (Buken et al., 2019), show that fibroblast plasticity is manifested even in unloading conditions. As closing the knowledge gap on fibroblast plasticity is considered an essential aspect of progress in the development of new therapeutic strategies, this topic should be investigated even in conditions of altered gravity, and these studies might contribute to better understanding of the subtle differences among various fibroblast subpopulations both in space and on Earth.

Therapeutic strategies to promote wound healing and tissue regeneration

Delayed wound closure, chronic ulcers, and fibrotic scars are important health and social problems. The wound-care market has been estimated to be over $ 6.5 billion worldwide (Cision, 2011). Unfortunately, it is destined to grow due to the increase in chronic diseases (e.g., diabetes and obesity) and chronic wound patients.

Hence, since ancient times, remedies have been sought to promote successful healing and regeneration of functional tissues. Traditional therapies based on natural origin compounds, such as plant extracts, honey, and products of animal origin, are still used for their low cost, making them the only possible therapy in some parts of the world. Natural compounds show some therapeutic properties, including anti-inflammatory, antimicrobial, and cell-stimulating activities. However, despite advances in extraction and purification methods, the exact mechanisms of action, side effects, and safety of most of these compounds have not been sufficiently studied (Pereira et al., 2013).

Among the pharmacological therapies, antimicrobial agents, including those based on silver, prevent or decrease wound contamination. Corticosteroids and other anti-inflammatory drugs can be useful in attenuating excessive inflammatory response, but they can inhibit fibroblast activity and collagen synthesis, affecting the remodeling phase. In diabetic ulcers, in addition to antidiabetic drugs, estrogens, β-blockers, and ACE inhibitors showed some promising results, acting on microcirculation, angiogenesis, and fibroblast function. Nevertheless, the clinical application of these therapies is often problematic due to the secondary effects of drugs and because the efficacy of some of them is not adequately supported by clinical studies (Spampinato et al., 2020). The possible use of these drugs during space missions will have to be carefully evaluated, as it is known that drug pharmacokinetics and pharmacodynamics can change in the space environment.

Important advances have been made in wound dressing, with the transition from the passive role of traditional protective bandages to the active one of advanced therapeutic dressing systems, such as antimicrobial dressings, anti-inflammatory and analgesic dressings, drug delivery dressings containing

pharmacological, biological, or naturally derived agents (Boateng and Catanzano, 2015). Advanced dressings could be useful in the management of serious wounds occurring during space missions. However, the activity of the products they release (antimicrobials, anti-inflammatories, growth factors, *etc*.) must be studied in the space environment, as it could be different from what has been assessed on Earth.

Another category of products that promote WH is blood derivatives such as PRP, platelet-rich fibrin (PRF), leucocyte-rich PRP (LR-PRP), and leucocyte PRF (L-PRF). These products have anabolic effects on fibroblasts, enhance their migration and proliferation, and modulate the expression of genes encoding for ECM and adhesion molecules (Devereaux et al., 2020; Hermida-Nogueira et al., 2020; Akbarzadeh et al., 2021). PRP has already been tested both *in vitro* and in animal model experiments under simulated μg conditions. The results show that PRP enhances the activity of fibroblasts, but its effectiveness in μg is lower than in normogravity (Cialdai et al., 2020). Therefore, even in the case of blood derivatives, studies are needed to define protocols and dosages for their use in spaceflight conditions.

Physical therapies are used as a single therapy or combined with other treatments to manage wounds and promote healing. One of the most widely used is laser therapy, whose effectiveness is based on anti-inflammatory and antimicrobial actions, as well as on the enhancement of cell energy metabolism. Although there are numerous studies demonstrating the beneficial effects of this therapy, the different types of laser sources and treatment parameters used make it difficult to compare the various studies and develop standardized guidelines and protocols (Suan et al., 2014). Defining the most effective laser sources and treatment protocols could help draw guidelines and achieve unambiguous outcomes. However, a recent study showed that NIR laser radiation effectively controls fibroblast activation induced by cytokine stimulation, thus damping excessive inflammatory response (Genah et al., 2021).

Some difficulties in defining mechanisms of action and treatment parameters affect the use of electromagnetic fields (EMF), whose application in WH has been the subject of numerous studies, with sometimes conflicting results (Pesce et al., 2013; Saliev et al., 2014).

Another therapy showing some effectiveness is Negative Pressure Wound Therapy (NPWT). It can reduce infections, maintain a moist environment, regulate blood flow and exudates, and, by applying pressure, promote wound closure (Orgill and Bayer, 2013).

Hyperbaric oxygen has been applied for the alleged ability to enhance fibroblast proliferation and angiogenesis and improve immune function. However, due to limited effectiveness and the significant side effects, today, this therapy is only considered for ulcers in which a highly hypoxic environment is demonstrated (Han and Ceilley, 2017).

In a space environment, the use of physical therapies could have some advantages. Physical therapies are non-invasive, are generally well tolerated, and have fewer side effects than medications. In addition, they are easy to apply and do not require highly specialized personnel, but only some training. The application of devices for NPWT and advanced laser sources (compact and very safe sources are already marketed and widely applied in clinics) could be feasible, provided that they meet the requirements for use aboard spacecraft or space stations. Furthermore, for physical therapies, studies are needed to evaluate the mechanisms of action in μg conditions and develop adequate protocols.

More recent research on advanced therapeutic approaches has been focused on inflammatory mediators, such as TGF-β1; growth factors release in more or less sophisticated ways, from gradual release bandages and scaffolds to gene therapy; modulation of myofibroblast integrins affecting mechanosensing; and regulation of myofibroblast differentiation and epithelial-to-mesenchymal transition (Eming et al., 2007; Schnittert et al., 2018; Sawant et al., 2021).

Despite the promising results in the study phase, the therapies focused on specific targets have not proved to be effective in clinical application. This is probably because WH is a very complex process in which many cell populations and biochemical mediators play their roles according to a precise timeline and often play different roles at different stages of the process. Various tools of regenerative medicine should most likely be used to address the different stages of WH. Furthermore, there are different types of injuries (burns of various degrees, wounds of different depths, etc.) whose healing is strongly influenced by the patient's condition, particularly in the case of serious diseases, such as diabetes and obesity (Chin et al., 2019). Therefore, the lesson learned is that therapies focused on a single target (or a few targets) have limited efficacy, and, probably, a more holistic approach considering the whole healing process and patient's conditions is needed.

Skin grafting, that is, skin transplantation, presents some major concerns because it is frequently associated with donor site morbidity, pain, discomfort, and hypertrophic scarring and often requires microsurgery and long healing times (You and Han, 2014). Techniques aimed at developing increasingly advanced skin substitutes utilize hydrogels and 3D bioprinted scaffolds, which can be shaped according to the wound and can incorporate bioactive molecules, such as growth factors, as well as cells, such as fibroblasts, keratinocytes, or stem cells (Murray et al., 2019; Tottoli et al., 2020). These very advanced and promising techniques are mostly in the development phase, and there are no established clinical applications yet. Skin grafting is still the gold standard for severe burns and extensive wounds, but its application in a space environment would be difficult to achieve. The prospects for skin substitutes and regenerative medicine look promising, but these technologies are not yet mature enough. Therefore, Space Agencies started to support research programs aimed at the development of regenerative medicine technologies in a

space environment. In summary, to effectively promote healing and prevent scarring, rather than single therapies, there is a need for multifactorial therapeutic strategies (El Ayadi et al., 2020), and further research efforts are needed.

In space, PRP used on a WH model in *Hirudo medicinalis* proved less effective than on the ground (Cialdai et al., 2020). Therefore, the effectiveness of existing therapies will have to be carefully verified in weightlessness, and it will probably be necessary to develop therapeutic strategies that consider the alteration of tissue repair mechanisms induced by *μg*.

Conclusion

Fibroblasts play a crucial role in WH. After hemostasis, they are involved in all three phases of the healing process: inflammation, proliferation, and remodeling, of which they are the absolute protagonists. Fibroblasts orchestrate the whole WH process through crosstalk with other cell types; production of growth factors, chemokines, MMPs, and ECM components; and the ability to transduce mechanical cues in biological responses and transdifferentiate into myofibroblasts, which are responsible for wound contraction. Therefore, it is not surprising that fibroblast dysfunction is a typical feature of WH impairment, from chronic non-healing ulcers to fibrotic scars, and that most therapeutic strategies aim at regulating and improving fibroblast function. Unfortunately, the many gaps of knowledge that remain regarding fibroblast function in WH negatively affect the development of new and more effective therapeutic strategies. The most important gaps of knowledge concern the plasticity of the fibroblast phenotype and the different fibroblast subpopulations, understanding the roles of the various subpopulations in each WH phase, the different ways fibroblasts use to crosstalk with other cell types, the interplay between mechanical cues, including the mechanical and elastic properties of ECM, and lastly the fibroblast response to them, which can generate a self-sustaining loop.

Studies on fibroblast behavior in weightlessness, a condition typical of the space environment, demonstrated that *μg* strongly affects fibroblast functions, in particular those involved in WH, such as the production of growth factors, pro-inflammatory molecules, MMPs and ECM components, migration/adhesion properties, fibroblast-myofibroblast transdifferentiation, and the crosstalk with other cell types, such as keratinocytes and endothelial cells. These *μg*-induced alterations in fibroblast functions show an amazing similarity with fibroblast dysfunctions observed on Earth in WH disorders ranging from chronic non-healing ulcers to scarring and fibrosis. Therefore, fibroblast cultures in space could represent a useful model to study fibroblast dysfunction on Earth. Moreover, exploring the behavior of fibroblasts in weightlessness is a unique opportunity to gain insights into the regulation of fibroblast functions by mechanical forces and the role of gravity in WH. An interesting effect observed in simulated *μg* is the presence, in the same cell culture, of two phenotypes that differ in the ability to adhere in monolayer or form 3D aggregates. This suggests that fibroblast models in *μg* might help better understand the morphological and functional differences among various subpopulations of fibroblasts.

Progress in the knowledge of tissue repair mechanisms orchestrated by fibroblasts is very important for the development of more effective strategies for wound management and care. Current therapies have limited effectiveness, probably because they target only one or a few steps of the healing process and cannot act on multiple key points. The main goals of current studies are, on the one side, to develop multifactorial therapeutic strategies able to address multiple aspects of the healing process and, on the other side, to identify among fibroblast subpopulations a "signature for nearly scarless healing," which could pave the way to perfect regeneration. Research on WH and fibroblast models in space, where mechanical stimuli and probably also the ECM properties change, could make a significant contribution to the achievement of these objectives.

Author contributions

Conceptualization and methodology (MM); literature review and investigation (MM, FC and CR); writing (MM and FC); figures and tables preparation (FC and RC) and supervision (MM); writing review and editing (MM, FC and CR); supervision and funding acquisition (MM). All authors haveread and agreed to the published version of the manuscript.

Acknowledgments

This review has been written within the activities of 1) the ESA Topical Team on "Tissue Healing in Space: Techniques for Promoting and Monitoring Tissue Repair and Regeneration" and 2) the ESA-MAP Project "WHISPER-Wound Healing In Space: problems and PErspectives for tissue Regeneration and engineering" SciSpacE Microgravity Application Promotion Programme. ESA Contract Number 4000130928/20/NL/PG/pt.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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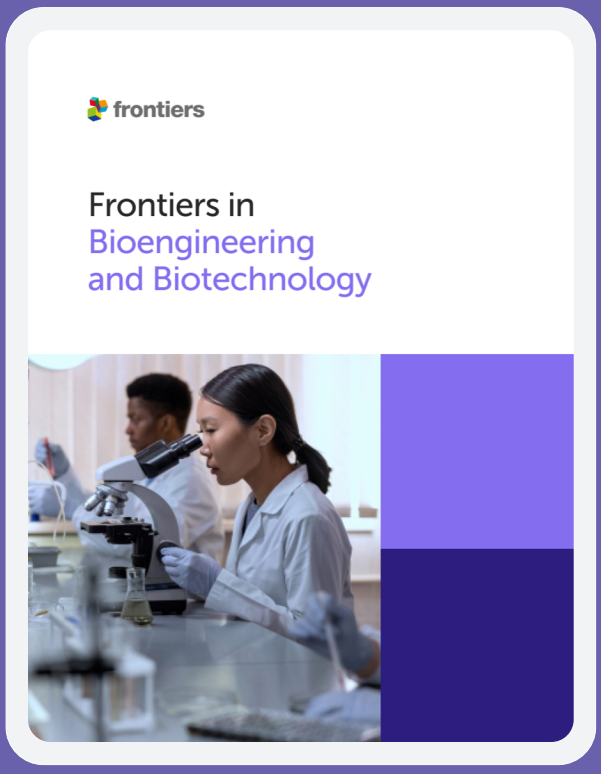

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