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Nutritional and physical activity strategies to boost immunity, antioxidant status and health, volume III

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Nutritional and physical activity strategies to boost immunity, antioxidant status and health, volume III

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Editorial: Nutritional and physical activity strategies to boost immunity, antioxidant status and health, Volume III

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KEYWORDS

exercise, nutritional supplement, skeletal muscle function, aging, overall health

Editorial on the Research Topic
Nutritional and physical activity strategies to boost immunity, antioxidant status and health, Volume III

This Research Topic comprised of research articles, reviews, and meta-analyses, emphasizes the beneficial effects of exercise or food-based interventions alone or in combination on skeletal muscle function, antioxidant status and wellbeing in both humans and animal models. Intake of various plant-based supplements from fruit, root or leaves has been shown to promote overall health against various diseases. For instance, supplementation of *Spondias mombin* leaves extract for 28 days exhibits antidiabetic property through decreased blood glucose and restored insulin levels in diabetic rats. This hypoglycemic effect was further accompanied by alleviation of dyslipidemia and improvement of liver function. The phytochemical in *Spondias mombin* leaves, namely flavonoids, anthraquinone, tannins, saponins, steroids, phenols and alkaloids may be contributed to these beneficial effects Gobinath et al. Another laboratory study stated that treatment of *Solanum torvum* fruit extracts to diabetic rats considerably reversed the hyperglycemia and dyslipidemia. Besides, this fruit extract decreased the elevated liver transaminases, and increased the number and size of pancreatic β-cells against streptozotocin-induced destruction Satyanarayana et al. These reports suggest that supplementation of plant-based extracts can effectively attenuate the diabetes-induced complications, and thereby protect the tissues.

Sarcopenia, loss of muscle mass and strength due to natural aging is an inevitable phenomenon leading to decline the quality of life among older people. Therefore, it is necessary to find out the alternative strategies to maintain the physical fitness and improve overall health of aged population. One of the best ways to delay the sarcopenia or improve fitness is regular exercise with or without proper dietary intake. In an aging study on rats, Su et al., demonstrated that age-induced progressive loss of skeletal muscle mass index and muscle fiber cross section area (CSA) was reversed by 32-week high-intensity interval training (HIIT) and resistance training (RT). The reactive oxygen species (ROS), which play a critical role in propagation of age-induced apoptosis, were found to be lower in the soleus of

aging rats after HIIT. Besides, both HIIT and RT modalities attenuated the age-associated pro-apoptotic signals Su et al. Another study reported that RT combined with isolated soy protein supplementation had greater beneficial effects on physical performance, muscle strength and muscle glycogen reserves in aging mice. The increased type II muscle fibers and CSA further evidenced that combination of soy protein plus RT had greater beneficial effect than that of soy protein or RT intervention alone. These results indicate that age-induced loss of muscle mass could be maintained and/or delayed through exercise plus soy protein supplementation Lee et al. Although exercise is effective in improving skeletal muscle mass and physical fitness, the repeated high-intensity exercise-induced oxidative stress, inflammation or muscle damage cannot be ruled-out when prescribing exercise for older adults. In view of this, quercetin, a phytochemical flavonoid has been tested for its beneficial effects against high-intensity exercise in healthy adults. Tsaoet al. reported that short-term quercetin supplementation (7 days) effectively improved the time-to-exhaustion of high-intensity cycling exercise in healthy adults. This beneficial effect of quercetin may be due to the increased glucose uptake and/or attenuation of high-intensity exercise-induced oxidative stress and pro-inflammatory response Tsaoet al. These findings emphasizes that exercise training combined with nutritional supplements are effective in improving the skeletal muscle function, endurance performance and antioxidant capacity.

A study on young male athletes examined the muscle damaging effects of acute RT under normoxia and hypoxia-hyperoxia conditions. This study reported higher muscle soreness, increased creatine kinase and myoglobin levels after acute RT under normoxia. However, athletes exposed to hypoxia-hyperoxia reported no such adverse effects following acute RT. These findings imply that hypoxia-hyeroxia alternative exposure prior to intense exercise can alleviate the muscle damage and muscle soreness in athletes Chen et al. On the other hand, blood flow restriction (BFR), which increases the mechanical pressure to working muscle during exercise, can cause local hypoxia/ischemia. In this context, a systematic review and meta-analysis summarized that exercise with BFR can increase the mRNA expressions of angiogenic factors, including vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) in healthy adults. To be specific, RT combined with BFR appears to be greater in promoting the skeletal muscle angiogenic factors (VEGF and HIF-1α) than aerobic exercise with BFR Li et al. Other than exercise-induced beneficial effects on skeletal muscle in healthy adults, it is important to know the influence of exercise on improving the balance capability in children/adolescents with hearing impairment. To address this, a systematic review included eligible studies which examined the intervention effect on balance ability of children and adolescent with hearing impairment. This systematic review concluded that exercise intervention is effective in improving the balance among children/adolescents with hearing impairments. Specifically, intervention with a duration of 8–16-week is more efficient than a duration of < 8-week in improving the balance ability Zhou et al. Depression is a worldwide health issue for people that commonly coexist with other debilitating chronic illnesses. The correlation between depression and low-levels of vitamin D is still debatable. A meta-analysis synthesized evidence from randomized controlled trials, and stated that vitamin D supplementation is not only beneficial to decline the incidence of depression, but also effective to treat depression. It is further emphasized that supplementation of vitamin D with a daily dose of >2,800 IU, and intervention duration of 8-week or more were effective in prevention and treatment of depression Xie et al. Our Research Topic highlights that exercise training with or without combination of nutritional supplements is capable of improving the skeletal muscle function, antioxidant homeostasis and maintain overall wellbeing.

Author contributions

MK and VL organized, drafted and finalized the editorial. AM and WY provided the essential points from the articles. All authors contributed to the article and approved the submitted version.

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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The Effect of Blood Flow Restriction Exercise on Angiogenesis-Related Factors in Skeletal Muscle Among Healthy Adults: A Systematic Review and Meta-Analysis

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Background: Blood flow restriction (BFR) exercise may be a potential exercise program to promote angiogenesis. This review aims to compare the effects of exercise with and without BFR on angiogenesis-related factors in skeletal muscle among healthy adults.

Methodology: Searches were made in Web of Science, Scopus, PubMed, and EBSCO databases from January 2001 to June 2021. Studies were screened, quality was evaluated, and data were extracted. The review protocol was registered at PROSPERO (PROSPERO registration number: CRD42021261367). Standardized mean differences (SMD) of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR-2), hypoxia inducible factor 1α (HIF-1α), peroxisome proliferator-activated receptorγcoactivator-1α (PGC-1α) and endothelial nitric oxide synthase (eNOS) were analyzed using Revman 5.4 software with a 95% confidence interval (95% CI).

Results: Ten studies fulfilled the inclusion criteria with a total of 75 participants for BFR group and 77 for CON group. BFR exercise elicits greater expression of VEGF (heterogeneity test, $P = 0.09$, $I^2 = 44\%$; SMD, 0.93 [0.38, 1.48], $P < 0.05$), VEGFR-2 (heterogeneity test, $P = 0.81$, $I^2 = 0\%$; SMD, 0.64 [0.08, 1.21], $P < 0.05$), HIF-1α (heterogeneity test, $P = 0.67$, $I^2 = 0\%$; SMD, 0.43 [0.03, 0.82], $P < 0.05$), PGC-1α (heterogeneity test, $P = 0.02$, $I^2 = 54\%$; SMD, 0.74 [0.21, 1.28], $P < 0.05$) and eNOS (heterogeneity test, $P = 0.88$, $I^2 = 0\%$; SMD, 0.60 [0.04, 1.17], $P < 0.05$) mRNA than non-BFR exercise. In the sub-group analysis, resistance exercise with BFR elicits greater expression of VEGF (heterogeneity test, $P = 0.36$, $I^2 = 6\%$; SMD, 1.66 [0.97, 2.35], $P < 0.05$) and HIF-1α (heterogeneity test, $P = 0.56$, $I^2 = 0\%$; SMD, 0.51 [0.01, 1.02], $P < 0.05$) mRNA than aerobic exercise with BFR.

Conclusion: Exercise with BFR elicited more angiogenesis-related factors mRNA expression than exercise without BFR, but not VEGF and PGC-1α protein expression. Therefore, BFR training may be a potential training program to improve vascular function.

Systematic Review Registration: [https://www.crd.york.ac.uk/prospero/], identifier [CRD42021261367].

Keywords: blood flow restriction, angiogenesis, skeletal muscle, resistance exercise, healthy adults

INTRODUCTION

Blood flow restriction (BFR) can limit the blood flow and increase the mechanical pressure to the working muscle during exercise, resulting in local hypoxia/ischemia. Low-load training with BFR is more conducive to increasing muscle strength (Lixandrão et al., 2018) and endurance (Kacin and Strazar, 2011) than that without BFR, which may be attributed to improved oxygen delivery and extraction of working skeletal muscles during BFR training. The combination of these mechanisms increases the peripheral vascular system adaptability (Kacin and Strazar, 2011). A recent meta-analysis demonstrated that resistance training impacts more positively on arterial compliance regulation as the capacity to restrict blood flow increases (Liu et al., 2021). Compared to traditional resistance training, this positive effect on vascular function was significantly higher when the training time does not exceed four weeks (Liu et al., 2021). Nevertheless, the relevant mechanism of BFR-training regulating the peripheral vascular system (PVS) remains unclear.

The neogenesis of peripheral capillaries has been suggested as one of the underlying mechanisms through which BFR-training regulates the PVS. A previous study that indirectly assessed the skeletal muscle microvasculature using the capillary filtration technique showed that BFR exercise enhanced capillary growth (Hunt et al., 2013). In the study of Mueller et al. (2014), 21 young men were divided into two groups for 8 weeks of resistance training intervention. Their results showed that the synergism of whole-body vibration and blood flow restriction could further improve the capillary-to-fiber ratio, which were not observed by resistance training alone. Furthermore, the expression of some angiogenic genes was reported to be significantly enhanced following low-load resistance exercise with BFR (Larkin et al., 2012). The increase of angiogenesis gene expression is closely related to angiogenesis after exercise (Olfert et al., 2016). Therefore, further analysis is required to determine the physiological or molecular mediators of this response.

One of the principal growth factors in the complex pathways involved in angiogenesis is the vascular endothelial growth factor (VEGF) (Olfert et al., 2010). During low-load resistance exercise with BFR, the resulting decrease in muscle oxygen levels may stabilize hypoxia-inducible factor 1α (HIF-1α) for targeted activation of VEGF transcription (Barjaste et al., 2021). Furthermore, VEGF efflux from the skeletal muscle was promoted through the activity of endothelial nitric oxide synthase (eNOS) after the generation of nitric oxide due to shear stress (Gielen et al., 2011). These events improved the availability of VEGF at EC-receptor sites for vascular endothelial growth factor receptor 2 (VEGFR-2) activation (Shen et al., 1998) and facilitated the angiogenic effect of VEGF (Milkiewicz et al., 2005), whereas the expression of skeletal muscle VEGF was mainly mediated by peroxisome proliferator-activated receptor-gamma co-activator alpha (PGC-1α) (Leick et al., 2009). Conclusively, VEGF secretion may be regulated by BFR in various ways to promote angiogenesis.

There is data paucity regarding the effect of BFR exercise on various angiogenesis-related factors in skeletal muscle. Research findings have demonstrated that BFR exercise could facilitate the expression of VEGF by enhancing HIF-1α (Larkin et al., 2012; Ferguson et al., 2018), whereas other researchers have contradicting views (Taylor et al., 2016; Preobrazenski et al., 2020). This review was conducted to compare the effects of BFR exercise and non-BFR on angiogenesis-related factors, and to explore the effects of various exercise programs on VEGF and HIF. The findings will improve the current body of knowledge on the role of BFR exercise in angiogenesis.

METHODOLOGY

Protocol and Registration

The review protocol was registered on June 18, 2021, with the International Platform of Registered Systematic Review and Meta-Analysis Protocols (PROSPERO registration number: CRD42021261367).

Data Sources and Study Selection

Because the test methods and experimental instruments in the early research are very different from those now, the research in recent twenty years is searched. The systematic search was conducted using four databases: Scopus, PubMed, Web of Science and EBSCO. Studies published between January 2001 and June 2021 were considered while the last retrieval date was June 18, 2021. The search terms used were "blood flow restriction," "kaatsu," "blood flow restricted," "HIF," "VEGF," "NOS," "PGC," "training," and "exercise." Both the search strategy for each database and the corresponding results are presented in **Appendix A**. To minimize bias during the literature search, the titles and abstracts retrieved from the databases were screened by two independent investigators.

Additional relevant information such as the first author's name, the year of publication, sample size, exercise program, age, BFR method and main findings were documented. Outcome variables included VEGF, VEGFR-2, PGC-1α, HIF-1α, and eNOS. When necessary, the corresponding authors were contacted via

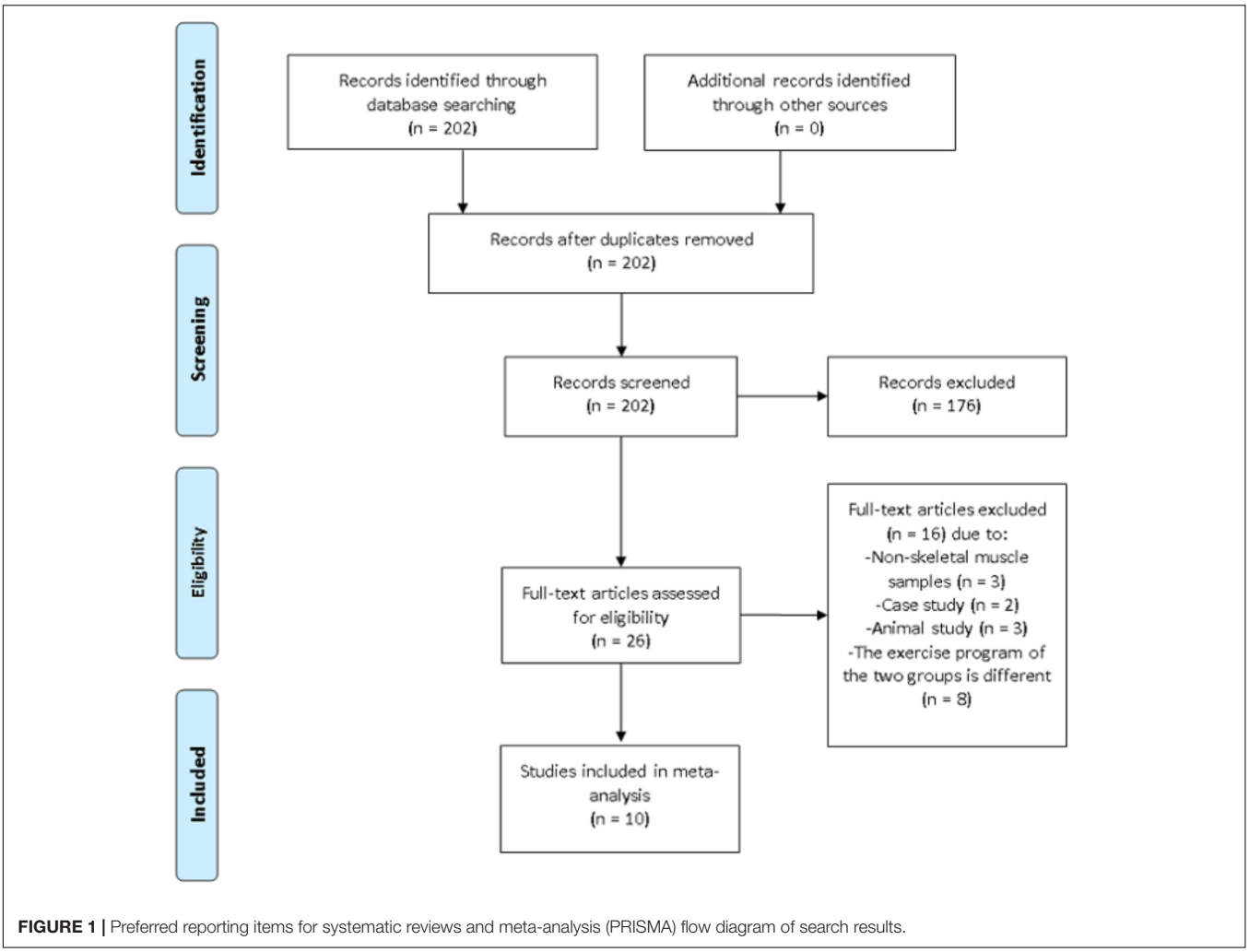

FIGURE 1 | Preferred reporting items for systematic reviews and meta-analysis (PRISMA) flow diagram of search results.

e-mail to clarify any unclear information. A third investigator was assigned to provide an opinion if the two principal investigators disagree on any information from the articles. On that note, the disagreement was resolved after discussing and reaching a consensus. The article selection process is summarized in **Figure 1**.

Inclusion and Exclusion Criteria

The inclusion criteria entailed full-text articles written in English, cross-sectional study design, participants were healthy individuals between 18 and 40 years old, the experimental group was exercise combined with BFR while the control (CON) group was exercise without BFR. Studies were also eligible for inclusion if the sampling method entailed vastus lateralis muscle biopsy taken either before exercise or two to four hours after exercise. Other literature such as abstracts, conference proceedings, posters, or presentations were excluded.

Quality Assessment

A pre-designed quality assessment tool, the NIH quality assessment tool for observational cohort and cross-sectional studies, was used to assess all the included studies. Study Quality Assessment Tools – NHLBI[1], accessed 15 June 2020) has a total of 14 questions. The quality of each study was classified as "poor," "fair," or "good."

Risk of Bias Assessment

A funnel plot was used to analyze the publication bias of the study. Sensitivity analysis was performed if more than five studies include the same indicator. Specifically, the sensitivity analysis was conducted by excluding each study sequentially to determine the stability of the meta-analysis results.

Data Analysis

The meta-analysis was executed by entering all the relevant outcome variables in the Review Manager (Version 5.4, Copenhagen: The Nordic Cochrane Center, The Cochrane Collaboration, 2014). All the studies included in the analysis had continuous outcome variables while the test units and methods were different. Therefore, standardized mean difference (SMD)

[1]https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools

was chosen as the effect scale index. Heterogeneity between studies was tested using the I (Kacin and Strazar, 2011) statistic. Heterogeneity was considered absent between studies when the I (Kacin and Strazar, 2011) is less than 40%. Therefore, a fixed-effect model can be used for analysis. Conversely, heterogeneity is present between studies if I (Kacin and Strazar, 2011) is equal to or greater than 40% and a random effect model must be used for analysis. Sub-group analysis was further performed to determine the heterogeneity. The level of statistical significance was adjusted to $P < 0.05$. According to the Cohen's guideline (Cohen, 1992), the effect size was explained as: 0.2 is a small effect, 0.5 is a medium effect and 0.8 is a large effect.

RESULTS

Eligibility of Studies

A total of 10 cross-sectional studies evaluating the effects of BFR training on angiogenesis-related factors in skeletal muscle among healthy adults were included in this review. The basic information in the included studies is shown in **Table 1**. Ethical approvals were obtained from the various institutions in which the studies were conducted. The level of agreement between the two principal investigators was high with a Cohen kappa coefficient of 0.884. A total of 74 men and 3 women participated in the studies, corresponding to the overall sample size in the BFR and CON groups. Seven studies focused on PGC-1α and VEGF. HIF-1α were reported in six articles while only three studies evaluated VEGFR-2 and eNOS. Six of the articles employed resistance exercises, whereas aerobic exercises were used in the remaining four studies. Overall, the BFR pressure ranged from 50 to 220 mmHg.

Sensitivity Analysis

Three steps were performed for the sensitivity analysis: changing the analysis model, effect size selection, and exclusion of individual studies. VEGF and PGC-1α indices were not significantly affected after the sensitivity test and the outcomes were stable. In contrast, the HIF-1α was highly sensitive and characterized by unstable results.

TABLE 1 | Overview of the included studies.

Author	Sample size	Age(y)	Exercise program	Blood flow restriction	Outcomes	Test timing (h)	Test method
AE							
Barjaste et al., 2021	5M	33.4 ± 1.0	5 × 2 min aerobic walking with 40%VO_{2max}; 1-min rest between each repetition.	7-cm wide Inflatable cuffs; 200 mmHg	PGC-1α; VEGF; HIF-1α	0&2	WB
Christiansen et al., 2018	6M	26.0 ± 5.0	9 × 2 min aerobic running with 12km/h; 1-min rest between each repetition; 34 min.	Inflatable cuff; 175 mmHg	PGC-1α	0&3	PCR
Preobrazenski et al., 2020	12M	22.4 ± 3.0	Cycled upright with legs below the heart;77%HR_{max}; 30 min.	Cycled supine with legs above the heart	PGC-1α; VEGFA; HIF-1α	0&3	PCR
Taylor et al., 2016	8M	32.0 ± 7.0	Four 30 s "all-out" sprints; 4.5 min recovery.	Inflatable cuff; 130 mmHg	PGC-1α; VEGF; VEGFR-2; HIF-1α; eNOS	0&3	PCR
RE							
Ameln et al., 2005	9M	22.0 ± 2.0	Knee extension; 45 min; 26% of peak load.	Pressure chamber; 50 mmHg	VEGF; HIF-1α	0&2	PCR
Drummond et al., 2008	6M	32.0 ± 2.0	Knee extension; 20%RM; 4 sets (30, 15, 15, 15); 30 s recovery.	Inflatable cuff; 200 mmHg	HIF-1α	0&3	PCR
Ferguson et al., 2018	6M	26.0 ± 2.0	Bilateral knee extension; 20%RM; 4sets (30:15:15: continued to fatigue); 30 s recovery.	13 cm wide Inflatable cuffs; 110 mmHg	PGC-1α; VEGF; VEGFR-2; HIF-1α	0,2&4	WB; PCR
Larkin et al., 2012	3M/3F	22.0 ± 1.0	knee extension;40%RM; 10sets × 12repetitions; 60s recovery.	Inflatable cuff; 220 mmHg	PGC-1α; VEGF; VEGFR-2; HIF-1α; eNOS	0&4	WB; PCR
Norrbom et al., 2004	9M	23.0 ± 2.0	Knee extension; 45 min; 26% of peak load.	Pressure chamber; 50 mmHg	PGC-1α	0&2	PCR
Norrbom et al., 2011	8M	24.0 ± 1.8	Knee extension; 45 min; 26% of peak load.	Pressure chamber; 50 mmHg	PGC-1α	0&2	PCR

M, Male; F, Female; RM, Repetition Maximum; PCR, Polymerase Chain Reaction; WB, Western Blot; VO_{2max}, Maximal oxygen uptake; HR_{max}, Maximum heart rate; PGC-1α, Peroxisome proliferator-activated receptorγcoactivator-1α; VEGF, ascular endothelial growth factor; VEGFR2, Vascular Endothelial Growth Factor Receptor 2; HIF-1α, Hypoxia inducible factor-1α; eNOS, Endothelial nitric oxide synthase.

Quality Assessment

Based on the NIH scale, nine of the 10 studies included in this review (Ameln et al., 2005; Drummond et al., 2008; Norrbom et al., 2011; Larkin et al., 2012; Taylor et al., 2016; Christiansen et al., 2018; Ferguson et al., 2018; Preobrazenski et al., 2020; Barjaste et al., 2021) recorded an overall quality rating of "Good" and only one study (Norrbom et al., 2004) had an overall quality rating of "Fair" as shown in **Table 2**.

Quantitative Synthesis

Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor-2

Figure 2A shows the six studies (Ameln et al., 2005; Larkin et al., 2012; Taylor et al., 2016; Ferguson et al., 2018; Preobrazenski et al., 2020; Barjaste et al., 2021) that evaluated the effects of BFR on VEGF, whereas the three studies (Larkin et al., 2012; Taylor et al., 2016; Ferguson et al., 2018) reporting the effects of BFR on VEGFR-2 mRNA are presented in **Figure 2B**. The meta-analysis revealed that BFR group (n = 58) improved VEGF more significantly ($P < 0.05$) compared to the CON group (n = 58) (heterogeneity test, P = 0.09, I^2 = 44%; SMD, 0.93 [0.38, 1.48]). VEGF protein (heterogeneity test, P = 0.80, I^2 = 0%; SMD, 0.33 [−0.52, 1.17]), VEGF mRNA AE (heterogeneity test, P = 0.71, I^2 = 0%; SMD, 0.40 [−0.23, 1.03]) or VEGF mRNA RE (heterogeneity test, P = 0.36, I^2 = 6%; SMD, 1.66 [0.97, 2.35]) showed greater homogeneity in the sub-group analysis. Likewise, a significant difference ($P < 0.05$) was observed in the VEGFR-2 mRNA between the BFR (n = 26) and CON (n = 26) groups (heterogeneity test, P = 0.81, I^2 = 0%; SMD, 0.64 [0.08, 1.21]).

Hypoxia Inducible Factor 1α

The effects of BFR on HIF-1α was investigated in six studies (Ameln et al., 2005; Drummond et al., 2008; Larkin et al., 2012; Taylor et al., 2016; Ferguson et al., 2018; Preobrazenski et al., 2020) as shown in **Figure 3**. HIF-1α was significantly improved ($P < 0.05$) in the BFR group compared to the CON group (n = 53) (heterogeneity test, P = 0.67, I^2 = 0%; SMD, 0.43 [0.03, 0.82]). A greater homogeneity was detected in the sub-group analysis for HIF-1α mRNA AE (heterogeneity test, P = 0.38, I^2 = 0%; SMD, 0.29 [−0.34, 0.91]) or HIF-1α mRNA RE (heterogeneity test, P = 0.56, I^2 = 0%; SMD, 0.51 [0.01, 1.02]).

Peroxisome Proliferator-Activated Receptorγcoactivator-1α

Seven studies (Norrbom et al., 2004, 2011; Taylor et al., 2016; Christiansen et al., 2018; Ferguson et al., 2018; Preobrazenski et al., 2020; Barjaste et al., 2021) reported the effects of BFR on PGC-1α (**Figure 4**). PGC-1α was significantly improved ($P < 0.05$) in the BFR group (n = 72) than the CON group (n = 74) (heterogeneity test, P = 0.02, I^2 = 54%; SMD, 0.74 [0.21, 1.28]). Likewise, the sub-group analysis demonstrated a greater homogeneity for PGC-1α protein (heterogeneity test, P = 0.23, I^2 = 32%; SMD, −0.07 [−0.92, 0.78]) or PGC-1α mRNA (heterogeneity test, P = 0.12, I^2 = 41%; SMD, 1.04 [0.49, 1.59]).

Endothelial Nitric Oxide Synthase

As shown in **Figure 5**, the effects of BFR on eNOS were evaluated in three of the reviewed articles (Larkin et al., 2012; Taylor et al., 2016; Ferguson et al., 2018). The meta-analysis revealed eNOS was more significantly improved ($P < 0.05$) in the BFR group (n = 26) compared to the CON group (n = 26) (heterogeneity test, P = 0.88, I^2 = 0%; SMD, 0.60 [0.04, 1.17]).

Analysis of Publication Bias

A funnel plot was employed in analyzing the publication bias. Using the minimum requirement of the funnel plot, the total sample size of all the 10 studies reflected the publication bias to a certain degree. The feasibility of performing funnel plot analysis using a small sample size has been demonstrated by Lu et al. (2020) in a previous study. **Figure 6** depicted the funnel plots of all the symmetrically-distributed indicators, reflecting a small degree of bias in the studies.

DISCUSSION

Studies in improving skeletal muscle capillary networks and functions emphasize the high intensity of either aerobic or resistance exercise (Prior et al., 2003; Gavin et al., 2007; Larkin et al., 2012). However, high-intensity exercise is restricted

TABLE 2 | Depiction of the risk of bias assessment.

NIH Tool	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
Ameln et al., 2005	Y	N	Y	Y	N	N	N	NA*	N	N	Y	Y	Y	NA*	7/12
Barjaste et al., 2021	Y	Y	Y	Y	N	N	N	NA*	Y	N	Y	Y	Y	NA*	8/12
Christiansen et al., 2018	Y	N	Y	Y	N	N	N	NA*	Y	N	Y	Y	Y	NA*	7/12
Drummond et al., 2008	Y	Y	Y	Y	N	N	N	NA*	Y	N	Y	Y	Y	NA*	8/12
Ferguson et al., 2018	Y	N	Y	Y	N	N	N	NA*	Y	N	Y	Y	Y	NA*	7/12
Larkin et al., 2012	Y	Y	Y	Y	N	N	N	NA*	Y	N	Y	Y	Y	NA*	8/12
Norrbom et al., 2004	Y	N	Y	Y	N	N	N	NA*	N	N	Y	Y	Y	NA*	6/12
Norrbom et al., 2011	Y	Y	Y	Y	N	N	N	NA*	N	N	Y	Y	Y	NA*	7/12
Preobrazenski et al., 2020	Y	Y	Y	Y	N	N	N	NA*	N	N	Y	Y	Y	NA*	8/12
Taylor et al., 2016	Y	Y	Y	Y	N	N	N	NA*	Y	N	Y	Y	Y	NA*	8/12

*Y = yes; N = no; NA = not applicable; * Not included in total score.*

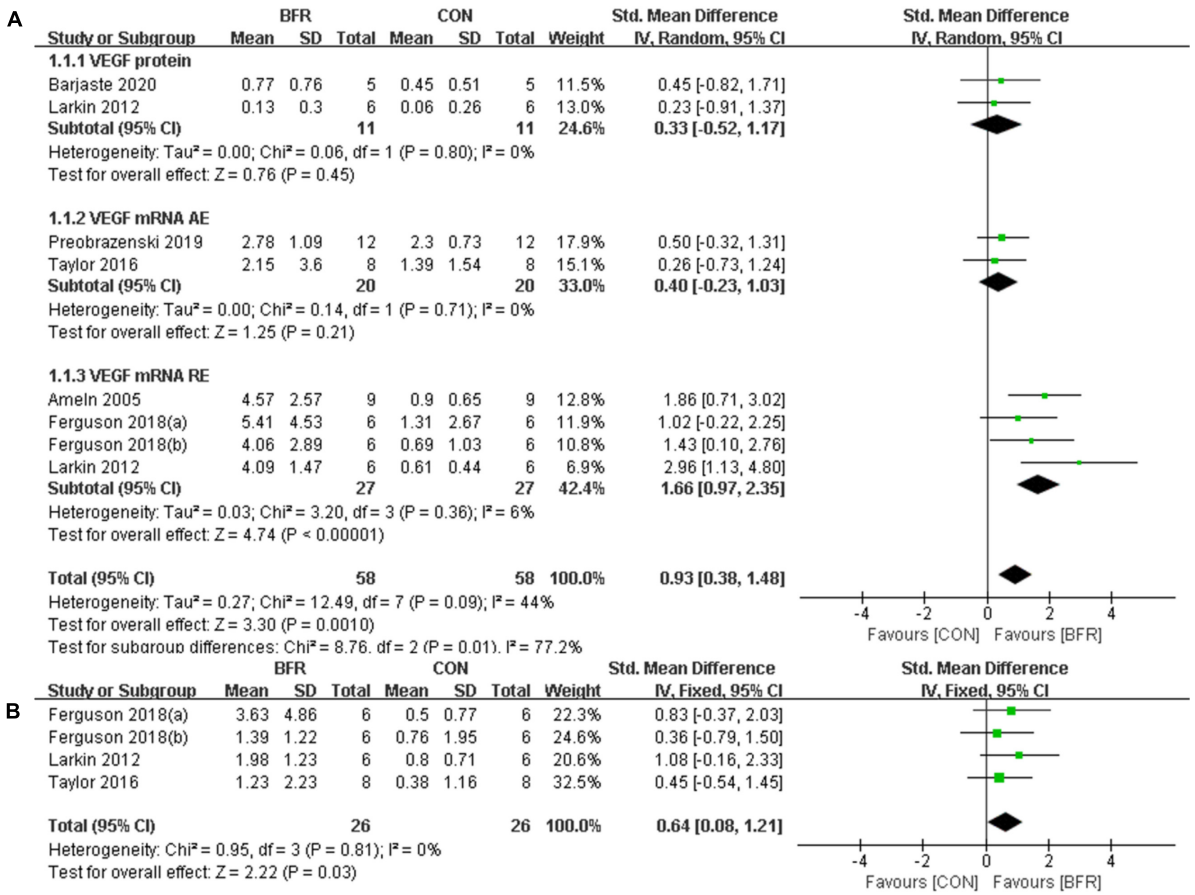

FIGURE 2 | The forest plot of the effect size for studies assessing the effect of blood flow restriction exercise on the VEGF **(A)** and VEGFR-2 **(B)** mRNA.

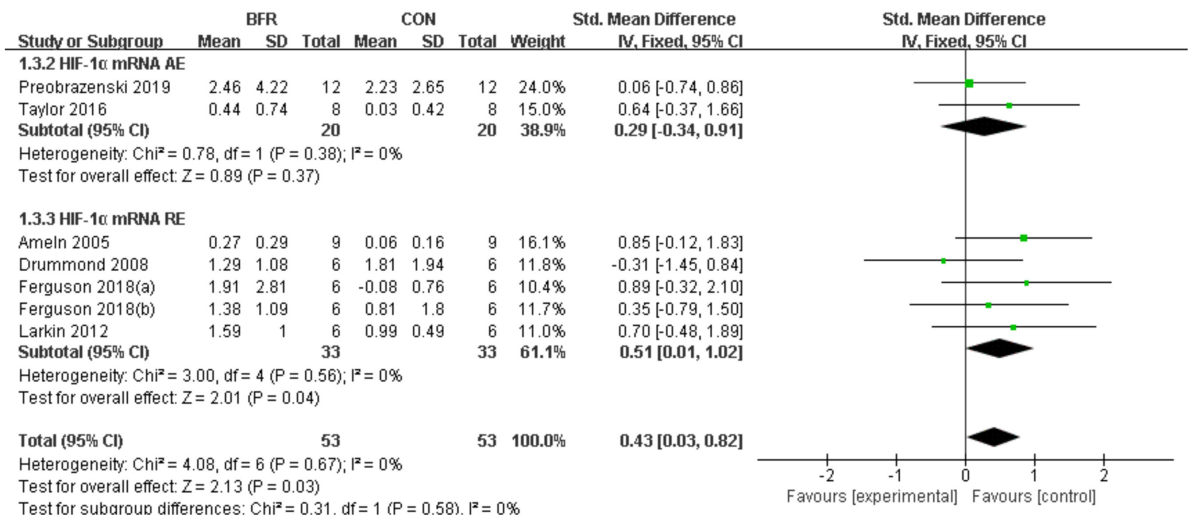

FIGURE 3 | The forest plot of the effect size for studies assessing the effect of blood flow restriction exercise on the HIF-1α mRNA.

to some special populations, including cardiovascular patients and older persons. BFR exercise is a proposed alternative for traditional high-intensity exercise (Drummond et al., 2008) and it is important to compare their effects on skeletal muscle capillary growth. Hence, the present meta-analysis investigated the expression of VEGF, VEGFR-2, PGC-1α, HIF-1α, and eNOS

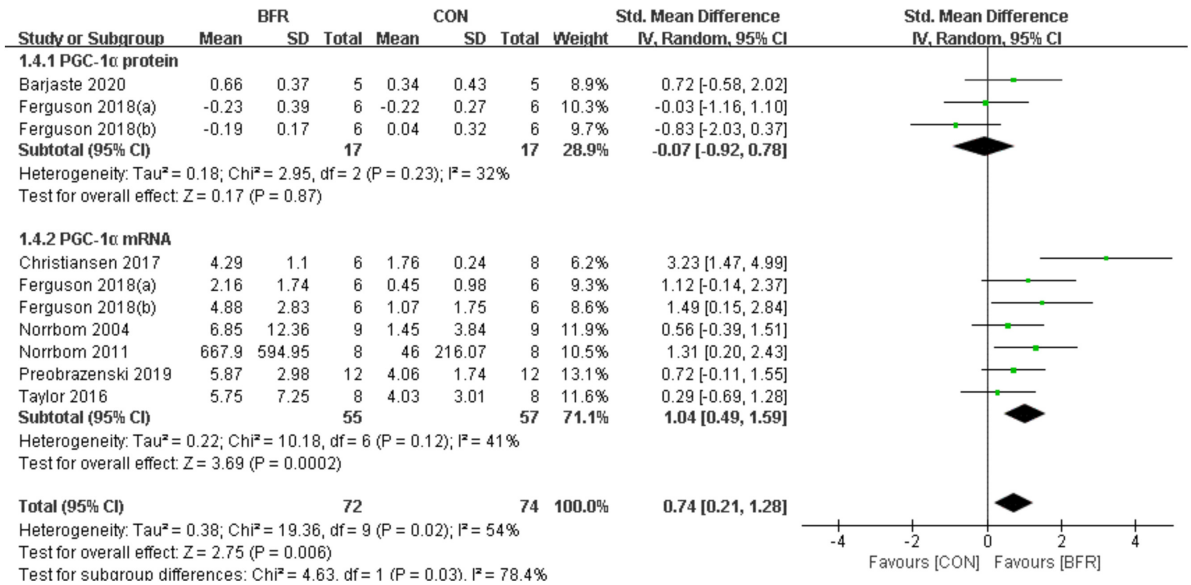

FIGURE 4 | The forest plot of the effect size for studies assessing the effect of blood flow restriction exercise on the PGC-1α.

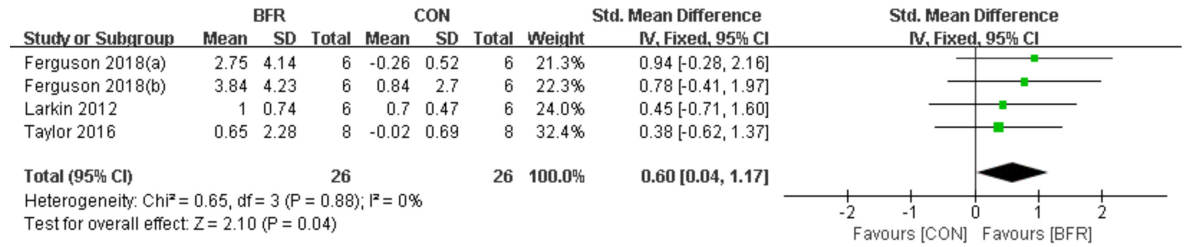

FIGURE 5 | The forest plot of the effect size for studies assessing the effect of blood flow restriction exercise on the eNOS mRNA.

in healthy adults after both exercise modalities. The results showed that the expression of the above mentioned angiogenic genes was significantly increased in the BFR group compared to the control group. However, different exercise programs may cause various changes in the mRNA expression of VEGF and HIF-1α. The present results revealed that resistance exercise can significantly increase the mRNA expression of HIF-1α and VEGF in healthy adults. Conversely, these positive effects were lacking for aerobic exercise.

It is speculated that this may be caused by different degrees of hypoxia, and the increase of HIF-1 may mainly depend on anaerobic metabolism (Lundby et al., 2009). In exercise with BFR, resistance exercise mainly stimulates the skeletal muscle system, which may lead to lower oxygen consumption than aerobic exercise (Ferrari et al., 2018). The lower oxygen exchange level may be the reason for the higher expression of HIF-1 when resistance exercise combined with BFR. Furthermore, some evidence suggests that resistance exercise with BFR may lead to better superposition effect on angiogenesis rather than aerobic exercise (Mueller et al., 2014; Conceicao et al., 2016; Mitchell et al., 2019). Mitchell et al. (2019) indicated that the change of capillary density after four weeks of aerobic training with BFR was similar to that without BFR. Briefly, resistance exercise combined with BFR may have a better effect on angiogenesis than aerobic exercise.

Interestingly, the included studies presented contradicting outcomes in the expression of VEGF (Larkin et al., 2012) and PGC-1α (Ferguson et al., 2018) protein from mRNA. The authors attributed the discrepancies to the limitations of sampling time as it might take several days after exercise for some proteins to be translated (Gustafsson et al., 2005; Perry et al., 2010). In contrast, Barjaste et al. (2021) reported that VEGF, HIF-1α, and PGC-1α protein expression were significantly increased at 3-h post-BFR-walking. This finding corroborates an animal-based study, which reported an increase in PGC-1α protein level in BFR-low intensity aerobic training (Bahreinipour et al., 2018). This might be explained by the different exercise modes adopted in the studies, reflecting diverse metabolic stress and mechanical tension between them. For instance, it was found the expression of HIF-1α, VEGF and PGC-1α proteins (Conceicao et al., 2016) was better stimulated by low-intensity BFR walking compared to low-intensity BFR cycling (Barjaste et al., 2021).

One of the central pro-angiogenic factors during exercise training is VEGF (Hellsten and Hoier, 2014) and its mRNA

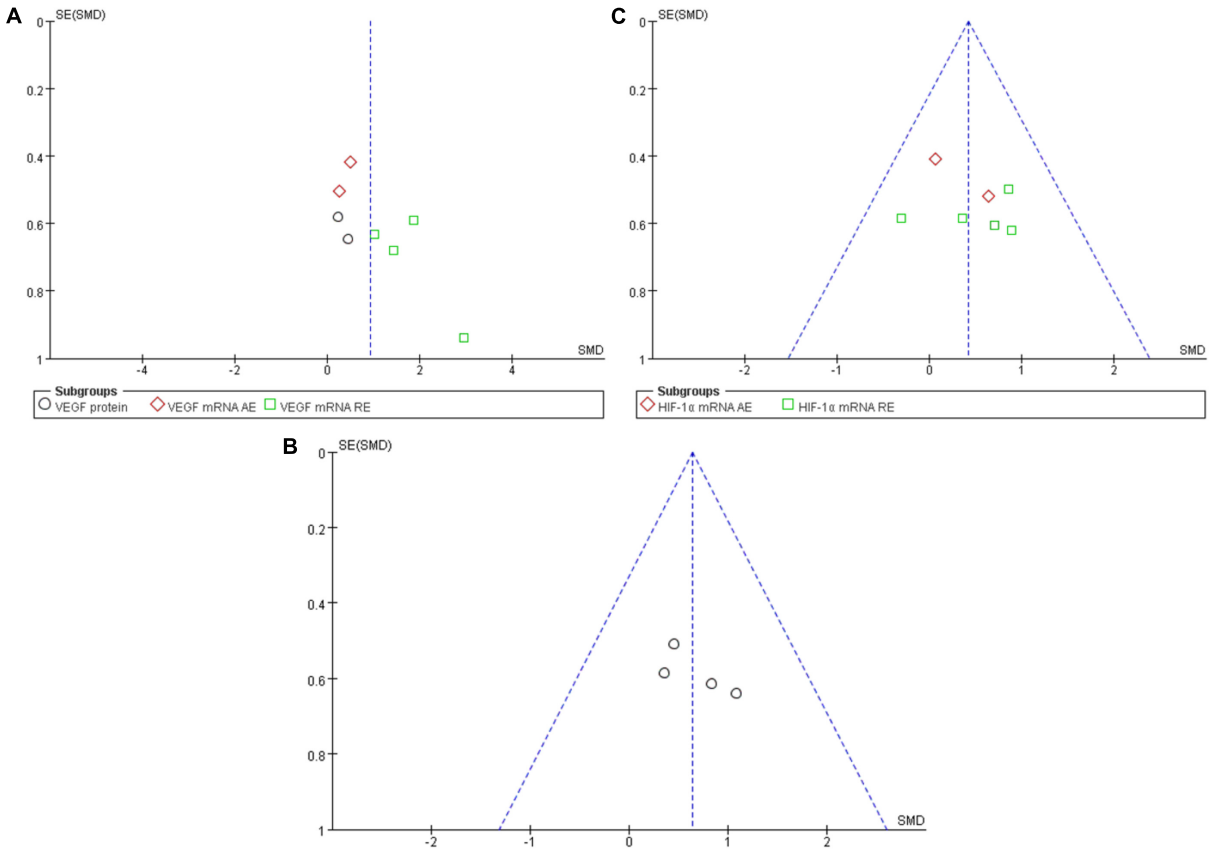

FIGURE 6 | Funnel plot of publication bias for the effect of resistance exercise on the VEGF **(A)**, VEGFR-2 **(B)** and HIF-1α **(C)**.

expression increases after a single bout of exercise (Gustafsson et al., 1999). This event was reported by Larkin et al. (2012) as the most notable transcriptional change among other angiogenic genes in response to the reduced blood flow and oxygen delivery in exercised skeletal muscle during BFR. The primary receptor of VEGF (VEGFR-2) mediates the VEGF-induced angiogenesis (Milkiewicz et al., 2005; Ferguson et al., 2018). Results from this meta-analysis indicated that VEGFR-2 mRNA increased after BFR-exercise, which may result from the increased VEGF binding (Ferguson et al., 2018) and the number of endothelial cells (Gustafsson et al., 2007) induced by BFR-exercise. Additionally, VEGF expression would be promoted by activating some of its key regulating factors (HIF-1α, PGC-1α, and eNOS) when BFR is combined with exercise, thereby eliciting an increased local muscular ischemia or hypoxia, shear-stress, and mechanical stress (Shweiki et al., 1992; Stein et al., 1995; St-Pierre et al., 2003; Bloor, 2005; Jager et al., 2007; Arany et al., 2008; Hawley et al., 2014; Paula et al., 2020).

HIF-1α is an important angiogenic regulator of hypoxia and metabolic stress (Semenza, 2006; Taylor et al., 2016). While skeletal muscle PO_2 is reduced by exercise, exercise-dependent reduction in oxygen tension might facilitate up-regulation of HIF-1α expression in skeletal muscles. The levels of HIF-1α is increased significantly after BFR-exercise compared to non-BFR exercise (Larkin et al., 2012; Taylor et al., 2016; Barjaste et al., 2021). This reflects a higher degree of hypoxia, followed by increased activation of VEGF, which is the downstream factor of the HIF-1α pathway. This is in line with the present pooled data meta-analysis but contradicts the findings from the analysis of some included studies. Ameln et al. (2005) found no significant differences between BRF and non-BFR conditions in HIF-1α protein levels although the lactate levels indicated a lower oxygen tension during BFR-exercise. The degree of hypoxia may be already low enough to stimulate HIF-1α expression at 50 to 60% of the maximum work rate during non-BFR exercise. Hence, the intensity of exercise protocols may also play an important role in activating the HIF-1α by BFR-exercise. Other possible reasons that may likely account for the negative results include subject variability, exercise modes and sampling time (Drummond et al., 2008; Ferguson et al., 2018; Preobrazenski et al., 2020). Given these negative results, the enhanced angiogenesis induced by BFR more likely depends on the PGC-1α pathway.

PGC-1α has recently emerged as an inducer of angiogenesis in skeletal muscle, which strongly induces VEGF expression (Jung and Kim, 2014) in response to ischaemia (Arany et al., 2008).

Therefore, increased oxidative stress (Kemp et al., 2003; Yu et al., 2008; Zhong et al., 2011) and fiber type-dependent AMPK signaling (Norrbom et al., 2011; Christiansen et al., 2018; Preobrazenski et al., 2020) may be associated with augmentation of BFR in exercise-induced PGC-1 mRNA. Four out of five studies including PGC-1α mRNA measurement showed a higher expression of the protein during BFR-exercise (Norrbom et al., 2011; Christiansen et al., 2018; Ferguson et al., 2018; Preobrazenski et al., 2020), which is consistent with the current pooled data meta-analysis. However, one study reported no differences between the two exercise modalities, which might be due to different exercise and BFR modes adopted (Taylor et al., 2016). Christiansen et al. (2018) found that muscle hypoxia was not a key factor for BFR upon comparing the effects of BFR and systemic hypoxia (∼3,250 m) on PGC-1α. Similarly, a previous study discovered that moderate-intensity cycling at simulated altitude (3,000 m) did not affect the PGC-1α mRNA in the skeletal muscle (Slivka et al., 2014). In addition, the alterations in the expression of PGC-1α protein might be influenced by the intensities of exercise with BFR (Bahreinipour et al., 2018; Ferguson et al., 2018; Barjaste et al., 2021). Moreover, this protein may also be affected by the sampling time because the increase in protein concentrations or levels may delay for hours or days after the exercise (Baar et al., 2002).

The binding of VEGF to VEGFR-2 activates a signaling cascade leading to NO production (Larkin et al., 2012). Furthermore, the production of NO is directly induced by the eNOS activity, which is controlled by either shear-stress dependent or independent VEGF expression (Larkin et al., 2012). Therefore, the increased eNOS mRNA expression after BFR-exercise (Ferguson et al., 2018) might result from the shear stimulus caused by BFR, as well as mechanical compression by skeletal muscle contraction. In contrast, Larkin et al. (2012) and Taylor et al. (2016) indicated that muscle expression of eNOS was not increased by the combination of BFR with acute low-intensity exercise. These inconsistent results may be due to variations in sampling time, as the peak expression of eNOS may appear before VEGF and VEGFR-2 (Milkiewicz et al., 2005; Ferguson et al., 2018).

Study Limitations

Most of the studies included in this review reflected a small sample size and only 10 articles met the inclusion criteria. This reflects the lack of large sample size literature on this subject. Secondly, differences in muscle fiber types may explain the differences in the expression patterns of angiogenic factors, but there is a lack of direct evidence. Only two indirect evidences showed that there was no difference in the expression of AMPK (Christiansen et al., 2018) and PGC-1α (Norrbom et al., 2004) after exercise with blood flow restriction compared without blood flow restriction. Additionally, the changes of angiogenesis related factors in skeletal muscle can not really represent the actual angiogenesis. Capillary-to-fiber ratio is an effective index to reflect vascular density, however the relationship between blood flow restriction training intervention and capillary-to-fiber ratio is not clear. This limits our further explanation and verification of the effect of blood flow restriction training. In future research, we can further explore the effect of blood flow restriction training on vascular density and its relationship with angiogenesis related factors. Furthermore, the majority of studies focused on male samples while only three females were included. This might be attributed to the fact that women are more reluctant to accept muscle biopsy. Moreover, the forms of BFR used in various studies vary greatly, including cuff, raised limb and pressure chamber. This may lead to differences in the actual pressure applied and some errors may occur in horizontal comparison. Finally, most studies focused on mRNA expression and only a few considered protein alterations. Future studies should further explore the effect of BFR training on the expression of angiogenesis-related proteins, especially after a few days of exercise.

CONCLUSION

This study revealed that exercise with BFR elicited more VEGF, VEGFR-2, HIF-1α, PGC-1α, and eNOS mRNA expression than exercise without BFR, but not VEGF and PGC-1α protein expression. Given that the combination of resistance exercise and BFR was more conducive to improving VEGF and HIF-1α mRNA expression compared to aerobic exercise. Therefore, BFR training may be more conducive to improve vascular function, the protocol should be considered when developing sports-based training programs. Results cannot be extrapolated to all individuals. Future studies should include samples from other populations to determine feasible training programs.

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.

AUTHOR CONTRIBUTIONS

LW conceptualized and designed the study, collected and organized the data, and drafted the initial manuscript. ShuL collected and organized the data, reviewed the included articles, and conducted the analyses. ShiL and WY collected and organized the data and reviewed the included articles. WL and HQ conceptualized and designed the study and critically reviewed and revised the manuscript. TL conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed and revised the manuscript. All authors read and approved the final manuscript.

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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.

The handling editor declared a shared affiliation with several of the authors LW, HQ, TL, and WL at time of review.

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APPENDIX

APPENDIX A | Search strategy.

Databases	Search strategy	Result (Approximately)
Scopus	#1: Title-Abs-Key (blood flow restriction or kaatsu or blood flow restricted) #2: Title-Abs-Key (exercise or training) #3: Title-Abs-Key (VEGF or HIF or PGC or NOS) #4: #1 and #2 and #3 Limiters - Published Date: 20010101-20210618	3,532 1,374,656 5,126,702 113
Pubmed	#1: [Title/Abstract] blood flow restriction or kaatsu or blood flow restricted #2: [Title/Abstract] exercise or training #3: [Title/Abstract] VEGF or HIF or PGC or NOS #4: #1 and #2 and #3 Filters: Publication date from 2001/01/01 to 2021/6/18	824 512,592 112,029 22
Web of Science	#1: TOPIC: (blood flow restriction or kaatsu or blood flow restricted) #2: TOPIC: (exercise or training) #3: TOPIC: (VEGF or HIF or PGC or NOS) #4: #1 and #2 and #3 Refined by: PUBLICATION YEARS: (20210618-20010101) Indexes=SCI-EXPANDED, SSCI, CCR-EXPANDED,	6,176 1,069,019 138,289 37
EBSCO	#1: Abstract: (blood flow restriction or kaatsu or blood flow restricted) #2: Abstract: (exercise or training) #3: Abstract: (VEGF or HIF or PGC or NOS) #4: #1 and #2 and #3 Year: 20010101-20210618	1136 2,983,417 221,179 30

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ORIGINAL RESEARCH
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Effects of Hypoxia–Hyperoxia Preconditioning on Indicators of Muscle Damage After Acute Resistance Exercise in Male Athletes

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Purpose: The purpose of this study was to investigate the effects of acute repeated hypoxia–hyperoxia preconditioning on resistance exercise (RE)-induced muscle damage in male athletes.

Methods: Eleven young male athletes participated in this randomized double-blind counter-balanced crossover study, and were divided into Normoxia (N) and Hypoxia–Hyperoxia (HH) trials. Subjects of the respective trials were supplied with normoxic (FiO_2 = 0.21), or alternating hypoxic/hyperoxic air (FiO_2 = 0.10/0.99, 5 min each) for 60 min. Thirty minutes after preconditioning, subjects performed acute bouts of RE consisting of bench press, deadlift, and squats. Each exercise included 6 sets of 10 repetitions at 75% one-repetition maximum (1RM) with 2 min rest between sets. After a 2-week washout period, subjects changed trials and completed the same study procedure after the alternate preconditioning. Muscle soreness, maximal voluntary contraction (MVC), and circulating biochemical markers were tested before preconditioning (baseline) and during recovery at 0, 24, and 48 h after exercise.

Results: Acute RE significantly increased levels of muscle soreness, creatine kinase (CK) and myoglobin (Mb), and decreased levels of peak knee extension torque in the N trial. Muscle soreness, CK, and Mb levels of the HH trial were significantly lower than that of the N trial after exercise. Interestingly, interleukin-6 (IL-6) levels of the HH trial increased significantly 0 h after exercise compared to baseline and were significantly higher than that of the N trial 0 and 24 h after exercise. However, no significant differences of thiobarbituric acid reactive substances (TBARS), cortisol, testosterone, peak torque, and average power levels were found between N and HH trials during recovery.

Conclusion: Our data suggest that pre-exercise treatment of alternating hypoxic/hyperoxic air could attenuate muscle damage and pain after acute RE, but has no effect on muscle strength recovery in young male athletes.

Keywords: muscle soreness, maximal voluntary contraction, creatine kinase, muscle strength, inflammation

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INTRODUCTION

In athletic training, resistance exercise (RE) effects are derived by controlling the loading, sets, repetitions, rest duration, and movement velocity of exercise, these stimulations result in physiological response and adaptation (Spiering et al., 2008). Heavy RE protocols that consist of high loads (<10 repetition maximum, RM), high volume (6 or more sets of 10 repetitions per exercise), short inter-set rests, and use of major muscle groups, are frequently used to induce muscle hypertrophy and improve strength in athletes (Tesch, 1988; Izquierdo et al., 2009). However, excess mechanical force during heavy RE protocols could injure muscle sarcomere, resulting in exercise-induced muscle damage (EIMD). Symptoms of delayed onset muscle soreness (DOMS) including increased muscle soreness and reduced muscle force appear during recovery periods 24–72 h after EIMD. In addition, because of increased cell membrane permeability and inflammatory response, levels of special muscular proteins and pro-inflammatory cytokines increase after EIMD (Smith, 1991; Cheung et al., 2003). Moreover, strenuous or unaccustomed exercise during training or competition also induce DOMS, negatively influencing exercise performance in athletes (Smith, 1992). Various protocols have been developed to alleviate DOMS induced by acute resistance or strenuous exercise, including the use of stretching (Dupuy et al., 2018), ice packs (Howatson and Van Someren, 2003), massage (Kargarfard et al., 2016), nutritional supplements (Harty et al., 2019) and anti-inflammatory drugs (Schoenfeld, 2012).

Systemic hypoxia–hyperoxia exposure is characterized by breathing several cycles of oxygen-depleted air (<20.9% inspired O_2) followed by oxygen-enriched air (>20.9% inspired O_2). Intermittent hypoxia–hyperoxia training (IHHT), resulting from chronic or prolonged hypoxia–hyperoxia exposure, has recently been found to positively impact a number of medical conditions. These include improving cardiorespiratory fitness (Dudnik et al., 2018); alleviating markers of diabetes and cognitive impairment (Serebrovska et al., 2019); and attenuating symptoms of coronary artery disease (Glazachev et al., 2017). In addition, IHHT has been used to accelerate the recovery of athletes with over training syndrome (Susta et al., 2017). The underlying physiological mechanism may be associated with upregulation of adaptive reactive oxygen species (ROS) signaling, because of increased ROS production during ischemic–reperfusion stress (Haffor and Al-Johany, 2005), resulting in better antioxidant capacity adaptation (Sazontova et al., 1994). However, the effects of acute systemic hypoxia–hyperoxia preconditioning on EIMD are still unclear.

Therefore, the main purpose of the present study was to investigate the effects of hypoxia–hyperoxia preconditioning on acute RE-induced muscle damage in male athletes. We hypothesized that acute hypoxia–hyperoxia preconditioning would attenuate the levels of muscle damage markers and improve the muscle function recovery following acute heavy RE in male athletes.

MATERIALS AND METHODS

Subjects

Eleven male collegiate swimmers were recruited as subjects. Subjects belonged to competitive swimming teams at a physical education university and were trained professionally at least four times per week for more than 1 year on their respective university teams. The exclusion criteria for participation were as follows: smoking, cardiac arrhythmia, chronic metabolic diseases (diabetes, cardiovascular disease, or metabolic syndrome), and musculoskeletal injury. In order to reduce the metabolic effects associated with these substances, subjects were asked to avoid use of tobacco, alcohol, or sports supplements (whey protein, branch chain amino acids, or Chinese herbs etc.) from 1 month before to the end of the experiment. In addition, subjects maintained moderate physical activity and avoided high intensity or RE during the experiment. Subjects were informed of the purpose and experimental protocol of the study before experiments began, and they were informed of potential risks of participation and then signed their consent. The procedure of this study was ethically approved by the Institutional Review Board of University of Taipei, Taipei, Republic of China (Taiwan; Approved number: IRB-2016-052). Prior to the beginning of the study, demographic and anthropometric information including age, height, and weight was collected or measured by the research staff. Based on relevant investigations (Franz et al., 2018), *a priori* sample size of 11 subjects was calculated using the Sample Size Calculator.[1] This group size was sufficient to detect a significant difference of CK levels between preconditioning and sham treatment groups 24 h after damaging exercise with $\alpha = 0.05$ and $\beta = 0.2$.

Study Design

In this randomized, double-blind, counter-balanced crossover study design, participants were randomly divided into Normoxia (N) or Hypoxia–Hyperoxia (HH) preconditioning trials. On the experiment day, subjects were supplied either normoxic air ($FiO_2 = 0.21$) or alternated between hypoxic ($FiO_2 = 0.10$) and hyperoxic ($FiO_2 = 0.99$) air for 60 min according to their trial. Thirty minutes after N or HH preconditioning, subjects performed an acute bout of heavy RE to induce muscle damage. Muscle soreness and isokinetic muscle strength were determined before preconditioning and at 0, 24, and 48 h after exercise. Blood samples were collected at the same time points and used to assess the levels of muscle damage [creatine kinase (CK) and myoglobin (Mb)], inflammation (interleukin-6, IL-6), oxidative damage (thiobarbituric acid reactive substances, TBARS), and catabolism/anabolism (cortisol and testosterone). After the first trial, subjects switched preconditioning trials and completed the same study procedure. The trials were separated by a 2 weeks washout period. The study procedure was completed during off-season.

[1] https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html

Anthropometric and One-Repetition Maximum Measurements

One week before acute RE, subjects attended the laboratory for anthropometric (height and weight) measurements [mean height 172.5 ± 1.4 cm; median weight 70.3 (62.0–71.9); **Table 1**]. All subjects were then familiarized with the RE to be used in the study. After standardized 5 min light running and dynamic stretches, 1RM of each subject was determined for bench press, deadlift, and squat using the Smith machine (Cybex Plate Loaded 16,120 Smith Press, Cybex, Medway, MA, United States) according to a previous study (Maud and Foster, 2006). In order to ensure the correct motion and safety of subjects, 1RM testing and RE procedures in this study were supervised by a specialist. 1RM in squat, deadlift, and bench press were 99.1, 86.9, and 58.0 kg, respectively (**Table 1**).

Normoxia and Hypoxia–Hyperoxia Preconditioning

PVC balloons were filled with normoxic, hypoxic, or hyperoxic air, and supplied to subjects by mask and two-way non-rebreathing valves (T-shape 2,700, Hans Rudolph Inc., Kansas, MO, United States). Oxygen concentration of normoxic ($FiO_2 = 0.21$), hypoxic ($FiO_2 = 0.10$), or hyperoxic ($FiO_2 = 0.99$) air was produced by mixing 100% oxygen and nitrogen gas, each resulting gas was then checked using Model GB300 Percent Portable Oxygen Analyzer (Teledyne Technologies Inc., City of Industry, CA, United States) before treatment. The HH preconditioning protocol consisted of 6 cycles of alternating hypoxia/hyperoxia (5 min each) for a total 60 min. In the HH trial, hypoxic and hyperoxic air were supplied, respectively, *via* Y tube and switched every 5 min using a switching valve. Normoxic air was continuously supplied in a blind manner to subjects of the N trial for 60 min also using the same type of Y tube in order to reduce the placebo effect.

Acute Heavy Resistance Exercise Protocol

All resistance exercises in this study were completed using a Smith machine (Cybex Plate Loaded 16,120 Smith Press, Cybex, Medway, MA, United States). After standardized dynamic stretches, participants performed the squat, deadlift, and bench press for 10 repetitions at 50% of their 1RM to warm up before the acute RE protocol. This protocol was modified from a previous study (Vingren et al., 2009) and consisted of 3 training sessions of squat, deadlift, and bench press in order. Each session included 6 sets of 10 repetitions of each exercise at 75% 1RM with 2 min static rest between sets. Subjects were asked to performed full range of motion and maintain constant velocity during each motion. If subjects were unable to complete 10 repetitions in a set, additional 2 min static rest periods were implemented. Subjects were then allowed to complete any remaining repetitions.

TABLE 1 | Physical characteristics of the subjects.

Variables	Values or numbers
N	11
Height (cm)	172.5 ± 1.4
Body weight (kg)	70.3 (62.0–71.9)
Age (years)	21.4 ± 0.3
BMI (kg/m^2)	22.7 ± 0.5
1RM (kg)	
Squat	99.1 ± 4.5
Deadlift	86.9 ± 8.3
Bench press	58.0 ± 4.4

The values are mean ± SE or median (interquartile range). BMI, body mass index; 1RM, one-repetition maximum.

Isokinetic Muscle Strength and Muscle Endurance

The maximum voluntary contraction (MCV) of knee extensor was assessed using a Biodex System 4 isokinetic dynamometer (Biodex Medical Systems Inc., Shirley, NY, United States). Before testing, subjects performed a 5 min walking warm up on a treadmill. The right leg of each subject was investigated. Subjects were fixed in a rigid chair with 90° hip to knee angle, then performed 20 repetitions of knee extension and flexion between 90° and 0°. The angular velocity was fixed at 180° per second. Subjects exerted maximum power against the mechanism during extension and flexion. Knee extension peak torque (Newton-metre, N-m) and average power (Watts, W) were calculated as muscle strength and endurance, respectively.

Muscle Soreness

Muscle soreness was scored using ratings of perceived muscle soreness (RPMS), a verbal rating scale (VRS) ranging from 0 to 6. The numbers corresponded with no soreness (0), dull feeling of soreness (1), light continuous soreness (2), more than light soreness (3), annoying soreness (4), severe soreness (5), and intolerable soreness (6; Rodenburg et al., 1993).

Biochemical Analysis

Blood samples were taken using vacutainer tubes and needles (22-gauge) from the median antebrachial vein of the left forearm. After centrifugation at 3000 *g* and 4°C, supernatant (serum) was obtained and stored at −80°C until biochemical analysis was performed. A bench top DT-60II analyzer (Johnson and Johnson, NY, United States) was used to enzymatically analyze CK levels. Mb, IL-6, cortisol, and testosterone levels were tested using commercially available enzyme-linked immunosorbent assay (ELISA) kits. These kits were the Human Myoglobin ELISA Kit (Immunology Consultants Laboratory Inc., Portland, OR, United States), IL-6 ELISA Kit (Thermo Fisher Scientific Inc., Waltham, MA, United States), Cortisol ELISA (Immuno-Biological Laboratories Inc., Minneapolis, MN, United States) and Testosterone (Free) ELISA (Immuno-Biological Laboratories Inc., Minneapolis, MN, United States), respectively, and were used following the manufacturers' protocols. The lipid peroxidation marker (malondialdehyde, MDA) was measured using a TBARS assay kit (Cayman Chemical Company, Ann Arbor, MI, United States).

Statistical Analyses

All statistical analyses were performed using the SPSS 22.0 software (SPSS, Chicago, IL, United States). The data in figures and tables were expressed as changes from baseline. The Shapiro–Wilk normality test was used to analyze the normal distribution of all indicators. The normally and non-normally distributed variables are presented as mean ± standard error and 1st quartile median–3rd quartile, respectively. For non-normally distributed variables (soreness, CK, Mb, IL-6, testosterone, peak torque, and average power), the Friedman non-parametric statistical test was used to analyze the results at different time points within groups. The Wilcoxon signed rank test was used to determine the difference between N and HH trial results at the same time point. For normally distributed variables (TBARS and cortisol), two-way ANOVA with repeated measure was used to analyze mean differences. The results were considered to be significant when $p < 0.05$.

RESULTS

Post-exercise Muscle Soreness Was Reduced by HH Preconditioning

Muscle soreness is shown in **Table 2**. In both trials, muscle soreness increased significantly ($p < 0.05$) 0 to 48 h after RE compared to baseline. The muscle soreness levels of the HH trial were significantly lower than those of the N trial at 24 h after RE.

Post-exercise Circulating Muscle Damage Markers Were Reduced by HH Preconditioning

The circulating biochemical markers are shown in **Figures 1–3**. The CK levels were significantly increased at 0 and 24 h after RE in the N trial and only significantly increased at 24 h after RE in the HH trial. The CK levels of the HH trial were significantly lower than those of the N trial at 24 and 48 h after RE (**Figure 1A**). In both trials, the Mb levels increased immediately after RE and returned to baseline 24 to 48 h after RE. The Mb levels of the HH trial were significantly lower than those of the N trial at 0 h after RE (**Figure 1B**). RE did not increase TBARS levels significantly in both trials. There was no statistically significant TBARS level difference between trials at all time points (**Figure 2A**). The IL-6 levels only increased significantly from baseline in the HH trial at 0 h after RE and were significantly higher than those of the N trial at both 0 and 24 h (**Figure 2B**). Cortisol and testosterone levels did not significantly change after RE in both trials. No statistically significant differences of cortisol and testosterone were found between the N and HH trials at all time points (**Figures 3A,B**).

TABLE 2 | The muscle soreness measured before preconditioning and post-exercise in N and HH trials.

	Baseline	0 h	24 h	48 h
N	0.0 (0.0–0.0)	5.0 (4.0–5.0)†	4.5 (4.0–5.0)†	4.0 (3.0–4.0)†
HH	0.0 (0.0–0.0)	4.0 (4.0–4.0)†	4.0 (4.0–4.0)†,*	3 (3.0–3.5)†

The values are median (interquartile range; n = 11). N, normoxia; HH, hypoxia–hyperoxia.
†p < 0.05 vs. Baseline.
**p < 0.05 vs. N trial.*

HH Preconditioning Had no Effect on Isokinetic Muscle Strength and Muscle Endurance Following Exercise

The peak torque and average power of knee extension are shown in **Figure 4**. Peak torque decreased significantly immediately after RE and returned to baseline 24 to 48 h after RE in both trials (**Figure 4A**). Average power increased significantly at 48 h after RE in both trials (**Figure 4B**). However, no significant difference was observed between trials at all time points (**Figures 4A,B**).

DISCUSSION

The biochemical and mechanical stresses caused by RE included inflammatory response, free radical generation, and muscle cell damage. These stresses temporarily cause reduced skeletal muscle function. To examine the potential of acute HH preconditioning to impact recovery from RE, we measured a number of markers including muscle soreness, cell damage, inflammation, oxidative damage, and isokinetic muscle function. The major findings of this study are as follows: (1) Acute HH preconditioning showed a protective effect resulting in reduced muscle damage after RE when compared to the N trial. This was evidenced by decreased circulating levels of CK and Mb, and reduced muscle soreness. (2) Interestingly, inflammatory cytokine levels after RE were increased with HH preconditioning, evidenced by elevation of IL-6 levels. (3) Catabolic, anabolic signal, and isokinetic muscle function response to RE were not affected by HH preconditioning. Taken together, our data suggest that the RE-induced muscle damage is partly blunted by HH preconditioning in young male athletes.

Levels of CK and Mb are important circulating indirect indicators of muscle damage response to strenuous or unaccustomed exercise (Sorichter et al., 1999). In agreement with previous RE studies (Bartolomei et al., 2017), increased levels of CK and Mb after RE protocols were observed in this study. In addition, RE-induced muscle damage levels were reduced by HH treatment according to CK and Mb data (**Figure 1**). A previous study reported that chronic HH treatment could reduce stress and damage under some conditions. Four days of IHHT pre-treatment could reduce serum troponin I levels after coronary artery bypass graft (CABG) surgery compared to sham treatment in patients with ischemic heart disease (Tuter et al., 2018). However, to the best of our knowledge, this is the first study using acute repeated HH treatment to blunt stress-induced physiological damage.

ROS plays an important role in muscle damage after exercise. However, the results demonstrated that circulating TBARS levels were not significantly elevated after RE in N trial (**Figure 2A**). This result is inconsistent with previous studies which reported

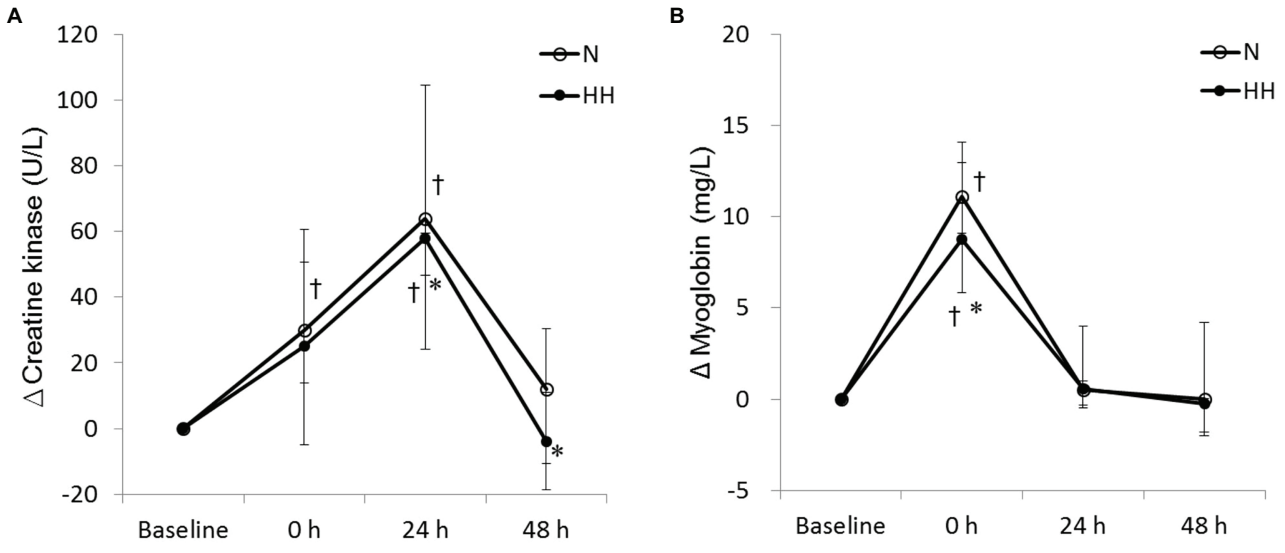

FIGURE 1 | Changes in circulating creatine kinase **(A)** and myoglobin **(B)** levels before preconditioning and post-exercise in N and HH trials. The values are 1st quartile median–3rd quartile ($n = 11$). N, normoxia; HH, hypoxia–hyperoxia. $^{\dagger}p < 0.05$ vs. Baseline. $^{*}p < 0.05$ vs. N trial.

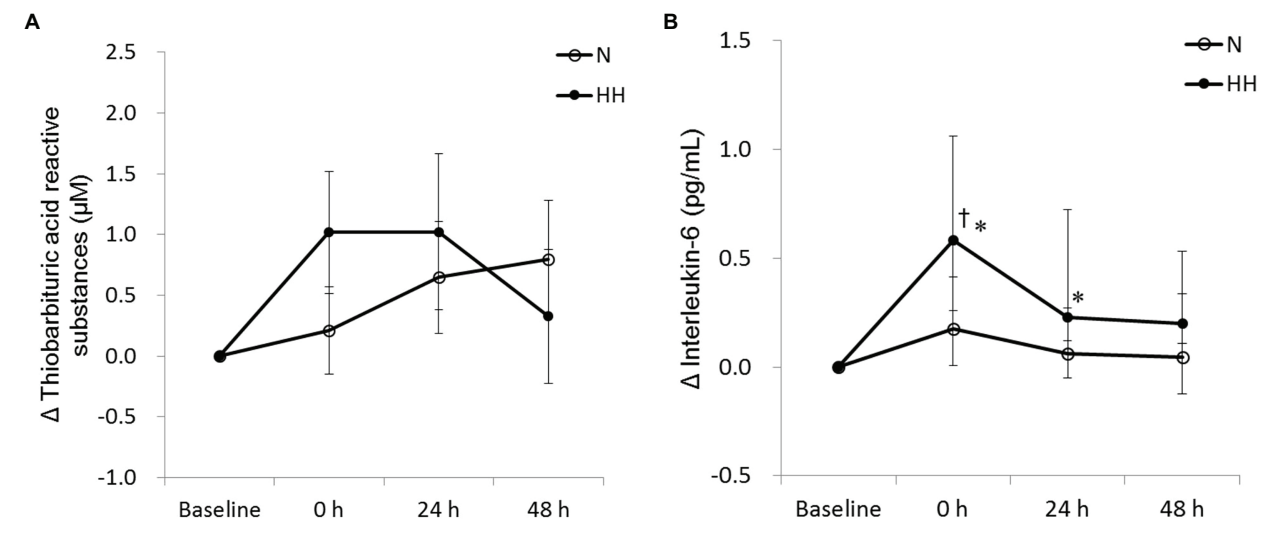

FIGURE 2 | Changes in circulating thiobarbituric acid reactive substances (TBARS; **A**) and interleukin-6 **(B)** levels before preconditioning and post-exercise in N and HH trials. The values of TBARS and interleukin-6 are presented mean ± SE or 1st quartile median–3rd quartile, respectively ($n = 11$). N, normoxia; HH, hypoxia–hyperoxia. $^{\dagger}p < 0.05$ vs. Baseline. $^{*}p < 0.05$ vs. N trial.

elevated lipid peroxidation after RE (Güzel et al., 2007; Deminice et al., 2010). The inconsistent results may be explained by low sensitivity of TBARS assay (Frijhoff et al., 2015). In addition, 2 weeks of daily hypoxia (10% O_2)–hyperoxia (30% O_2) treatment can reduce acute hypoxia (Gonchar and Mankovska, 2012a) or Fe^{2+}/ascorbate (Gonchar and Mankovska, 2012b)-induced lipid peroxidation. However, in this study, results showed that lipid peroxidation increased marginally ($p = 0.071$ by t-test) in the HH trial immediately after exercise, evidenced by increased TBARS levels after exercise (**Figure 2A**). It is possible that this TBARS response may be due to the stress induced by HH preconditioning, but without oxidative stress measurements during HH preconditioning this conclusion is uncertain.

Circulating IL-6 often increases after an acute bout of RE, which indicates an inflammatory response following exercise. In this study, circulating IL-6 levels of N trial were not changed significantly after acute RE (**Figure 2B**). This result was inconsistent with previous studies (Nieman et al., 2004; Kraemer et al., 2014). Similar to the results presented here, a report indicated that acute RE at varying volume loads could not elevate plasma IL-6 levels significantly in human subjects (Raines et al., 2020). Intriguingly, in the present study, RE-induced

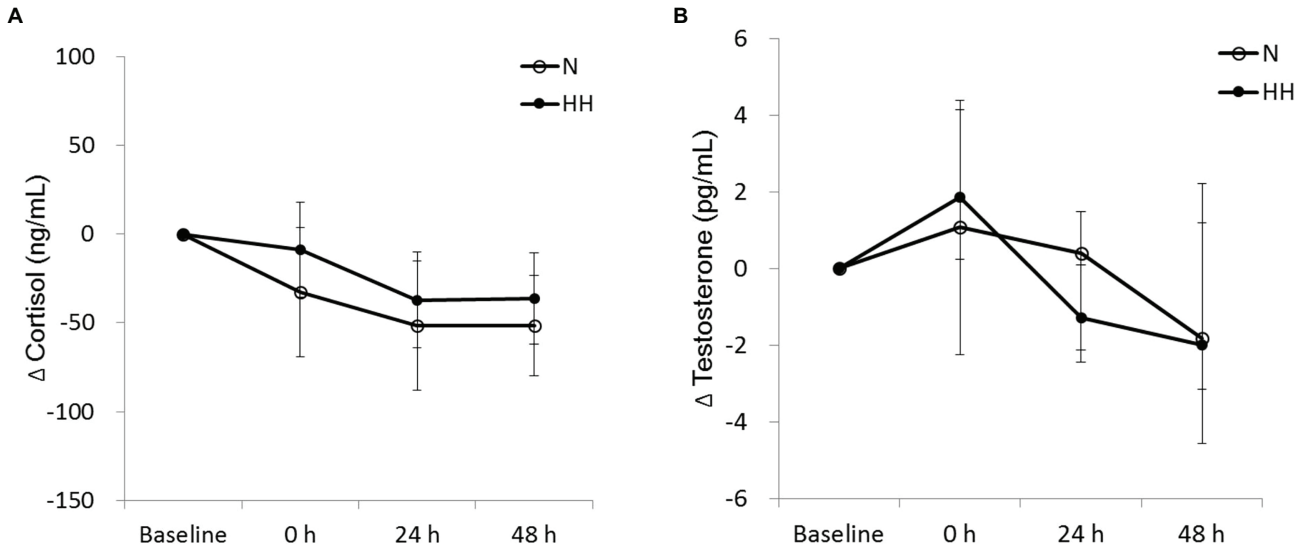

FIGURE 3 | Changes in circulating cortisol **(A)** and testosterone **(B)** levels before preconditioning and post-exercise in N and HH trials. The values of cortisol and testosterone are presented mean ± SE or 1st quartile median–3rd quartile, respectively ($n = 11$). N, normoxia; HH, hypoxia–hyperoxia.

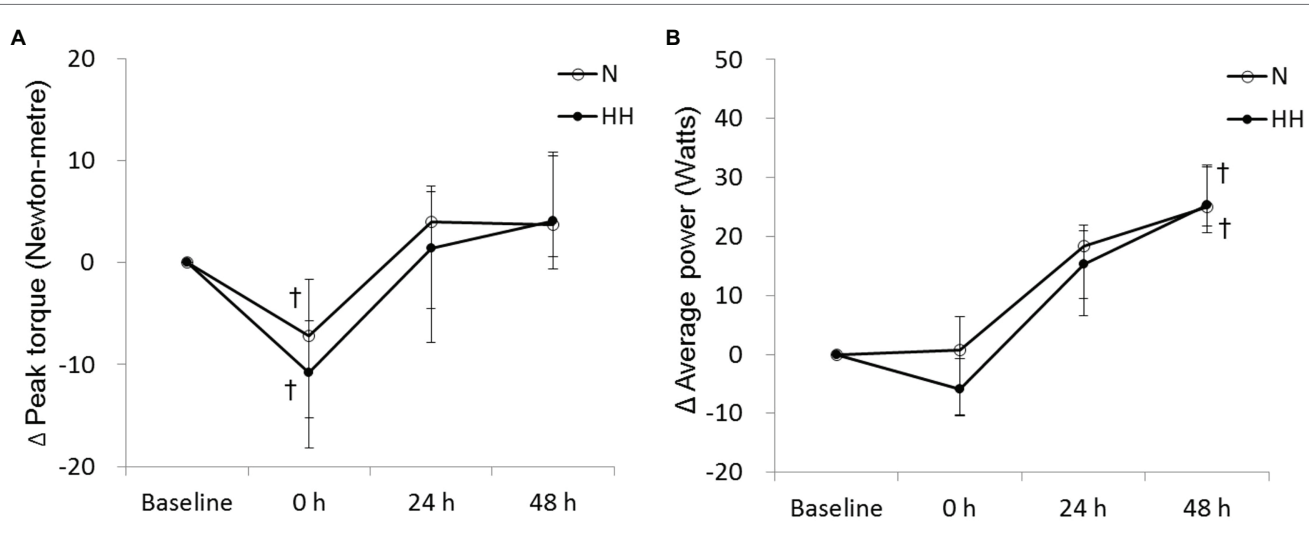

FIGURE 4 | Changes in peak torque **(A)** and average power **(B)** levels during maximal voluntary contraction (MVC) test before preconditioning and post-exercise in N and HH trials. The values are 1st quartile median–3rd quartile ($n = 11$). N, normoxia; HH, hypoxia–hyperoxia. †$p < 0.05$ vs. Baseline.

IL-6 level increases only appeared in HH treatment trial (**Figure 2B**). IL-6 in circulation after exercise is released mainly from the contracting muscle (Fischer, 2006). Increased IL-6 expression in conjunction with higher levels of muscle damage indicators is usually associated with the disruption of myofibers after exercise (Tomiya et al., 2004). However, in this study, lower levels of CK and Mb in HH trial showed the decreased muscle damage as expected, but unexpectedly IL-6 levels increased. It is speculated that higher release of IL-6 in the HH trial may be explained by activation of the IL-6-related signaling pathway during HH treatment, such as NFκB, Ca^{2+}/NFAT, or p38 MAPK. Exercise activates these redox-sensitive intercellular signaling pathways, which in turn cause intramuscular IL-6 release and expression. This signaling pathway activation and IL-6 release during exercise has been shown to be blunted by antioxidant supplementation (Fischer et al., 2004). Therefore, we suggest that HH treatment-induced oxidative stress may activate more IL-6-related signaling pathways and cause more IL-6 release and production. Unfortunately, the related signaling pathway levels in muscle tissue were not measured in this study.

In addition, in this study, elevated IL-6 is proposed as the cause of attenuated muscle damage in HH trial (**Figure 2B**). IL-6 has been reported as an anti-inflammatory cytokine

(Petersen and Pedersen, 2006). For example, anti-IL-6 receptor monoclonal antibody (IL-6r mAb) treatment increased inflammatory response in the mouse model of Duchenne Muscular Dystrophy (mdx; Kostek et al., 2012). Recombinant human IL-6 (rhIL-6) infusion could attenuate *E. coli* lipopolysaccharide endotoxin-induced increase in tumor necrosis factor α (TNF-α) in healthy humans (Starkie et al., 2003). Inflammation plays an important role in muscle damage after an acute bout of RE. Anti-inflammatory treatment, such as Ibuprofen, a non-steroidal anti-inflammatory drug, has been shown to decrease exercise-induced muscle damage (Fraga et al., 2020). The results of this study suggest that the increased IL-6 release in the HH trial will reduce post-exercise inflammation and result in reduced secondary damage of skeletal muscle after RE.

Release of IL-6 is an essential mediator of satellite cell-mediated skeletal muscle regeneration and hypertrophy. IL-6 induced signaling is associated with satellite cell proliferation following acute contraction-induced muscle damage in human subjects (Toth et al., 2011). In cardiotoxin-induced muscle injury/regeneration (Zhang et al., 2013) and compensatory hypertrophy (Serrano et al., 2008) models, myoblast proliferation and muscle regeneration are attenuated in IL-6 knock-out mice compared to wild type mice. The magnitude of IL-6 response after acute resistance is positively correlated with chronic changes in muscle fiber cross-sectional area (CSA) following 16 weeks of resistance training (Mitchell et al., 2013). Accordingly, we hypothesize higher post-exercise IL-6 response induced by HH preconditioning may accelerate skeletal muscle adaptation after chronic resistance training. However, long-term adaptation to chronic HH preconditioning combined with resistance training was not investigated in the present study and remains to be explored.

In the present study, our data show that isokinetic muscle strength decreased significantly immediate after RE in both trials (**Figure 4A**), but returned to baseline after 24 h. These results are consistent with a previous study (Paulsen et al., 2014). However, the decreased muscle strength after exercise was similar for N and HH trials, and no significant muscle strength difference was found between HH and N trials. The positive effects of HH preconditioning on muscle damage and soreness were strangely not reflected in the resulting muscle strength. We suggest that the HH preconditioning-induced excessive oxidative stress, evidenced by increased TBARS levels, may be contrary to force generation (Powers and Jackson, 2008). Alternatively, influence by other fatigue-related factors cannot be excluded, such as levels of phosphocreatine or glycogen.

Post-exercise circulating cortisol and testosterone levels indicated catabolism and anabolism balance, respectively. Cortisol and testosterone levels often increased significantly immediately after strenuous exercise (Hug et al., 2003). However, in this study, the data showed that when compared to baseline cortisol and testosterone levels do not change significantly immediately after RE in both trials (**Figure 3**). Cortisol and testosterone levels in blood have large diurnal variation, decreasing from morning to evening (Hayes et al., 2010). Because the time gap between the baseline (before preconditioning) and 0 (immediately after exercise) points is about 2.5 h, we suggest that exercise-induced cortisol and testosterone variations were hidden by larger changes within this period because of the diurnal cycle. In addition, testosterone is a signal for muscle protein synthesis, resulting in muscle growth and other adaptations to resistance training (Hayes et al., 2010). In this study, testosterone levels of HH trial marginally ($p = 0.062$ by Wilcoxon signed rank test) decreased 24 h after exercise (**Figure 3B**), indicating reduced anabolic signaling. Consequently, we cannot exclude the possibility of decreased training adaptation after chronic resistance training with HH preconditioning.

The underlying mechanism for this HH preconditioning benefit remains unclear. Similar to the hormesis effect, appropriate stressor pre-exposure can result in reduced muscle damage during exercise. For example, preconditioning using hyperthermia (Nosaka et al., 2007), ischemia (Franz et al., 2018), or light exercise (Nausheen et al., 2017) have been shown to reduce RE-induced muscle damage and inflammation. In a previous cell culture study, intermittent hypoxia–hyperoxia treatment elevated oxidative stress production in human airway smooth muscle cells (Bartman et al., 2021). Chronic intermittent hypoxia–hyperoxia also increased oxidative damage in lung tissue of neonatal rodents (Dylag et al., 2017; Mohamed et al., 2020). Thus, we hypothesize that slight oxidative stress induced during HH preconditioning may be responsible for attenuating the subsequent RE-induced muscle damage. In addition, an increased antioxidant protein expression was induced by repeated HH treatment in murine cells (Wohlrab et al., 2021), which might improve the function of the oxidative defense systems. Consequently, we speculate that HH-induced oxidative stress might activate the redox-sensitive signaling system and then enhance the antioxidant defense system. Furthermore, in order to optimize the favorable effect of HH preconditioning, the exposure time, oxygen concentration, and numbers of repetitions of HH intervention could be modified in future research.

The purpose of this study was to analyze the protective effects of acute repeated HH preconditioning on RE-induced muscle damage. The results are ambivalent because a favorable effect of HH treatment on muscle damage and soreness markers was observed, but muscle performance was not significantly affected by the different protocols. It appears that the degree of muscle damage reduced by HH treatment is not enough to influence exercise performance. Muscle damage is induced during sports training and competition and influences training quality and exercise performance (Smith, 1992). In conclusion, we suggest that acute repeated HH treatment has the potential to be developed and optimized as a physical intervention strategy for sports training and competition to prevent muscle damage. The future studies should focus on underlying mechanisms and impact in chronic training applications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The procedure of this study was reviewed and approved by the University of Taipei Institutional Review Board, Taipei, Republic of China (Taiwan; Approval number: IRB-2016-052).

AUTHOR CONTRIBUTIONS

P-WC contributed by recruiting subjects, collecting data, and analyzing statistics. C-CH, L-FL, and C-PC contributed by recruiting subjects and collecting data. S-HY contributed by conceiving the study, interpreting the results, and writing the manuscript. All authors contributed to the article and approved the submitted version.

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Short-Term Oral Quercetin Supplementation Improves Post-exercise Insulin Sensitivity, Antioxidant Capacity and Enhances Subsequent Cycling Time to Exhaustion in Healthy Adults: A Pilot Study

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Aim: Quercetin has been reported to have antioxidant and anti-inflammatory properties on health promotion in human studies. The main purpose of this study was to investigate the effect of short-term oral quercetin supplementation on post-exercise whole-body energy metabolism. This study also aimed to determine the effects of supplementation on oxygen stress, inflammation, muscle damage, and high-intensity cycling exercise performance.

Method: Twelve healthy participants, physically active students, were recruited to perform a randomized, single-blind crossover study. All subjects completed 7-days of quercetin (quercetin:1,000 mg per day for 7-days) and placebo supplementation in a randomized order. Supplement/placebo was combined with exercise consisting of 70% $\dot{V}O_{2max}$ cycling for 60-min, followed by 3-h of recovery, then a subsequent single bout of cycling exercise with 75% $\dot{V}O_{2max}$ to exhaustion. Time to exhaustion, indicators of muscle damage, as well as blood and gaseous parameters relating to energy metabolism, oxidative stress, inflammatory response, respectively, were determined.

Results: The results showed that 7-day quercetin supplementation significantly attenuated the post-exercise glucose-induced insulin response, increased total antioxidant capacity (TAC) and superoxidase dismutase (SOD) activities, and mitigated malondialdehyde (MDA) levels during the recovery period ($p < 0.05$). While subsequent 75% $\dot{V}O_{2max}$ cycling performance was significantly improved after quercetin treatment

and accompanied by lower responses of interleukin 6 and creatine kinase at 24-h. However, it's noted that there were no significant responses in glucose, respiratory exchange rate, tumor necrosis factor-α (TNF-α), myoglobin, and high sensitivity C-reactive protein between quercetin and placebo trials.

Conclusion: Our findings concluded that 7-day oral quercetin supplementation enhances high-intensity cycling time to exhaustion, which may be due in part to the increase in whole-body insulin-stimulated glucose uptake and attenuation of exercise-induced oxygen stress and pro-inflammation. Therefore, quercetin may be considered an effective ergogenic aid for enhancing high-intensity cycling performance among young adults.

Keywords: exercise, time to exhaustion, ergogenic aids, quercetin, insulin-stimulated glucose uptake

INTRODUCTION

Attenuating oxidative stress, inflammation, and muscle damage induced by high-intensity exercise is the key factor for improving exercise performance (1). These effects have been shown in both animal and human models. Uchiyama et al. reported that rats had elevated levels of reactive oxygen species (ROS) and malondialdehyde (MDA) following repeated maximal resistance exercise until fatigue (2). This same study also indicated that muscle damage to the plantar flexor was highly associated with creatine kinase (CK) concentrations (2). As for humans, high-intensity exercise such as running up and down hills for 60 min has been shown to increase oxidative stress, inflammation, and muscle damage (3). Yet another study, involving 18 athletes, found that the concentration of thioredoxin (TRX) and myeloperoxidase (MPO) increased after completing a triathlon (1). In addition, an increase was also observed in CK levels in response to high-intensity triathlon exercise (1). Interestingly, 3-week supplementation of lactobacillus Plantarum PS128, an antioxidant supplement, attenuated exercise-induced oxidative stress and muscle damage levels which resulted in improved performance among the triathletes (1). Therefore, Huang's study suggests that assuaging oxidative stress, inflammation, and muscle damage induced by mid- and high-intensity exercise may be an important physiological factor for enhancing performance.

Quercetin is a type of phytochemical belonging to the flavonoid family, hence it has antioxidant effects and the ability to decrease free radicals in the body (4). Quercetin can be purified from fruits and vegetables including onion, blueberry, and broccoli. Results from animal cell and human experiments show that quercetin has beneficial physiological effects such as improving insulin-stimulated whole-body glucose uptake, antioxidant capacity, and anti-inflammatory responses (5, 6). Additionally, Nieman et al., demonstrated the positive influence of fourteen days of quercetin (1,000 mg/day) exercise performance of 12-min time trial on the treadmill walking immediately after 60 min of moderate exercise preloads at 60% maximal oxygen uptake ($\dot{V}O_{2max}$) (7). McAnulty et al., evaluated the chronic quercetin effect on exercise-induced oxidative damage and inflammation, they recruited fourteen trained male cyclists who consumed 1,000 mg quercetin each day for 6 weeks before and during 3 days of cycling at 57% work maximum for 3 h (8). As compared to Nieman's and McAnulty's studies, we observe the participants in the present study performing 75% $\dot{V}O_{2max}$ cycling exercise and recorded the time to exhaustion for all subjects following a 3-h recovery period at 70% $\dot{V}O_{2max}$ exercise preload for 60 min under the shorter term of quercetin challenge (1,000 mg/day for 7 days). The questions of short-term quercetin and human exercise study need clarity regarding simulated cycling exercise challenge and post-exercise recovery during multiple races for cyclists in a one-day competition. However, some papers showing the ergogenic properties of quercetin were contrary (9–12). No effect of acute quercetin supplementation (2,000 mg) was found on 15 min cycling time trial following 30 min cycling exercise at a 50% $\dot{V}O_2$ peak under environmental circumstances of 40°C in non-heat-acclimated male volunteers by Cheuvront (9). Short-term (14 days) and long-term (6 weeks) quercetin supplementation (1,000 mg/d) both showed no ergogenic effect on the non-invasive measure of muscle oxidative capacity, physical fitness in healthy males, and $\dot{V}O_2$ peak measurement (10, 11). Moreover, Pelletier indicated that quercetin is unlikely to be evidence ergogenic for aerobic-oriented exercises in trained and untrained individuals by meta-analysis study (12). Nevertheless, whether the supplementation of quercetin alone can also improve the antioxidant enzymes and eliminate the oxidative stress caused by exercise remains unclear. Yet, no published human data are available regarding the effect of quercetin on post-exercise observation on whole-body insulin-stimulated glucose uptake, and indicators of oxidant stress, inflammation, muscle damage is a favor to improve subsequent cycling time to exhaustion. Especially, we wanted to demonstrate the effect of short-term quercetin supplementation (1,000 mg per day for 7 days) on exercise-induced oxidant damage and inflammation levels under simulating cycling competitions after exercise preloading.

It has been shown that the rate at which energy is replenished post-exercise, as well as decreased oxidative stress, inflammation, and muscle damage, is important for subsequent endurance exercise performance (13). Thus, athletes commonly use antioxidant supplements to accelerate energy recovery and eliminate oxidative stress and inflammation induced by exercise. In the present study, we explored whether

7-day quercetin supplementation can affect the blood energy metabolism index [glucose, insulin, and non-esterified fatty acids (NEFA)], oxidative markers [total antioxidant capacity (TAC), superoxidase dismutase (SOD), and MDA], inflammation markers [interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α)], muscle damage markers [CK, myoglobin (MB), and high sensitivity C-reactive protein (hs-CRP)], and time-to-exhaustion during two consecutive high-intensity cycling exercise bouts.

MATERIALS AND METHODS

Subjects

The sample size was calculated in the present study using G* power (3.1.9.4) software assuming an effect size of 1.07 as observed previously in the human study regarding the ergogenic property of exercise time to exhaustion (14, 15). Therefore, 12 healthy male participants, physically active students, were recruited for this study supposing that the alpha error and statistic power are set at 0.05 and 90% (16). On average, their age was 20.79 ± 0.53 years old, the height of 171.10 ± 2.52 cm, bodyweight of 66.64 ± 2.67 kg, body mass index (BMI) of 22.86 ± 0.56 kg/m^2, and the $\dot{V}O_{2max}$ of 45.18 ± 1.74 mL/kg/min, respectively. Prior to the experimental period, all participants were asked to familiarize with the bicycle ergometer, and perform the $\dot{V}O_{2max}$ test. During the recruiting process, the participants who were excluded included those whose chronic medical history such as cardiovascular disease, diabetes, musculoskeletal, or neuromuscular and were unable to complete the $\dot{V}O_{2max}$ test under trouble respiration. All participants kept their dietary habits and avoid consuming food, supplements, and beverages not including antioxidant and anti-inflammation (e.g., coffee, tea, cola, chocolate, drugs, and nutritional products) during the experimental period. The experimental protocol was approved by the Institutional Review Board at the University of Taipei, Taipei, Taiwan (UT-IRB-2018-085). The testing protocol was thoroughly explained to the participants and informed consent forms were signed in order to participate. Participants were allowed to voluntarily remove themselves from the study at any time without reason.

Experimental Design and Procedure

Twelve healthy participants, physically active students, were recruited for the single-blind crossover study design. Participants were randomly assigned the placebo (P) or Quercetin (Q). The trial order for the 12 subjects was randomized so that six subjects with quercetin trial, while six subjects stared with the placebo. The crossover trial was repeated after completion of the first trial with the 14-day washout period, all subjects accept the implementation of the random allocation sequence occurring without the knowledge of which supplements were received by themselves using the identical appearance of the capsule with quercetin and placebo. The participants received placebo or quercetin for seven consecutive days with a 14-day washout period between the two trials, as shown in **Figure 1**, the participants' $\dot{V}O_{2max}$ was measured, and the power for the cycle ergometer was calculated individually. During the trial, subjects were instructed to keep their diet habits, control the energy intake, receive the meals provided by investigators, and avoid strenuous exercise until the day of the experiment. Caffeine intake, smoking, ergogenic supplements, anti-inflammatory drugs, and food items including polyphenol and vitamins were not allowed. All participants received the light breakfast diet and the identical lunch and supper (total energy per meal of lunch/supper is 700.60 kcal, carbohydrate, 77.90 g; fat, 26.60 g; protein, 37.10 g) during the 24 h before trial. To achieve the simulating glycogen depletion was performed in the first cycling exercise challenge, participants fasted for 12-h before the day of the experiment. At 7 am on the next morning, participants reported to the lab and their height and body weight were measured, followed by a cycling challenge at 70% of $\dot{V}O_{2max}$ for 60 min while maintaining 60 rpm (17). Immediately after the first exercise challenge, participants consumed the light diet contained 60% carbohydrate with 1,000 mg quercetin/placebo (60% carbohydrate, 24.12 ± 0.20 g; 25% fat, 16.82 ± 0.21g; 15% protein, 16.12 ± 0.20 g; 304.12 ± 1.20 kcal). Approximately 3 h (post-exercise recovery) later, participants performed a cycling test at 75% of $\dot{V}O_{2max}$ until exhaustion. Time-to-exhaustion was recorded. Blood and gas samples were collected before and after the first cycling bout, during post-exercise recovery, and after exhaustion.

Maximal Oxygen Uptake Test

Participants performed the $\dot{V}O_{2max}$ test on a cycle ergometer (Monark, Varberg, Sweden) while each breath was assessed with gas analyzers (Cortex Biophysik, Non-nenstrasse, Leipzing, Germany). The participants maintained 60 RPM's throughout the test. The primary workload was at 0.5 kg for 4 min and then increased by 0.5 kg every 2-min until exhaustion. The determination of $\dot{V}O_{2max}$ was based on three criteria: (1) Respiratory exchange ratio (RER) > 1.10; (2) $\dot{V}O_{2max}$ variance < 2 mL/kg/min; and (3) target heart rate reaches a theoretical maximum value at "220-age" (18). Accordingly, the value obtained from the Y-axis was oxygen consumption (ml/kg/min); the value obtained from the X-axis was the workload that corresponded to the oxygen uptake. $\dot{V}O_{2max}$ (100%) was determined when the curve reached the plateau. The value multiplied by 0.70 was defined as 70% $\dot{V}O_{2max}$ as the intensity for the cycling challenge; multiplied by 0.75 was defined as 75% $\dot{V}O_{2max}$ as the intensity for the subsequent time-to-exhaustion test, and the corresponding value from X-axis was the workload used during the formal experiments (19).

Quercetin and Placebo Supplement

The participants received placebo or quercetin for seven consecutive days with a 14-day washout period between the two trials. During seven consecutive days, the participants ingested either two quercetin capsules or two placebo capsules after breakfast at 8 am in the lab. On the exercise challenge experimental morning, the participants ingested either two quercetin capsules or two placebo capsules immediately after the first exercise challenge. Each quercetin capsule (GNC Holdings Inc., United States) was 500 mg while each placebo capsule was 500 mg of cellulose. Thus, the participants received 1,000 mg

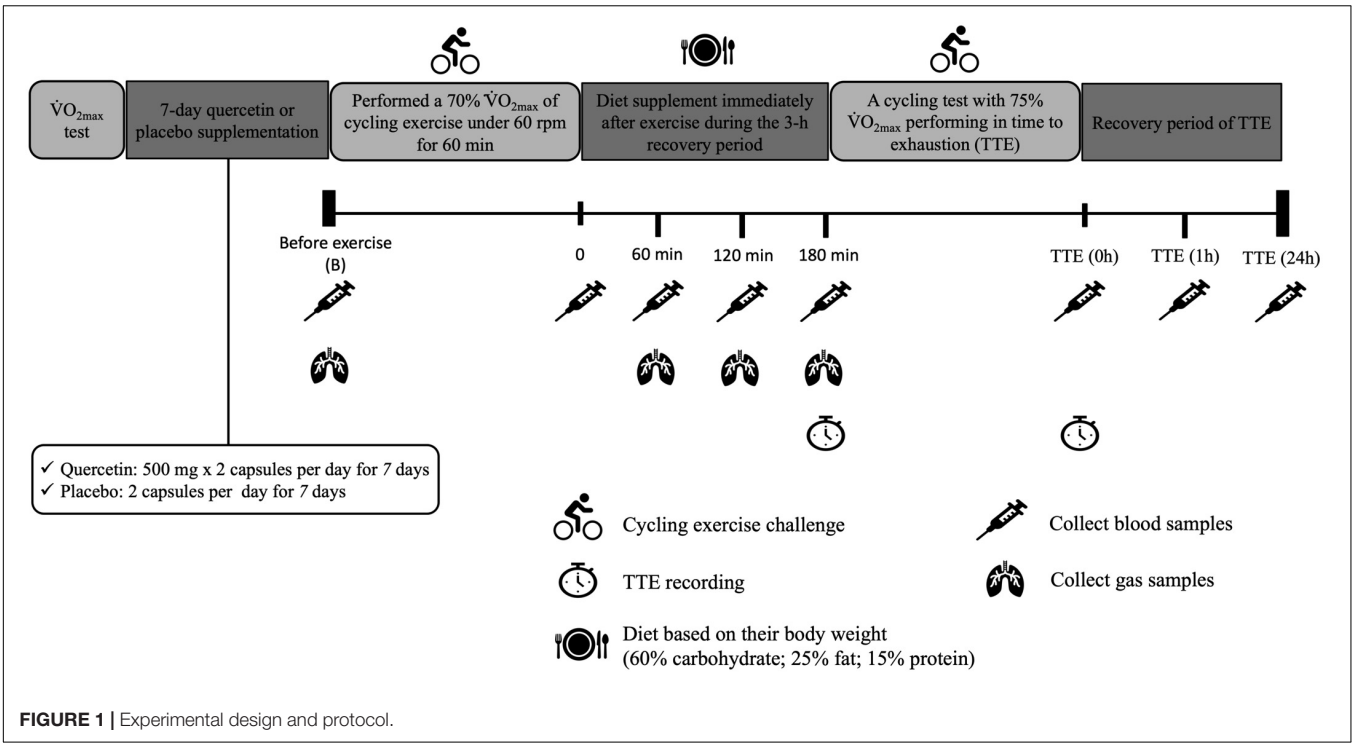

FIGURE 1 | Experimental design and protocol.

quercetin or cellulose per day. This dosage of quercetin was within safe limits for human intake (20).

Blood Sample Collection and Analysis

Blood samples were collected before exercise, 1st exercise recovery period, and 2nd post-exercise 0, 1, and 24 h. All blood samples were tested for the exercise-induced status of oxygen stress, inflammation, and muscle damage. Blood samples were collected and centrifuged at 1,000 *g* for 10 min. Afterward, the supernatant was collected and placed in a freezer at –20°C. The supernatant was used to measure the following: insulin, NEFA, TAC, SOD, MDA, IL-6, TNF-α, CK, MB, and hs-CRP. Blood glucose concentration was determined by an automated glucose analyzer (YSI Life Sciences). Plasma insulin levels were determined using a commercial kit (Roche Diagnostics, Mannheim, Germany). Streptavidin microparticle interacted with anti-insulin AB-biotin and anti-insulin AB-Ru (bpy) 32+ to emit chemiluminescence (Roche Elecsys 1010/2010, Roche Diagnostics; MODULAR ANALYTICS E170, Roche Diagnostics) for analyzing the insulin levels and calculation of insulin sensitivity index (ISI) (21). Plasma NEFA was measured using a commercial kit (Wako, Neuss, Germany) in combination with an automated biochemical analyzer (Hitachi Science Systems, Ltd., Lbaranki, Japan). TAC, MDA, SOD, IL-6, and TNF-α can be used to determine the status of oxidative stress and inflammation. TAC, MDA (Sigma, St. Louis, MO, United States), and SOD (Cayman Chemical Company, ANN Arbor, MI, United States) were measured using commercial kits (Tecan GENios, A -5082, Austria). The levels of TAC, MDA, and SOD were determined by absorbance at 734, 532, and 570 nm wavelength using the standard curve. Cytokine IL-6 (Bio-Legend Inc., San Diego, CA, United States) and TNF-α (Bio-Legend Inc., San Diego, CA, United States) were measured using a commercially available reader (Tecan GENios, A-5082, Austria) to read the absorbance at 450 and 570 nm wavelengths to calculate concentrations in the serum samples. Additionally, CK, MB, and hs-CRP served as the biomarker for muscle damage. CK (Cayman Chemical Company, ANN Arbor, MI, United States), MB (Wuhan Fine Biotech Co., Ltd., Hubei, China), and hs-CRP (Denka Seiken Co., Ltd., Tokyo, Japan) were measured using commercial kits. The

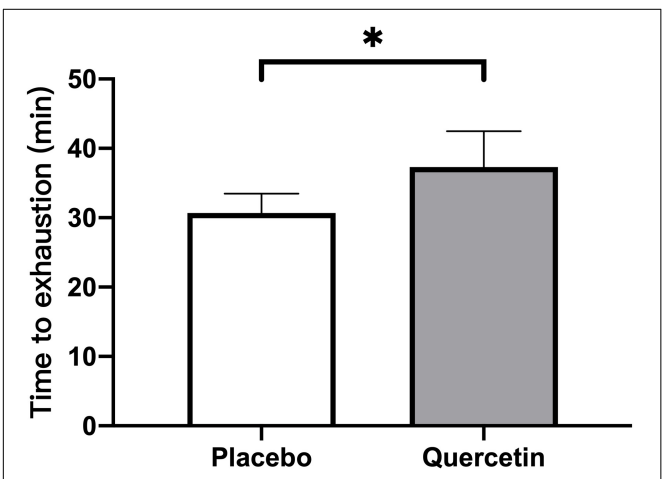

FIGURE 2 | Mean values data of all participants for the time-to-exhaustion tests of the second cycling exercise with 75% VO_{2max}. *Significant difference between quercetin and placebo ($p < 0.05$). Values are expressed as mean ± SE, $N = 12$.

concentration was determined using the reader (Tecan GENios, A-5082, Austria) to obtain absorbance at 450 and 570 nm wavelengths as mentioned in the previous section.

Gas Sample Collection and Analysis

Gas samples were collected via the gas analyzer every 60 min for 3 hours (for example, 60, 120, and 180 min) to determine RER during the post-exercise recovery period after subjects were given a carbohydrate-rich diet. RER was calculated based on the amount of CO_2 generated/VO_2.

Statistics

Values were presented as mean ± standard error (mean ± SE). SPSS software was used for statistical analysis. Paired t-test was used to analyze time-to-exhaustion. Repeated measure two-way ANOVA (trial × time) was used to determine the intra- and inter-difference at different time points and between the two groups with power set at 80%. If significant interaction was observed within and between groups, a simple main effects analysis was conducted. Fisher's least significant difference (LSD) was used for *post hoc* analysis. The α value was set at $p < 0.05$.

RESULTS

Quercetin Enhanced the Subsequence High-Intensity Cycling Performance to Exhaustion After 3-h Post-exercise Recovery of the First Exercise Challenge

We found that seven days of quercetin significantly enhanced the second cycling exercise performance to exhaustion (Quercetin: 37.29 ± 5.20 min; Placebo: 30.72 ± 2.16 min, $p < 0.05$) (**Figure 2**). Significantly lower insulin responses were found in quercetin than those of placebo at 60, 120, and 180-min after the first cycling exercise challenge (**Figure 3B**, $p < 0.05$). However, there was no significant difference in glucose and NEFA (**Figures 3A,C**) during the experimental procedures. No significant difference in whole-body carbohydrate and fat oxidation were found between the two trials calculated by gaseous samples during the first cycling exercise recovery period (**Figure 4**, $p > 0.05$).

Quercetin Enhanced Antioxidant Capacity and Resulted in Decreased Oxidative Stress After the Cycling Exercise Challenge

Total antioxidant capacity activity was enhanced by quercetin, evidenced by the response after the cycling exercise challenge (**Figure 5A**, $p < 0.05$). Similarly, the SOD concentrations were significantly higher in the quercetin than those of placebo (**Figure 5B**, $p < 0.05$). In addition, we measured oxidative stress MDA markers, which were significantly lower at 60, and 180-min during the first exercise recovery period and at the end of the second cycling exercise performance to exhaustion (**Figure 5C**, $p < 0.05$).

Quercetin Attenuated IL-6 and CK Levels Induced by the High-Intensity Cycling Exercise to Exhaustion After 3 h Post-eercise Recovery of 75% VO_{2max} Exercise Challenge

The effect of quercetin on circulating response regarding inflammation and muscle damage indicators was measured before and after two bouts of cycling exercise challenge. **Figures 6A**, **7A** showed the significant response of pro-inflammatory markers IL-6 and muscle damage indicators CK after Quercetin supplementation ($p < 0.05$). In this human study, IL-6 concentrations were significantly lower immediately, and 1-h after the second cycling exercise (**Figure 6A**, $p < 0.05$). The peak serum CK responded at 24-h after the second cycling challenge (**Figure 7A**). Identically, the concentrations of the serum CK were significantly lower at 24-h in the quercetin trial compared to the placebo trial (**Figure 7A**, $p < 0.05$). However, there were no differences in TNF-α (**Figure 6B**), MB (**Figure 7B**), and hs-CRP (**Figure 7C**) between the two trials. Therefore, we speculate that oral quercetin supplementation tends to attenuate IL-6 and CK concentrations after high-intensity cycling exhaustion challenges but no other inflammation and muscle damage indicators.

DISCUSSION

Quercetin is a polyphenolic flavonoid with antioxidant and anti-inflammatory properties (22–25). In the present human study, we hypothesized that 7-days of oral quercetin supplementation can boost antioxidant and anti-inflammatory responses as well as mitigate oxidative stress and inflammation caused by high-intensity cycling for 60 min. Furthermore, we hypothesized that supplementation would increase time to exhaustion during the second high-intensity cycling challenge. The major finding in the present study was significantly increased time-to-exhaustion during the second cycling challenge after a short-term (3 h) break following the first cycling bout (**Figure 2**). The compatible papers showing the ergogenic effect of quercetin are reported (7, 22, 23, 26). Nevertheless, some comparisons were made where the quercetin supplement in four published papers indicating the ergogenic properties of quercetin on exercise performance was contrary to the present study (9–12). The short-term model of quercetin supplementation in the present study is not the same as the acute condition as Cheuvront's study and the blood sampling about oxidant stress, inflammation, and muscle damage indicators in the present study are different from the non-invasive measurement of muscle oxidative capacity, physical fitness in Cureton or Bigelman studies. Nonetheless, we conservatively inferred that 1,000 mg/day of quercetin supplementation for a week improved a time trial performance following a moderate aerobic exercise, evidenced on post-exercise blood samples regarding increased systemic insulin-stimulated glucose uptake, antioxidant capacity, and decreased IL-6, CK levels.

As shown in **Figure 3B**, insulin levels by quercetin were significantly lower than those of placebo during the first

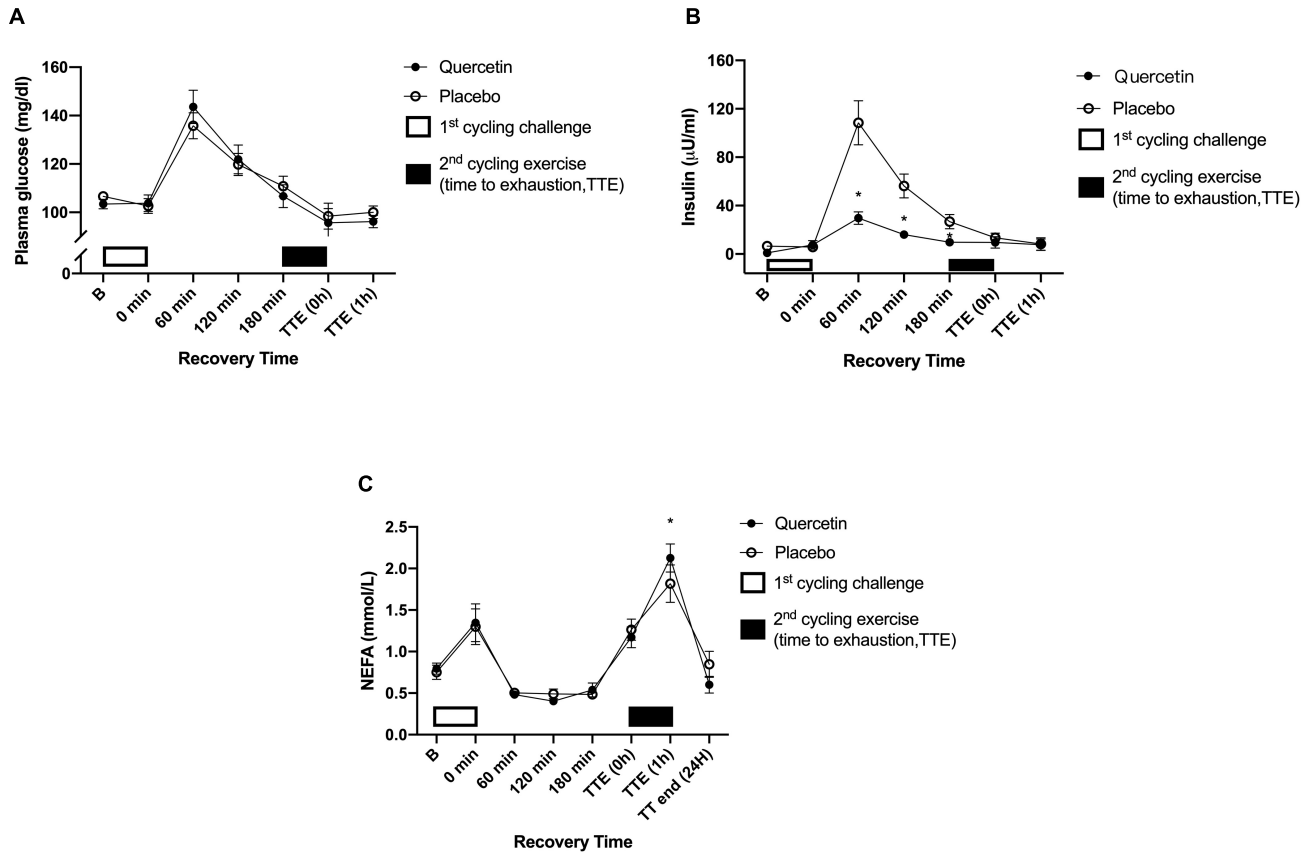

FIGURE 3 | Blood glucose **(A)**, plasm insulin **(B)**, and plasma non-esterified fatty acids (NEFA) **(C)** concentrations in (-●-) quercetin and (-○-) placebo trials. B represents before the first cycling challenge. TTE (0,1 h,24 h): represents immediately, 1-h, and 24-h after the second cycling exercise, respectively. *Significant difference between quercetin and placebo ($p < 0.05$). Values are expressed as mean ± SE, $N = 12$.

post-exercise recovery period. The calculation of area under the curve for glucose/insulin and insulin sensitivity index (ISI) are shown (IACU: Quercetin:3248.88 ± 1643.56 min × μU/ml; Placebo:13085.78 ± 6178.03 min × μU/ml, $p < 0.05$; GACU: Quercetin:671.50 ± 13.63 min × mg/ml; Placebo: 670.70 ± 17.51 min × mg/dl, $p > 0.05$; ISI: Quercetin: 24.85 ± 14.7; Placebo: 4.95 ± 1.32, $p < 0.05$). Presumably, these data led to increased muscle glycogen levels and endurance performance during subsequent exercises (27). In previous studies, Cheng et al. performed a single-blind, randomized, crossover study that involved post-exercise supplementation of hydroxycitric acid or placebo for a group of male subjects. The supplement significantly accelerated the rate of glycogen synthesis twofold in exercised human skeletal muscle compared to placebo; this increase occurred in parallel with increased whole-body insulin-stimulated glucose uptake, evidenced by reduced post-meal insulin response to the same carbohydrate meal challenge (28). Therefore, the synthesis of glycogen from glucose was promoted which is especially meaningful to energy replenishment during post-exercise recovery (13, 29). Guo et al. study showed that quercetin can positively regulate insulin receptor substrate 1 (IRS-1) serine/tyrosine phosphorylation and regulate insulin signaling through the downstream protein kinase B (Akt)/endothelial nitric oxide synthase (eNOS)-phosphoinositide 3-kinase (PI3K) pathway (30). Unfortunately, the present study did not perform the muscle biopsy to assess muscle glycogen results, and systemic fat oxidation utilization was not significantly enhanced by quercetin during post-exercise recovery (**Figure 4**). Therefore, we suggest that the higher insulin-stimulated glucose uptake found in this study enhanced replenishment function which would be conducive to the rapid restoration of muscle glycogen. This would then have a synergistic effect to improve time-to-exhaustion during exercise.

Following 7-days of quercetin supplementation antioxidant enzymes, TAC and SOD were changed significantly in the present study. Quercetin has been shown the most potent scavenger of ROS (31). The anti-oxidative capacities of quercetin are due to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e., the catechol group in the B ring and the OH group at position 3 of the AC ring (32). In the present study, the SOD activity and TAC by quercetin were significantly higher than those of placebo during the experimental period in parallel with significantly decreased levels of MDA, a plasma lipid peroxidation product caused by exercise (**Figures 5A–C**). TAC and SOD are considered the first line of defense against ROS, catalyzing the decomposition

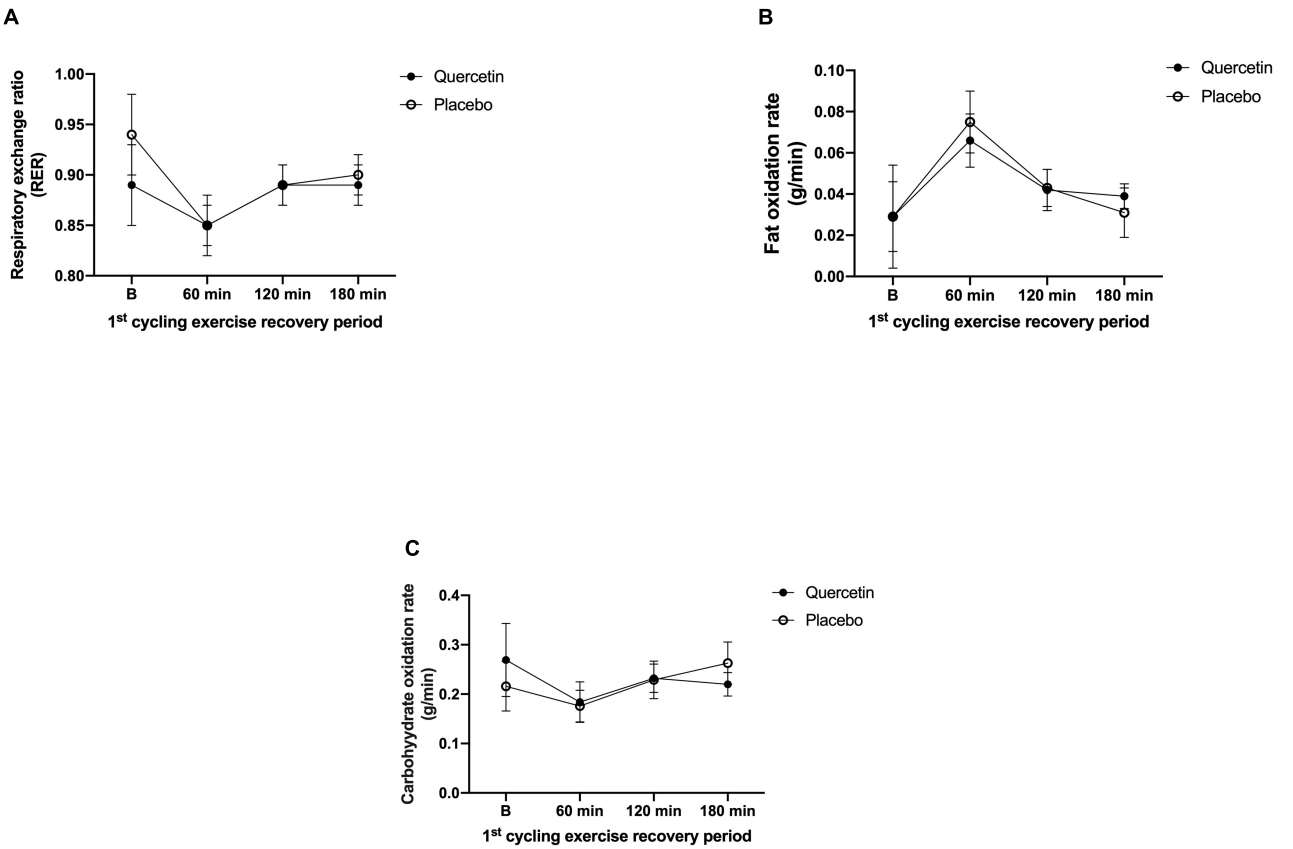

FIGURE 4 | Respiratory exchange rate **(A)**, fat oxidation rate **(B)**, and carbohydrate oxidation rate **(C)** in (-●-) and quercetin trials (-○-) placebo. B represents before the first cycling challenge; *Significant difference between quercetin and placebo ($p < 0.05$). Values are expressed as mean ± SE, $N = 12$.

of superoxide anions into hydrogen peroxide (33). These findings appear to be in line with the supplement itself. Quercetin, a natural polyphenolic flavonoid, has been shown to provide a combination of antioxidant and anti-inflammatory properties in both animal and human studies (5, 6). The antioxidant capacity of quercetin has been attributed to its chemical structure, especially the presence and position of the catechol group in the B ring and the hydroxyl (–OH) group, which regulate the redox mechanism and enhance the ability of free radical scavenging (34). It has been reported that high oxidative stress in the human body significantly postpones or reduces the mitochondrial synthesis in skeletal muscle (35) and affects the rate of cellular adenosine triphosphate (ATP) synthesis (36). Previous human studies showed that mitochondrial synthesis and ATP production play important physiological roles to improve human exercise performance (7). Additionally, Paschalis et al. reported that antioxidant supplements significantly decrease oxidative stress as measured by F2-isoprostanes and protein carbonyls, which effectively improved the aerobic performance ($\dot{V}O_{2max}$ test), anaerobic power test (Wingate), and 5-min time trial test (37). Almeida et al. reported that quercetins absorption was variated by their gut microbiota in the small intestine and low bioavailability in humans (38). Diksha et al., study was to investigate the absorption of quercetin in 18 healthy human subjects administered. The C_{max} of quercetin was highest achieved within 3.3 h (39). The present study was designed to perform multiple exercises over a long period of time, consisting of an initial 60-min 70% $\dot{V}O_{2max}$ test followed by a 3 h post-exercise recovery period, and finishing with high-intensity cycling exercise to exhaustion. Similar to Paschalis' human study, the increased antioxidant enzymatic activity, coinciding with reduced oxidative stress, supports quercetin supplementation for improving high-intensity exercise performance.

Physiological inflammation levels and increased muscle damage are related to both the muscles exercised and for how long the muscles were actively engaged. For instance, whole-body muscle movement has been reported to significantly increase the concentration of IL-6, TNF-α, CK, MB, and hs-CRP (40–42). A previous study from our lab indicated that IL-6 levels significantly increased in response to a cycling challenge at 80% $\dot{V}O_{2max}$ (43). The same exercise was applied in the present and significantly increased levels of IL-6 after exhaustion for both the quercetin and placebo groups (**Figure 6A**), indicating that treatment with exercise at 75% $\dot{V}O_{2max}$ could induce pro-inflammatory marker IL-6 levels in the human body. Quercetin has been shown the property of anti-inflammation via the inhibition of activation of the transcript factors such as nuclear factor-κB (NF-κB) and activator protein (AP-1) (44,

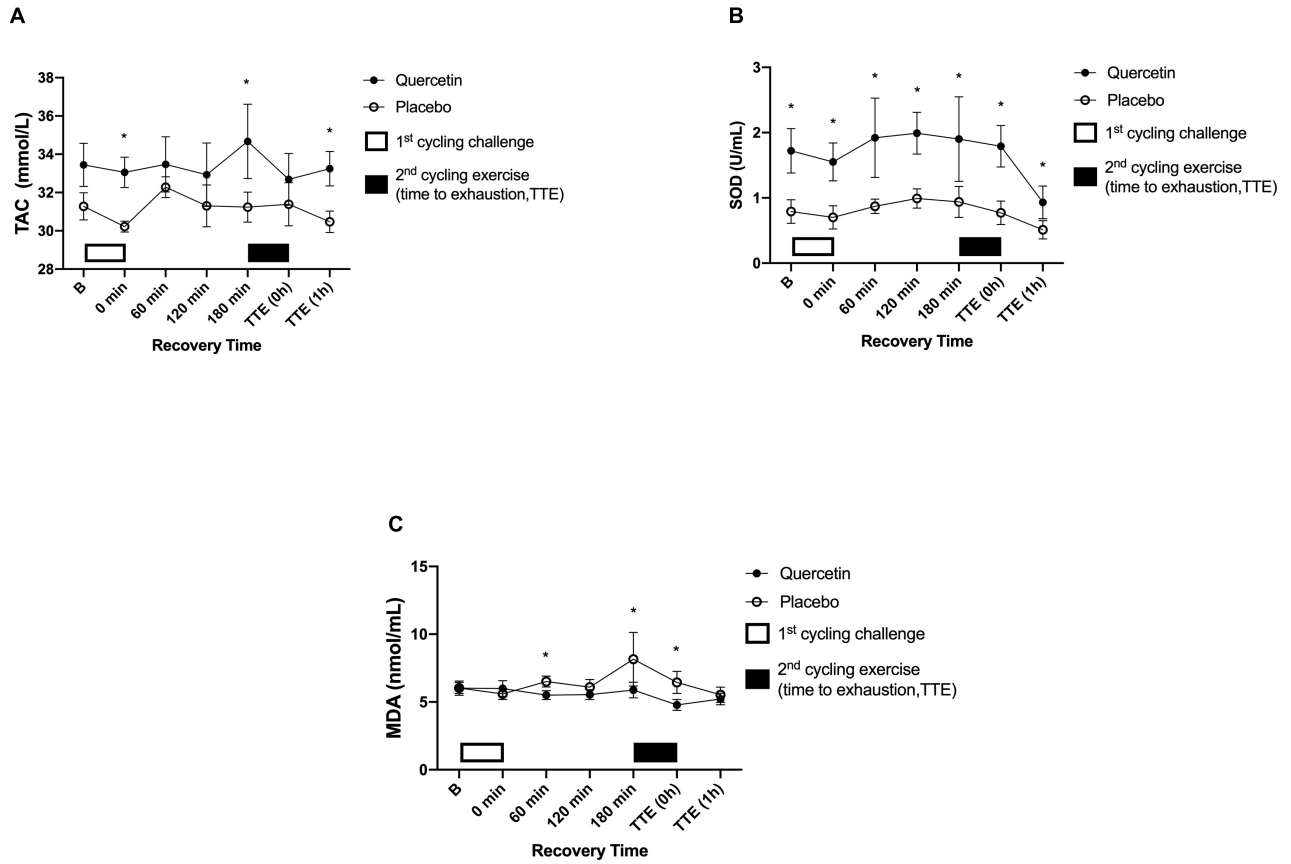

FIGURE 5 | Serum total antioxidant capacity (TAC) **(A)**, superoxidase dismutase (SOD) **(B)**, and malondialdehyde (MDA) **(C)** concentrations in (-○-) placebo and (-●-) quercetin trials. B represents before the first cycling challenge. TTE (0h,1h): represents immediately, and 1-h after the second cycling exercise, respectively. *Significant difference between quercetin and placebo ($p < 0.05$). Values are expressed as mean ± SE, $N = 12$.

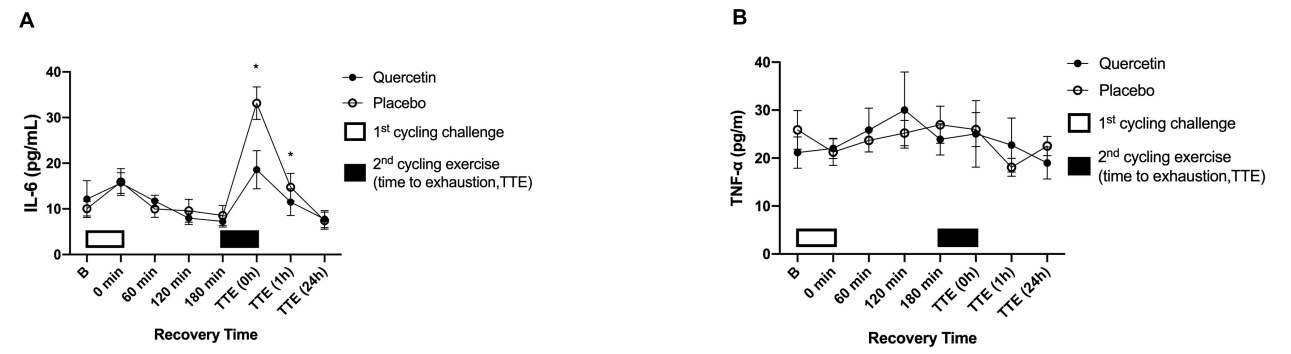

FIGURE 6 | Serum interleukin-6 (IL-6) **(A)** and tumor necrosis factor-α (TNF-α) **(B)** concentrations in (-○-) placebo and (-●-) quercetin trials. B represents before the first cycling challenge. TTE (0 h, 1 h, 24 h): represents immediately, 1-h, and 24-h after the second cycling exercise, respectively. *Significant difference between quercetin and placebo ($p < 0.05$). Values are expressed as mean ± SE, $N = 12$.

45). Consequently, the property of quercetin on scavenging ROS would not only prevent the occurrence of oxidative stress but also help mitigate inflammation. Indeed, it has already been shown that quercetin can inhibit pro-inflammation via modulation of NF-κB in human peripheral blood mononuclear cells (46). The present study monitored physiological reactions throughout the trial in participants treated with quercetin (1,000 mg/day) for 7 days. The IL-6 levels caused by exercises were significantly reduced by 7-day quercetin supplementation (**Figure 6A**, $p < 0.05$). Together, we were able to infer that 7-day quercetin supplementation was capable of inhibiting the muscle pro-inflammatory cytokine IL-6 concentration

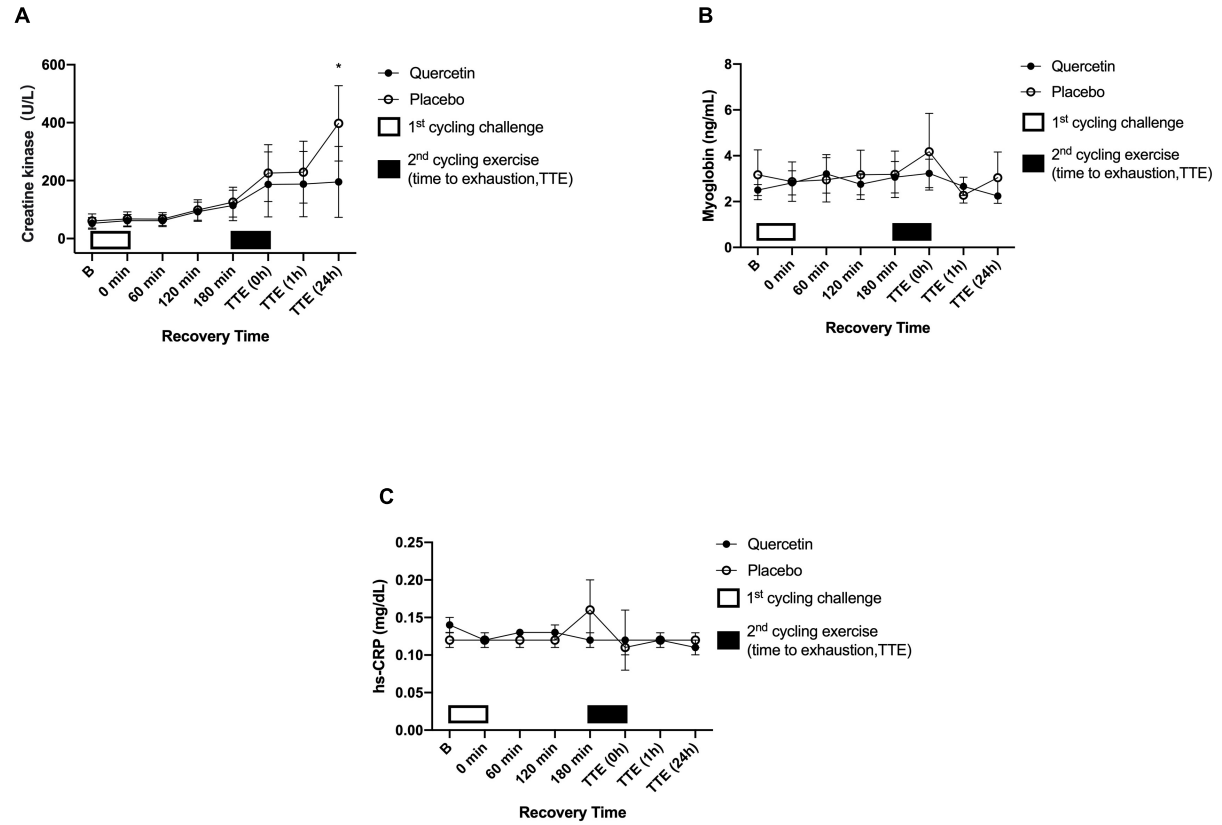

FIGURE 7 | Serum creatine kinase (CK) **(A)**, myoglobin **(B)**, and hs-CRP **(C)** concentrations in (-○-) placebo and (-●-) quercetin trials. B represents before the first cycling challenge. TTE (0 h, 1 h, 24 h): represents immediately, 1-h, and 24-h after the second cycling exercise, respectively. *Significant difference between quercetin and placebo ($p < 0.05$). Values are expressed as mean ± SE, $N = 12$.

induced by high-intensity exercise to exhaustion, suggesting that quercetin supplementation can assert positive physiological effects. In addition, blood CK and MB are sensitive to muscle damage and serve as biomarkers for such damage. Creatine kinase participates in the reversible reaction that catalyzes the phosphorylation of creatine, ultimately forming new ATP, which occurs primarily in skeletal muscle (47), the effect of quercetin on CK response involving AMP activated protein kinase (AMPK) itself a regulation in the heart muscle of rats (48, 49). In the present study, we speculated the conservation of the adenine nucleotide pools in the skeletal muscle may be a supplementary advantage of quercetin regarding ergogenic strategy for athletes. The present study investigated the positive effects of quercetin supplementation for 7 days on muscle damage biomarkers and showed that CK was significantly lower than placebo at 24-hr after the exercise-to-exhaustion challenge (**Figure 7A**, $p < 0.05$), not in MB, and hs-CRP (**Figures 7B,C**, $p > 0.05$). The authors wondered that unsuitable blood sample timing to correctly measure MB, and hs-CRP was likely the reason for our inability to detect changes. Cunniffe et al. showed that serum cortisol and IL-6 levels increased immediately after an International Rugby League game. While CK and the CRP index reached their peaks 14 and 38 hours after exercise respectively, perhaps revealing the true pattern of these physiological biomarkers in the acute phase post-exercise (50), suggesting that the time at which blood samples are obtained is crucial. Nevertheless, the last time-point of blood sampling in the present human study was 24 h after exhaustion, which may not be appropriate to observe the course of change among inflammation and muscle damage markers such as MB, and hs-CRP.

In conclusion, this study examined the effects of quercetin (1,000 mg/day for 7 days) supplementation on insulin sensitivity, antioxidant capacity, muscle damage, and exercise performance in human subjects. Quercetin supplementation was found to improve whole-body insulin-stimulated glucose uptake and antioxidant capacity, which attenuated exercise-induced oxygen stress during two consecutive high-intensity cycling bouts. Following a 3-h recovery period from the first high-intensity exercise session, quercetin improved time to exhaustion for the subsequent cycling test. Actually, there were limitations in the present study, including without performing a muscle biopsy, due to the COVID-19 pandemic, the muscle biopsy could not be performed on the exercised subjects to obtain muscle glycogen data, therefore, we speculated the effect of muscle glycogen use on cycling time to exhaustion only based on insulin data without to outline the possible underlying mechanisms for a relationship between muscle glycogen and cycling time to exhaustion. Of course, we also were lack of

records about physical activity and dietary diaries during the experimental periods for all participants. This useful information could elucidate the exclusive benefit effect of quercetin in the ergogenic property on exercise competition. However, the novel findings and new knowledge the present study provided was quercetin supplementation enhanced the post-exercise whole-body insulin-stimulated glucose uptake and antioxidant capacity, in parallel with a decrease in exercise-induced MDA, IL-6, and CK levels throughout the post-exercise period. The beneficial effects of quercetin are likely due to its glucogenic, antioxidant, and anti-inflammatory properties. Thus, short-term quercetin supplementation may be considered an effective ergogenic aid for enhancing high-intensity cycling performance among young adults.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at the University of Taipei, Taipei, Taiwan (UT-IRB-2018-085). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

I-SC designed the experiments. J-PT, S-FL, and H-CH carried out the laboratory experiments. J-PT and C-LH contributed reagents, materials, and analysis platforms. S-FL, C-LH, and I-SC analyzed the data. JB helped with the polishing of English writing. J-PT and I-SC interpreted the results, prepared the figures, and wrote and revised the manuscript. All authors are qualified and approved this final submitted version of this study.

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Effect of 32-Weeks High-Intensity Interval Training and Resistance Training on Delaying Sarcopenia: Focus on Endogenous Apoptosis

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Sarcopenia caused by aging is an important factor leading to a decline in the quality of life of older people. Apoptosis in muscle atrophy accelerates the process of muscle loss in older populations. The present study aimed to investigate the effects of 32 weeks of high-intensity interval training (HIIT) and resistance training (RT) on the skeletal muscle-related indices and provide a theoretical basis for regulating the mitochondrial-mediated pathway to delay sarcopenia. We randomly selected 10 from eight-month-old male SD rats (N = 130) as the baseline group; after 1 week of adaptive feeding, the rats were sacrificed. The remaining rats were randomly assigned to one of three groups: control group (C, N = 40, natural aging for 32 weeks), HIIT group (H, N = 40, performed six loops of 3 min at 90% and 3 min at 50% VO2 max speed treadmill running, with 5 min at 70% VO2 max speed at the beginning and the end of the training, 3 times a week for 32 weeks), and resistance group (R, n = 40, 46 min per day, 3 days per week, with a 30% maximum load on a treadmill with a slope of 35°, 15 m/min). The soleus muscles were collected for analysis at baseline and every 8 weeks. Aging resulted in decreased soleus muscle mass and Bcl-2 levels in the mitochondria, while the levels of reactive oxygen species (ROS) and Bax did not change. HIIT reversed the age-associated activation of pro-apoptotic processes, but RT did not. In addition, when rats were aged from 8 to 16 months, the level of Cyt-C did not change, the Caspase-9 levels and Caspase-3 levels decreased gradually in the soleus muscles, the rats of both the HIIT and RT groups had these indices decreased at 32 weeks. The results suggest that the age-associated loss of muscle mass was reversed by training, and the effect of RT was better than that of HIIT. Both the HIIT and RT rats showed a decrease in the apoptosis of skeletal muscle cells after 32 weeks of intervention. HIIT performed better for long-term intervention regarding the pro-apoptotic factors. This study warranted further research to delineate the underlying mechanism of effects of different exercise methods on the changes of aging skeletal muscle at *in vivo* level.

Keywords: aging, sarcopenia, HIIT, resistance exercise, endogenous apoptosis

INTRODUCTION

Populations are rapidly aging worldwide. Data from the World Health Organization on aging and health projects show that the population over the age of 60 will double from 11%, in 2000, to 22% by 2050, increasing from 605 million to 2 billion (Organization, 2016). Due to the dramatic growth of the elderly population, the proportion of the elderly in society is increasing; therefore, the issue of aging is of great concern globally.

Sarcopenia caused by aging is an important factor leading to a decline in the quality of life of older people (Cosquéric et al., 2006; Swan et al., 2021). Sarcopenia is the muscle failure associated with aging. Studies have shown that exercise is an effective way to delay sarcopenia, which can be achieved by balancing skeletal muscle synthesis and catabolism, improving the skeletal muscle mitochondrial density and activity, reducing apoptosis, among other ways (Ziaaldini et al., 2017).

Apoptosis in muscle atrophy accelerates the process of muscle loss in older populations, which may be the key mechanism leading to muscle performance impairment (Dupont-Versteegden, 2005; Marzetti et al., 2013; Faitg et al., 2017). With aging, the mitochondrial-mediated pathways may induce apoptosis in skeletal muscle, playing an important role in sarcopenia (Dirks & Leeuwenburgh, 2002; Marzetti & Leeuwenburgh, 2006; Song et al., 2006; Kob et al., 2015; Ziaaldini et al., 2015). The over-opening of the mitochondrial permeability transition pore (MPTP) leads to the release of many pro-apoptotic proteins into the cytoplasm, such as cytochrome C (Cyt-C). It forms an apoptotic body with apoptosis protease-activating factor-1 and caspase-9. The apoptotic body causes caspase-9 to transform into Caspase-9 and to activate Caspase-3, causing apoptosis (Feldstein and Gores, 2005). Caspase-3 can degrade the actomyosin complex, and the degraded products are degraded by other protein systems in cells, resulting in a decline in skeletal muscle mass and strength (Du et al., 2004).

Mitochondrial caspase-dependent apoptosis (endogenous apoptosis) is regulated by Bcl-2 family proteins. Bcl-2 and Bcl-XL are anti-apoptotic proteins, and Bax is a pro-apoptotic protein (Ashkenazi et al., 2017). In addition, ROS are closely related to "oxidative stress." A large amount of oxidative stress produces high levels of ROS, which breaks the balance between oxidation and antioxidants, damages the genetic material, changes the permeability of the mitochondrial membrane, and then induces apoptosis (Schieber and Chandel, 2013). Compared with young individuals, the expression of endogenous apoptosis-related proteins is increased in the skeletal muscle of older individuals (Dirks and Leeuwenburgh, 2002; Marzetti and Leeuwenburgh, 2006; Song et al., 2006; Kob et al., 2015; Ziaaldini et al., 2015).

So far, most of the research has mainly focused on the changes in endogenous apoptosis after sarcopenia transformation, and there is a lack of research on the temporal changes of the proteins related with the endogenous apoptosis pathway during aging. Compared with traditional exercise intervention methods, such as aerobic exercise and RT, HIIT, which is a new training method, is characterized by alternating short cycles of intense exercise with less intense periods of recovery (Bartlett et al., 2011; Heinrich et al., 2014). On role of physical activity in sarcopenia, many studies have shown that resistance exercise, aerobic exercise and HIIT can delay sarcopenia (Luo et al., 2013; Li et al., 2019; Neto et al., 2020). At present, there have been many studies on the effects of HIIT and aerobic exercise on skeletal muscle (Chavanelle et al. Sci Rep 2017; Martinez-Huenchullan et al., 2018; Martinez-Huenchullan et al., 2019). However, there are still are lacunae in the literature on role of compare different physical activity in sarcopenia, especially, research on the difference in the effects of RT and HIIT in sarcopenia on the aging process is rare. Therefore, In present study, the natural aging model of 32 weeks rats was established, and the rats were intervened with HIIT and RT during the aging process. The materials were taken every 8 weeks to observe the morphological changes of skeletal muscle and the changes of cytochrome c, caspase-9 and caspase-3 activities in the caspase dependent apoptosis pathway mediated by mitochondria of skeletal muscle cells, And the changes of Bcl-2 protein, Bax protein and ROS affecting caspase apoptosis pathway. The aim of the present study was to explore the effects of different exercise methods on the changes in the endogenous apoptotic pathway in the process of aging in order to provide a theoretical basis for exercise to delay the degeneration of aging skeletal muscle by regulating the endogenous apoptotic pathway. It was hypothesized that both HIIT and RT could both effectively reduce sarcopenia during aging, and improve endogenous apoptosis signaling pathway. And for endogenous apoptosis the effect of HIIT may be batter.

MATERIALS AND METHODS

Experimental Animals

All experimental protocols were approved by the Institutional animal care and use committee of the Beijing Sport University (Ref. No: 2019026A). A total of eight-month-old male Sprague-Dawley rats ($N = 130$), weighing 650–700 g, were provided by Sipeifu Biotechnology (Beijing, China). According to Sengupta's research, eight-month-old rats are approximately equivalent to twenty-year-old humans (Sengupta, 2013). After 1 weeks of acclimatization to the laboratory environment, 10 rats were randomly selected and sacrificed as the baseline group. Remaining rats were randomly divided into control group (C), HIIT group (H) and resistance group (R). Each group contained 40 rats.

Rats were given free access to standard food (includes water ≤10%, protein ≥18%, fat ≥4%, fiber ≤5%, fiber ≤8%, calcium ~1.4%, phosphorus ~0.8%) and water in the animal room of the Beijing Sport University (Certificate no. JDXT0029). The temperature of the animal room was 25 °C, with alternating light/dark cycles every 12 h (Specific Pathogen Free, SPF). Group C was fed for 32 weeks without exercise intervention; group H received HIIT intervention, and group R received RT intervention, which also lasted for 32 weeks.

Training Protocol

Rats of group H performed a maximal oxygen uptake test before the intervention and every 4 weeks subsequently, in order to

TABLE 1 | Training plan for RT.

Program	Training content
1	Weight bearing run for 15 s
2	Rest for 30 s
3	Repeat program 1–2 four times and rest for 3 min
4	Repeat program 3 three times and rest for 10 min
5	Repeat program 3 three times and finish the training

determine and appropriately adjust the speed of the treadmill for the HIIT. The tests were performed using an OxyMax Deluxe system (Columbus Instruments, USA). The rats were subjected to treadmill running at a speed corresponding to 70% VO2max for 5 min. Then, six loops of 3 min at 90% and 3 min at 50% VO2max speed treadmill running. Then, finishing with a speed corresponding to 70% VO2max for 5 min. HIIT was conducted for 32 weeks, 3 days per week, with each training session lasting for 46 min.

RT was performed on a treadmill (Weng et al., 2013) at a speed of 15 m/min and a slope of 35°. Rats were outfitted with a specially designed vest with 30% of max-weight bearing. The weight was adjusted according to the maximum weight-bearing capacity of rats, which was tested before intervention and every 4 weeks subsequently, to avoid adaptation to the intervention. Rats' max-weight bearing capacity were the weight they could barely moving on a treadmill, at a speed of 15 m/min and a slope of 35°.The training plan for group R is shown in **Table 1**. RT lasted for 32 weeks, 3 days per week, with each training session lasting for 46 min.

Tissue Collection and Preservation

Before the training intervention, 10 rats were randomly selected, fasted for 24 h, and euthanized as the baseline group. After beginning the exercise intervention, every 8 weeks, 10 rats (if there was no mortality) in each group were rested and fasted for 24 h and were then euthanized to provide experimental samples. The rats were weighed and anesthetized using an intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg). The soleus muscles of both legs were stripped and weighed, and the proximal fragment was used for the cross-sectional area (CSA) and the distal part was used for the ROS level and western blot tests.

Soleus Muscle Mass Index Analyses

At the time of sampling, the bilateral soleus muscles of rats were stripped and weighed, and the SMI was obtained by dividing the sum of the bilateral soleus muscles of each rat by the corresponding rat body weight.

CSA Analyses

The muscles were immersed in a paraformaldehyde stationary solution (Cat. No. G1101, Servicebio, China) for 24 h. Paraffin sections were prepared from the tissues soaked in the fixative, and then a H&E Staining Kit (Cat. No. G1005, Servicebio, China) was used for H&E staining. After taking pictures of the slices using a microscope (Nikon, Japan), a caseviewer (3DHISTECH, Germany) was used to scan the pictures. Five fields of view were selected from the center and four corners of each slice and saved in the TIF format. Image pro Plus6.0 (Media Cybernetics, Inc. United States) was used to calculate the skeletal muscle area in each visual field, which was then divided by the number of skeletal muscle fibers in the visual field to obtain the CSA.

Muscle ROS Detection

The total protein in the muscle tissue was quantified using a protein assay kit (Thermo Fisher Scientific, United States). ROS levels were measured using kits from Jianglai Biotechnology (Cat. No. JL21051) using an enzyme-linked immunosorbent assay. After the muscle tissues were stored at 4°C, PBS was added and the mixture was homogenized. After centrifugation at 4°C and 5,000 $\times g$ for 10 min, the supernatants were aspirated. Subsequently, the tests were performed according to the manufacturer's instructions.

Extraction of Mitochondria From Skeletal Muscle

A tissue mitochondria isolation kit (Beyotime Biotechnology, China, Cat. No. C3606) was used to extract the mitochondria from the skeletal muscle. We weighed 50 mg of soleus muscle and washed once with 600 μl of PBS. The soleus muscle was placed in a centrifuge tube, minced with ophthalmic scissors on ice, 1 ml of PBS was added to the centrifuge tube and ice bathed for 3 min. Put the centrifuge tube into a low temperature centrifuge, centrifuge at 600 $\times g$ at 4°C for 10–20 s, and discard the supernatant. Add 800 μl of trypsin digestion solution to the centrifuge tube, ice bath for 20 min, put the centrifuge tube into a low temperature centrifuge at 4°C and centrifuge at 600 $\times g$ for 10–20 s, and discard the supernatant. Add 200 μl of separation reagent to the centrifuge tube, resuspend the tissue, put the centrifuge tube into a low temperature centrifuge at 600 $\times g$ for 10–20 s at 4°C, and discard the supernatant. 800 μl of separation reagent and 8 μl of PMSF were added to the centrifuge tube, and homogenized with a homogenizer. Put the centrifuge tube into a low temperature centrifuge and centrifuge at 600 $\times g$ for 5 min at 4°C, take the supernatant and transfer it to another centrifuge tube. Put the centrifuge tube into a low temperature centrifuge and centrifuge at 3,500 $\times g$ at 4°C for 10 s, discard the supernatant, and the precipitate is mitochondria.

Western Blot Analysis

The total protein in the muscle tissue and muscle mitochondria tissue was extracted and quantified using a protein assay kit (Thermo Fisher Scientific, United States). Proteins were separated on 15 wells of 12% SDS-PAGE gels, 20 μg in each well, by electrophoresis. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes. Using Bovine serum albumin (BSA) as the blocking reagent and the target proteins were blocked and probed overnight at 4 °C using a Bax antibody (1:1,000, Cat. No. 2772T, CST, United States), Bcl-2 (1:4,000, Cat. No. ab196495, Abcam, United States), Cyt-C (1:5,000, Cat. No. ab133504, Abcam, United States), Caspase-3 (1:1,000, Cat. No. 9662S, CST, United States), Caspase-9 (1:2,000, Cat. No. ab184786, Abcam, United States), GAPDH (1:3,000, Cat. No. ab9485,

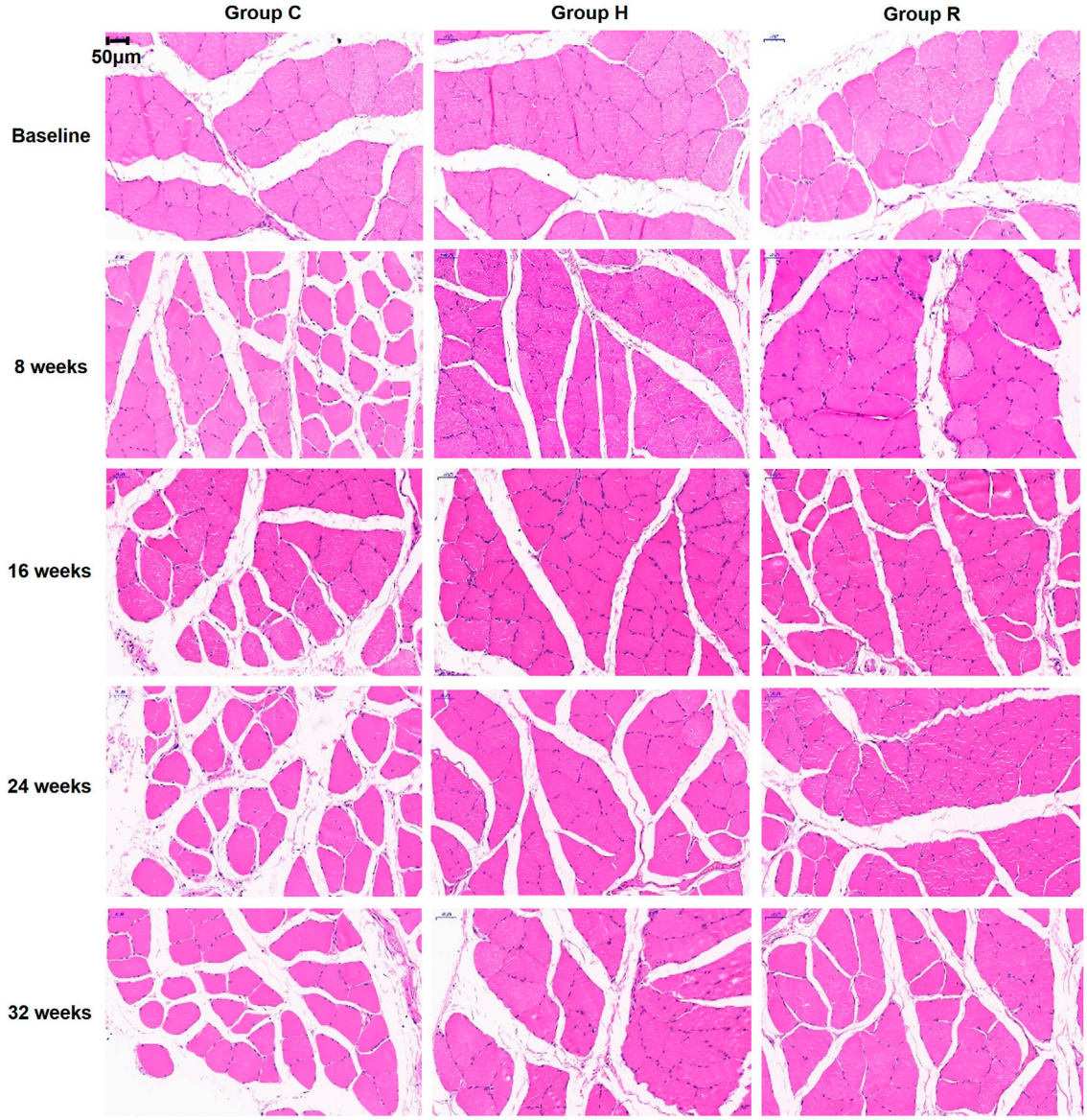

FIGURE 1 | HE staining sections of soleus muscle of rats in each group were observed under 400 times microscope.

Abcam, United States), and COXIV (1:2,000, Cat. No. 4850, CST, United States). All primary antibodies are from Rabbit. The following day, after washing with a TBST solution three times for 10 min each, the membranes were incubated with goat horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000, Cat. No. ab205718, Abcam, United States) at 25°C for 1 h. The membranes were washed six times with TBST for 5 min each. Signals were detected using an enhanced chemiluminescence (ECL) reagent. All bands were analyzed semi-quantitatively using ImageJ software and Total Lab Quant V11.5 (Newcastle upon Tyne, United Kingdom).

Statistical Analyses

Statistical analysis was performed using SPSS 22.0 (IBM SPSS Statistics, Armonk, NY, United States). All data are presented as mean ± SEM. All indices were analyzed using two-way ANOVA, time and exercise patterns were assessed as independent variables. The significance level was set at $p < 0.05$.

RESULTS

Skeletal Muscle Morphology and Weight

The results, as shown in **Figure 1**, indicate that age-related muscle fiber CSA loss occurred at 32 weeks in group C ($p < 0.001$). The muscle fiber CSAs of groups H and R were higher than those of group C at 8, 16, and 32 weeks ($p < 0.05$) and that of group R was higher than that of group H at 8 and 32 weeks ($p < 0.001$). At 8 and 32 weeks, the muscle fiber cross-sectional area of the R group rats was higher than at

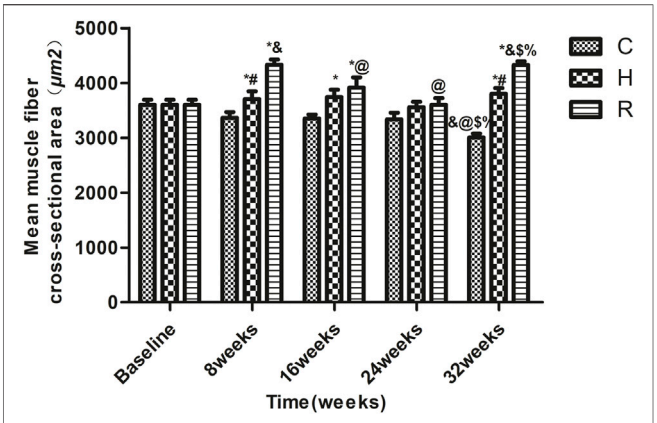

FIGURE 2 | The soleus muscle fiber CSA of rats in each group. * Significant different from C and other group; # Significant different from H and R; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks ($p < 0.05$).

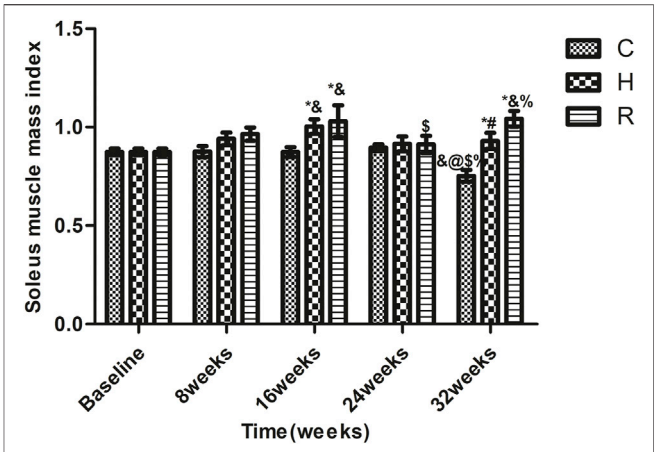

FIGURE 3 | The soleus muscle mass index of rats in each group. * Significant different from C and other group; # Significant different from H and R; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks ($p < 0.05$).

other time points ($p < 0.05$) (**Figure 1**, ×400 magnification; **Figure 2**).

Age-related SMI loss occurred at 32 weeks in group C ($p < 0.05$). The SMI values of the rats of groups H and R were higher than those of group C rats at 16 and 32 weeks ($p < 0.05$) and those of group R rats were higher than those of group H rats at 16 and 32 weeks ($p < 0.05$). The SMI values of group H rats were higher than the baseline level at 16 weeks ($p < 0.05$), and those of group R were higher at 32 weeks than the baseline level and the levels at 24 weeks ($p < 0.05$) (**Figure 3**).

Factors Affecting Apoptosis

The level of ROS in group C rats at 8 and 32 weeks was higher than that at 16 and 24 weeks ($p < 0.05$). The ROS levels of H group decreased significantly at 8 weeks of training ($p < 0.05$) and remained stable during the 8–32 weeks period. Those of group R decreased significantly at 8 weeks ($p < 0.05$) but increased significantly at 32 weeks ($p < 0.001$). The ROS levels of groups H and R were significantly lower than those of group C at 8 weeks ($p < 0.001$), but only those of group H were lower than those of the other groups at 32 weeks ($p < 0.001$) (**Figure 4**).

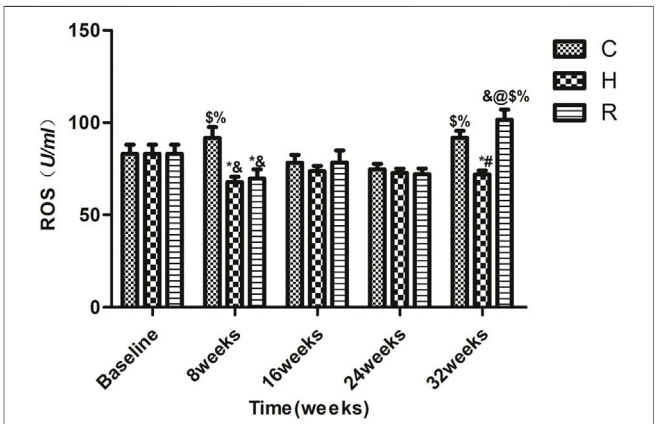

FIGURE 4 | The ROS level of soleus muscle in each group. * Significant different from C and other group; # Significant different from H and R; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks. ($p < 0.05$).

The levels of Bcl-2 in the mitochondria of the soleus muscle in group C decreased significantly at 8 and 24 weeks ($p < 0.001$), and showed a gradual downward trend. The Bcl-2 levels in group H showed a downward trend in the 8–24 weeks period of training, but increased at 32 weeks ($p < 0.05$). It is apparent from this table that the Bcl-2 levels in group H were significantly higher in groups C and R at 24 and 32 weeks of training ($p < 0.001$). In addition they were decreased in group R at 16 and 24 weeks of training ($p < 0.05$), and were higher than in other groups at 8 and 16 weeks ($p < 0.05$) but were equal to the baseline value at 24 and 32 weeks ($p < 0.05$) (**Figure 5**).

The level of Bax in the mitochondria of the soleus muscle in group C increased at 16 and 24 weeks, and decreased at 32 weeks, but there was no significant difference at each time point ($p > 0.05$). The Bax level in group R increased at 16 weeks and decreased at 24 weeks ($p < 0.05$). Group H presented a similar Bax level trend as group R, but none of these differences were statistically significant ($p > 0.05$) (**Figure 6**).

Endogenous Apoptotic Protein

There was no age-related change in the level of Cyt-C protein in the soleus muscle of group C at 32 weeks of aging. The Cyt-c level increased significantly in group H at 24 weeks ($p = 0.001$) and decreased significantly at 16 and 32 weeks ($p < 0.05$) and that of group R increased significantly at 8 weeks and decreased significantly at 16 and 32 weeks ($p < 0.05$). The Cyt-c level in group H was significantly lower than that in

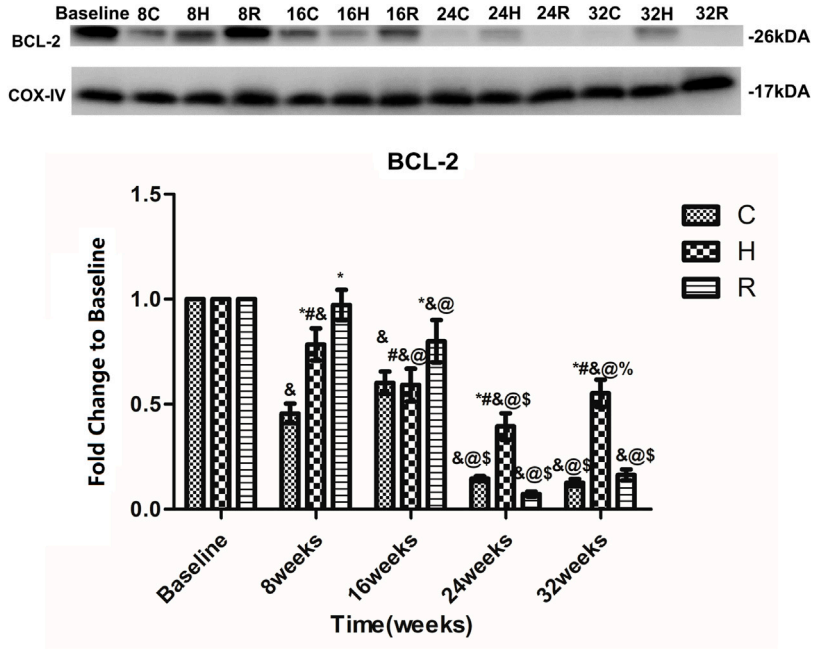

FIGURE 5 | Age and exercise training effects on Bcl-2 levels in mitochondria of soleus muscle, were evaluated by Western blot. * Significant different from C and other group; # Significant different from H and R; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks. ($p < 0.05$).

group C at 16 weeks ($p < 0.05$), and that of group R was significantly higher than that of group C at 8 weeks, but was significantly lower than that of group C at 32 weeks ($p < 0.05$) (**Figure 7**).

An age-related decrease in the caspase-9 level in the soleus muscle was observed in all groups. Interestingly, the decrease in H group was observed to occur mainly at 16 and 32 weeks ($p < 0.05$). At 16 weeks, the caspase-9 protein level in group H was higher than that of group R ($p < 0.05$). At 24 weeks, that of group H was higher than those of groups C and R ($p < 0.05$). However, at 32 weeks, those of groups H and R were lower than those of group C ($p < 0.05$) (**Figure 8**).

The level of caspase-3 in the soleus muscle in group C decreased at 8 weeks ($p < 0.001$), and then remained stable. In group H, the level of Caspase-3 in the soleus muscle decreased with age. It was significantly higher than that of groups C and R at 8 weeks, but lower than that of group C at 32 weeks ($p < 0.001$). In group R, the caspase-3 level increased at 16 weeks and decreased at other time points ($p < 0.001$), being significantly higher than that of groups C and H at 16 weeks, but lower than that of group C at 32 weeks ($p < 0.001$) (**Figure 9**).

DISCUSSION

In this study, through continuous observation of naturally aging rats, it was confirmed that the decrease in the muscle mass index and in the CSA of the soleus muscle in naturally aging rats was the earliest at 16-months-old of aging, which updated the time of age-related muscle atrophy. Several reports have shown that the SMI of rats over 24 months of age is significantly lower than that of rats aged 4–6 months (Rice et al., 2005; Song et al., 2006; Liu, 2018). The earliest age-related decrease in the soleus CSA has occurred at 25 months of age (Wang et al., 2015). Most of these studies are cross-sectional studies, which cannot demonstrate when age-related atrophy of the rat soleus muscle occurs. In this study, a longitudinal observation of aging rats (8–16-months-old) was conducted to explore the problem and yielded different results, which filled the gap in existing research.

Exercise can improve the age-related atrophy of muscles (Cui et al., 2021; Luo et al., 2013; Zhao et al., 2016; Ribeiro et al., 2017; Li et al., 2019; Neto et al., 2020); however, most current studies have been performed on elderly rats undergoing 12 weeks of exercise training, in comparison with the skeletal muscle of the control group rats. The results of this study suggest that both HIIT and RT can effectively increase the SMI of soleus muscle in rats after 16 and 32 weeks of training, and both methods can effectively increase the CSA of the rat soleus muscle at 8 and 32 weeks of training. One interesting finding is that the increase of SMI and CSA caused by exercise occurred at different times. However, this result has not previously been described. It may support the hypothesis that exercise induced increases in muscle fiber number (Gonyea et al., 1986). In addition, the muscle enhancement effect of 32 weeks of RT intervention was better than that of HIIT, which may be because RT can better activate the pathways related with muscle synthesis (Ribeiro et al., 2017; Neto et al., 2020).

In conclusion, the earliest age-related loss of the soleus muscle occurred at 16 months of age, a 32-weeks exercise intervention

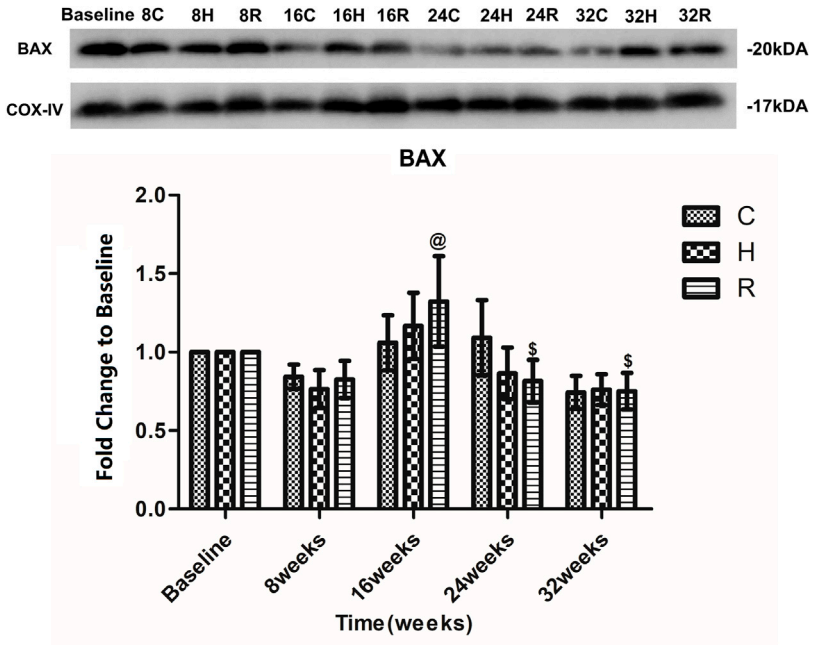

FIGURE 6 | Age and exercise training effects on BAX levels in mitochondria of soleus muscle, were evaluated by Western blot. @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks. ($p < 0.05$).

can improve the aging atrophy of the soleus muscle in rats, and the effect of RT is better than that of HIIT.

High concentrations of ROS can damage the outer membrane of mitochondria, leading to MPTP, which increases the release of Cyt-C to promote apoptosis (Schieber and Chandel, 2013). Many studies have discussed the age-related increase in ROS levels (Muller et al., 2007; Sullivan-Gunn and Lewandowski, 2013; Ziaaldini et al., 2015; Boengler et al., 2017; Damiano et al., 2019; Jiang, 2019), but it is uncertain when this phenomenon first occurs. In this study, in contrast to other studies, however, there were no differences in the ROS levels between each time point and the corresponding baseline value in the control group. This may be due to the different strains and living environments of the rats. In addition, this study found that 32 weeks of HIIT reduced the ROS levels. These relationships may partly be explained by HIIT improves the ability of the skeletal muscle to scavenge ROS and the mitochondrial respiration ability or other pathway (Alhadlaq et al., 2019; Chrois et al., 2019). Although many studies have shown that exercise can reduce the ROS levels (Vezzoli et al., 2019; Bartlett et al., 2020), in this study, 32 weeks of RT intervention increased the ROS levels. It is speculated that a higher exercise intensity of RT leads to oxidative stress (Liu et al., 2008) and ROS accumulation at 32 weeks. This indicates that HIIT may be a better intervention option for reducing the ROS levels.

Bax, which is a pro-apoptotic protein, can also induce MMPT. This can lead to the release of Cyt-C and, ultimately, promote apoptosis. Bcl-2 can reduce apoptosis by inhibiting the activity of Bax (Ashkenazi et al., 2017; Hikita and Takehara, 2017). This study confirmed that the Bcl-2 level in the mitochondria of skeletal muscle cells decreases with aging, and that the Bax level does not change with aging. Many studies have focused on the aging changes in the Bcl-2 and Bax levels, but the results are contradictory (Dirks and Leeuwenburgh, 2002; Baker and Hepple, 2006; Song et al., 2006; Liao et al., 2017). In general, the gene expression of Bcl-2 and Bax in the skeletal muscle decreases with aging. The protein expression of Bcl-2 in skeletal muscle showed an age-related decrease, whereas the expression of Bax showed an opposite trend. In skeletal muscle mitochondria, the expression of these proteins does not change with age. In this study, we not only found an age-related decrease in the Bcl-2 level in skeletal muscle mitochondria, but also found that it first occurred at 10 months of age. What is surprising is that this time point is much earlier than that observed in other studies, suggesting that the age-related changes in the levels of Bcl-2 family proteins in skeletal muscle mitochondria may occur earlier. Unfortunately, there were no age-related changes in Bax expression. In addition, some studies found that exercise training resulted in adaptations in the apoptotic signaling by Bcl-2 family proteins in the skeletal muscle of old rats (Song et al., 2006; Lin et al., 2013; Liao et al., 2017; Li et al., 2019); however, they did not compare the effect of HIIT and RT on the Bcl-2 levels. We found that RT was more effective before 16 weeks of aging, and that HIIT was more effective after the 16-weeks time point. This finding, while preliminary, suggests that HIIT is a more suitable long-term form of exercise for increasing the Bcl-2 level in skeletal muscle mitochondria. It is worth noting that RT can significantly increase the expression of Bcl-2 in the first 16 weeks (12-months-old), which may be the reason why the drastic changes in the early stage activated the feedback

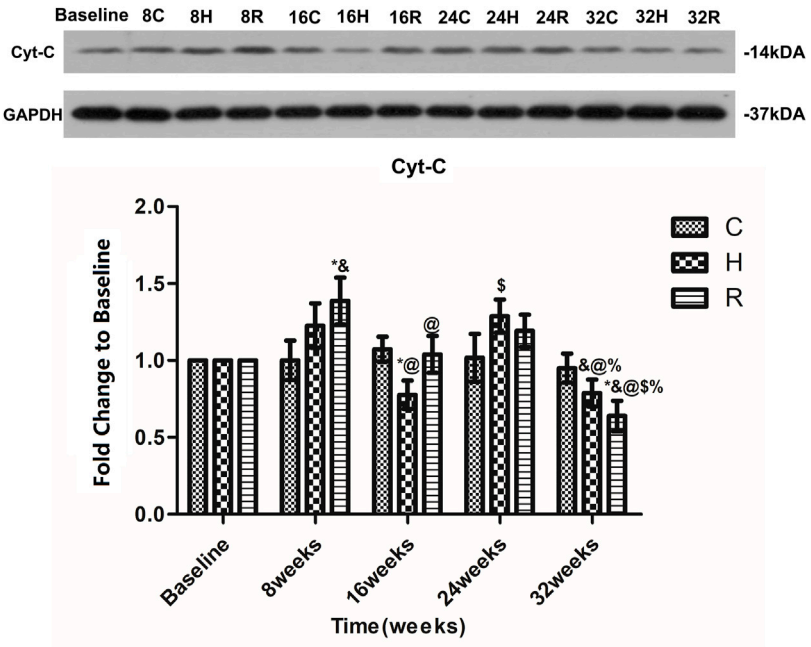

FIGURE 7 | Age and exercise training effects on Cyt-3 levels in mitochondria of soleus muscle, were evaluated by Western blot. * Significant different from C and other group; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks. ($p < 0.05$).

regulation of the body or HIIT activates other pathways (Ahamed et al., 2020)and, finally, caused it to lose its function after 16 weeks. In addition, the expression of Bax in mitochondria was not affected by exercise. The specific reasons and mechanisms require further studies. Overall, HIIT performed better as a long-term intervention regarding the ROS and Bcl-2 levels.

Cyt-C initially exists on the cristae of mitochondria and plays an important role in mitochondrial respiration. When the mitochondrial membrane permeability changes, Cyt-C is released into the cytoplasm and induces apoptosis. The release of Cyt-C is considered to be a marker of the activation of the mitochondrial apoptotic pathway (Dirks and Leeuwenburgh, 2002). We detected the level of Cyt-C in the rat soleus muscle every 8 weeks, and found that it remained unchanged during the 32 weeks of aging (8–16-months-old); some studies have drawn a similar conclusion (Dirks and Leeuwenburgh, 2002; Chung and Ng, 2006), but they only tested young and old rats. It is not yet clear when the aging-associated increase in the Cyt-C level occurs. It is worth noting that some studies found that, compared with 3-month-old rats, the content of Cyt-C in the skeletal muscle of 8-month-old rats increased (Ziaaldini et al., 2015) and, compared with 4-month-old rats, the content of Cyt-C in the skeletal muscle of 22-month-old rats increased (Kang et al., 2013). This study showed that there was no age-related change in the Cyt-C content in skeletal muscle from 8 to 16 months of age. According to these data, we can infer that the aging changes in the Cyt-C content in skeletal muscle may occur in rats under 8 months of age. We observed that 16- and 32-weeks HIIT interventions can reduce the release of Cyt-c. However, eight- and 24-weeks HIIT interventions increased the release of Cyt-C, which is consistent with the results of previous studies (Wang, 2018). This may be due to the fact that long-term HIIT intervention results in skeletal muscle adaptation regarding Bcl-2 and ROS in old rats. In addition, HIIT intervention can downregulate hist1h1c (Li et al., 2019) and improve the respiratory capacity of skeletal muscle mitochondria (Chrois et al., 2019), which can reduce the release of Cyt-c. RT can reduce the release of Cyt-C in the skeletal muscle of aged rats (Luo et al., 2013; Lin et al., 2014). We found that 32 weeks of RT intervention could achieve this effect. More importantly, at 32 weeks, the effect of HIIT was not as good as that of RT, in contrast with the results obtained for ROS and bcl-2. We speculate that RT can achieve this effect by changing the mitochondrial membrane potential and the levels of mitochondrial fusion protein (Su et al., 2020).

Caspase-9 can activate caspase-3 and promote apoptosis, and some studies have found an age-related increase in the caspase-9 level in skeletal muscle (Alway et al., 2002; Baker & Hepple, 2006). However, it is not known when it happens. In this study, no age-related increase in the caspase-9 level was found in the skeletal muscle of rats aged 8–16 months. Therefore, the specific time of this phenomenon requires further study. After 32 weeks of HIIT and resistance exercise intervention, the expression of caspase-9 decreased in the skeletal muscle of rats. The difference is that the caspase-9 level in the skeletal muscle of rats treated with HIIT from 8 to 24 weeks showed no significant changes, but it decreased at 32 weeks, which is similar to the trend observed for Cyt-C in group H. However, resistance exercise

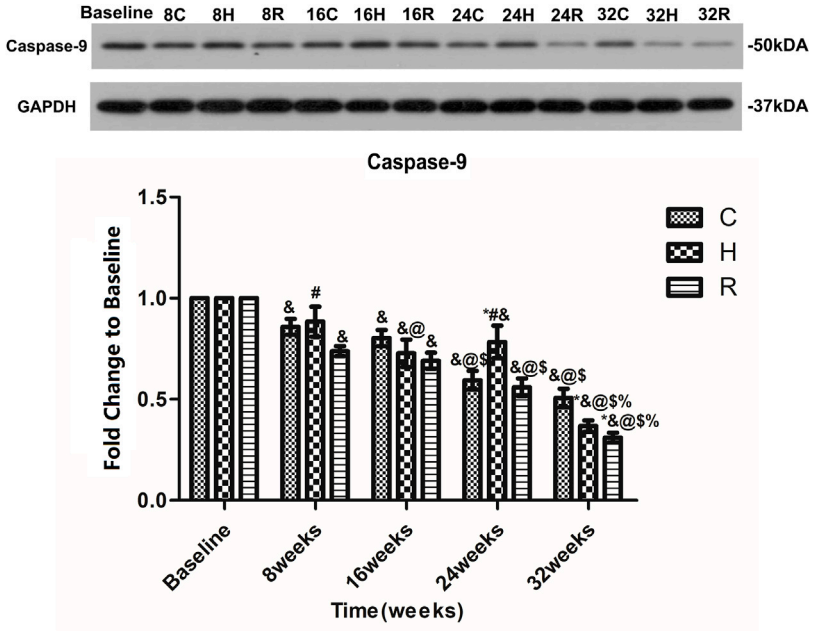

FIGURE 8 | Age and exercise training effects on Caspase-9 levels in mitochondria of soleus muscle, were evaluated by Western blot. * Significant different from C and other group; # Significant different from H and R; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks. ($p < 0.05$).

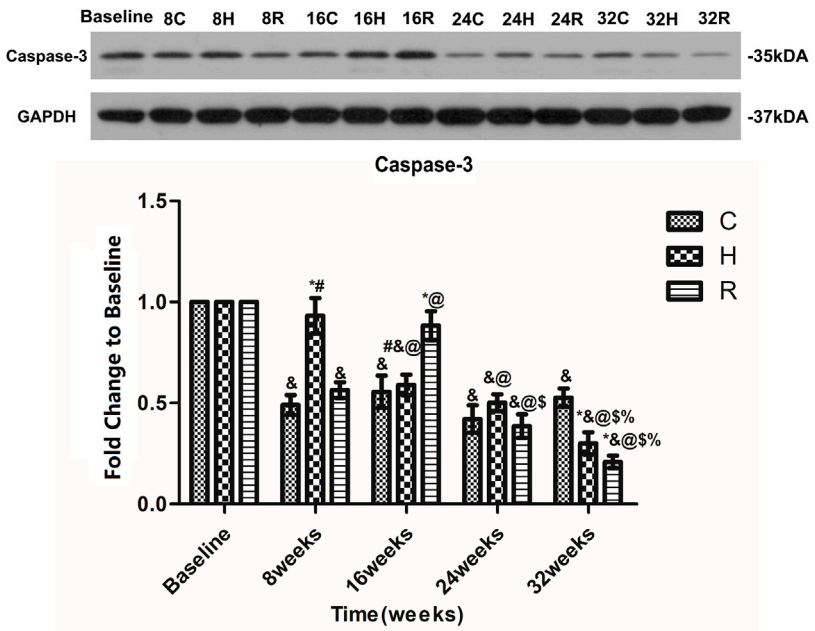

FIGURE 9 | Age and exercise training effects on Caspase-3 levels in mitochondria of soleus muscle, were evaluated by Western blot. * Significant different from C and other group; # Significant different from H and R; & Significant different from Baseline and other weeks; @ Significant different from 8 weeks and other weeks; $ Significant different from 16 weeks and other weeks; % Significant different from 24 weeks and other weeks. ($p < 0.05$).

can cause the skeletal muscle mass of rats to decrease gradually during aging, which indicates that resistance exercise will play a bigger role in this in the beginning. But, the changes in the levels of Cyt-C and caspase-9 observed in group R were different during aging, indicating that resistance exercise may also reduce the

level of caspase-9 through other factors (Mejías-Peña et al., 2017; Ribeiro et al., 2017; Neto et al., 2020), which requires further study.

This study confirmed that the content of Caspase-3 in the soleus muscle of aging rats decreased at 10-months-old and remained unchanged at 10–16- months-old. Some studies have shown that the level of Caspase-3 in skeletal muscle remains unchanged during aging (6-month-old rats were compared with 24-month-old rats) (Dirks and Leeuwenburgh, 2002; Chung and Ng, 2006). However, Some outcomes are contrary to that of Dirks et al. who found that compared with the 6–8-month-old rats, the level of caspase-3 was significantly increased in 27–35-month-old rats (Baker and Hepple, 2006; Song et al., 2006). Therefore, we speculate that the age-related increase in the level of Caspase-3 in the skeletal muscle of rats occurs after 24 months of age. In this study, the reason for the decrease in the caspase-3 level at the age of 10 months may be that the metabolism of cells in young rats is exuberant, and the cell renewal is fast; thus, apoptosis is active. At the age of 10–16 months, the metabolic rate decreases with the increase in age; therefore, the level of caspase-3 decreases and remains stable, compared with that at the age of 8 months (Ying and Liang, 2015). In addition, this study confirmed that 32 weeks of HIIT and RT intervention can reduce the level of Caspase-3 in the skeletal muscle of aging rats. However, in the aging process, HIIT can gradually reduce the caspase-3 level in skeletal muscle, while RT can increase and decrease the caspase-3 level. Therefore, the effect of HIIT appeared to be more stable than that of RT. We found that the results of the levels of caspase-9 and caspase-3 were consistent after 32 weeks of intervention. However, the changes in the Caspase-3 level in the three groups presented a different trend than that observed for the caspase-9 level during aging. It is speculated that aging and exercise may regulate the caspase-3 level through the death receptor pathway and endoplasmic reticulum pathway, and this process may affect the levels of Bad (BCL-XL/Bcl-2-associated death promoter), TNF-α, Caspase-8, caspase-12, and so on (Marzetti & Leeuwenburgh, 2006; Luo et al., 2013; Mejías-Peña et al., 2017; Ribeiro et al., 2017; Ahamed et al., 2018; Ahamed et al., 2021).

In conclusion, the level of Cyt-C in the soleus muscle remained unchanged, while the levels of caspase-9 and caspase-3 decreased during the 32 weeks of aging. Exercise training for 32 weeks reduced the level of Caspase-3 through Cyt-C and caspase-9. The effects of HIIT and RT were the same at 32 weeks.

CONCLUSION

This study set out to find a better way to delay sarcopenia and explore the effects of different exercise methods on the changes in the endogenous apoptotic pathway in the process of aging. We demonstrated that the age-associated loss of muscle mass was reversed by training, and that the effect of RT was better than that of HIIT. There was no age-related increase in skeletal muscle apoptosis in 8–16-month old rats. However, both HIIT and RT reduced the apoptosis level of skeletal muscle cells after 32 weeks of intervention. HIIT performed better in long-term intervention regarding the pro-apoptotic factors, and there was no difference in the effect of HIIT and RT on apoptosis at 32 weeks. Although these results generally support the idea that exercise reduces skeletal muscle apoptosis in aged rats, we could not find the specific time of the age-associated increase in apoptosis; therefore, further studies aimed at observing the apoptosis of skeletal muscle for a longer period are required to assess this.

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 Institutional animal care and use committee of the Beijing Sport University.

AUTHOR CONTRIBUTIONS

HS and QG conceived and designed the project. TW, and JS performed experiments. TW, DL, JS, and QG analyzed data and prepared figures. HS, TW, LZ and QG drafted the manuscript. TW and HS edited and revised the manuscript. QG was independently checked the included studies and resolved disagreements. All authors contributed to the article approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.811369/full#supplementary-material

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Antidiabetic and Antihyperlipidemic Effects of Methanolic Extract of Leaves of *Spondias mombin* in Streptozotocin-Induced Diabetic Rats

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Objective: *Spondias mombin* is a plant that reported to have anticonvulsant, antimicrobial, antioxidant, antiulcer, antiasthmatic, and wound healing activities. Diabetes dyslipidemic effect of *Spondias mombin* leaves is not clear. Hence, current study planned to evaluate the antidiabetic and antihyperlipidemic effects of methanolic extract of leaves of *Spondias mombin* (MESM) in streptozotocin (STZ) induced diabetic rats.

Methods: Phytochemicals were determined by standard method and antioxidant activity was determined by DPPH free radical scavenging and FRAP assay. Diabetes was induced by injecting a single dose of STZ (55 mg/kg) into female sprague dawley rats. After 3 days of induction of diabetes, the diabetic animals were treated for 28 days with MESM (125, 250, and 500 mg/kg) and glibenclamide (20 mg/kg) orally. The body weight of rats and blood glucose levels were monitored at regular intervals during the experiment. At the end of study, blood sample was collected from all the animals and subjected to biochemical, lipid profile, and they were sacrificed and their organs such as pancreas, liver and kidney were used for histopathological analysis.

Results: Quantitative analysis of MESM showed the presence of anthraquinone, tannins, saponins, steroid, phenols, flavonoids, alkaloids, and reducing sugars. Reduction in body weight and elevated blood glucose were observed in diabetic rats. Treatment with MESM in a concentration of 125, 250, and 500 mg/kg significantly reversed the elevated levels of blood glucose, reduced aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, urea, creatinine, total serum cholesterol (TC), serum triglyceride (TG), low-density lipoprotein (LDL), Very low-density lipoprotein (VLDL), and increased plasma insulin, total protein, albumin, globulin, A/G ratio, and high-density lipoprotein (HDL).

Conclusion: MESM exhibited a significant antidiabetic and antihyperlipidemic activities against STZ-induced diabetes in rats.

Keywords: antidiabetic, antihyperlipidemic, *Spondias mombin*, steptozotocin, diabetes

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1 INTRODUCTION

Diabetes is a heterogeneous endocrine and metabolic illnesses characterised *via* hyperglycemia due to deficiency or diminished effectiveness of insulin action, insulin secretion or both. Hyperglycemia causes long-term health damage and failure of various organs particularly nerves, blood vessels, kidneys, eyes, and heart (Sneha and Gangil, 2019).

Diabetes mellitus can be classified into two main types, type 1 and type 2 (Roussel et al., 2021), with type 1 resulting from the body's failure to produce insulin, and requires one to be injected with insulin (Smyth, 2021). Type 2 diabetes mellitus describes a condition of fasting hyperglycemia that occurs despite the availability of insulin (Effah-Yeboah et al., 2015). International Diabetic Federation (IDF) reported there are currently around 463 million people living with diabetes worldwide which is projected to increase to 700 million by 2045 (IDF Diabetes Atlas 9th edition 2019). Around 3.6 million people in Malaysia are confirmed with diabetes and seven million adults in Malaysia are predicted to get diabetes by end of 2025 (Institute for Public Health—NHMS 2019). This significant rise is due to Type 2 diabetes and consequence of excess body weight and physical inactivity. In type 2 diabetes mellitus, formation of Reactive Oxygen Species (ROS) resulted from impaired insulin synthesis due to pancreatic β-cell death by apoptosis. Hence, the body's antioxidant system enhanced through usage of supplements and plant compounds which may reduce oxidative stress and prevents the disease at the beginning stage (Tangvarasittichai, 2015). Medicinal plants believed to provide efficient against health problems with minimum side effects with reasonable cost and easily available. Plant products may play a vital role to discover new therapeutic agents and gained recognition as origin of bioactive compounds such as hypoglycemic, antioxidants, and hypolipidemic agents (Zhang and Reddy, 2018).

Spondias mombin L. (Anarcadiaceae) is generally referred as "Hog plum" in English (Ezuruike and Prieto, 2014). In traditional medicine, *Spondias mombin* claimed as efficient in treating psychiatric disorders, duodenal disorders, gonorrhoea, inflammatory conditions, wounds, infections, diabetes, and to remove placenta during childbirth (Iwu, 1993). Decoction of the powdered flowers and leaves of *Spondias mombin* are used to reduce stomach pain, cystitis, urethritis, biliousness, enhance eye, and throat inflammation (Temitope et al., 2017). *Spondias mombin* roots and stem bark exhibited anti-inflammatory, antimicrobial, anti-mycobacterial, antiviral, hematinic, anthelmintic, and sedative activities (Nwidu et al., 2018). Antidiabetic and antihyperlipidemic activity of *Spondias mombin* is not clear. Hence, the present study is planned to evaluate the antidiabetic and antihyperlipidemic effect of methanolic extract of *Spondias mombin* (MESM) on streptozotocin (STZ)-induced diabetes mellitus in *Sprague dawley* rats.

2 MATERIALS AND METHODS

2.1 Plant Material

Fresh and matured *Spondias mombin* leaves were collected from Semeling, Sungai Petani, Kedah. The plant was identified and authenticated by a botanist from herbarium department, (USM 11767/09/2018). The plant materials were washed; dried under the shade and pulse grinded using a grinding machine. The powder was kept in an air tight container for further use (Namani et al., 2016).

2.2 Chemicals

STZ was purchased from Sigma chemical company, Malaysia. All other solvents used in the experiments were purchased locally from Merck or SD fine Chemicals and were of analytical grade.

2.3 Extraction

Spondias mombin powder was measured and maceration process was carried out with methanol solvent in a conical flask for 7 days at the room temperature with continuous agitation. After maceration process completed, the leaves extract filtered using muslin cloth. The extract then dried by evaporation using rotary evaporator (Rotavapor® R-210, BUCHI Corporation). The MESM was stored at −80°C until further use (Samuggam et al., 2021). The percentage yield of MESM is 91% w/w.

2.4 Phytochemical Screening

The MESM was tested for the presence of secondary metabolites like anthraquinone (hydrochloric acid and sulphuric acid), tannins (ferric chloride), saponins (distilled water), steroid (chloroform and acetic anhydride), phenols (ferric chloride), flavonoids (ammonia and sulphuric acid), alkaloids (hydrochloric acid and Dragendorff reagent), and reducing sugars (Fehling's solution A and B) (Yadav and Agarwala, 2011; Sunitha et al., 2018).

2.5 Total Phenol and Flavonoid Content

Total phenolic content in methanol was measured using spectrophotometric method. 0.5 ml of plant sample added with 2.5 ml of 0.75% sodium carbonate solution and 2.5 ml of 1% Folin-Ciocalteu reagent added in a test tube. The mixture was incubated for 15 min at a temperature of 45°C and absorbance was measured at 765 nm. The standard calibration curve was plotted based on absorbance at 765 nm against concentration of gallic acid. Based on the calibration curve, total phenolic content was calculated and the results were expressed as gallic acid equivalent in mg/g (Kumari et al., 2016).

Total flavonoid content in methanol was measured using aluminium chloride colorimetric assay. 1 ml of plant sample added with 1 ml of standard quercetin solution, 4 ml of distilled water and 0.3 ml of 5% sodium nitrite solution followed by 2 ml of 1 M sodium hydroxide. The mixture was incubated for 15 min at a temperature of 45°C and absorbance was measured at 510 nm. The standard calibration curve was plotted using standard quercetin. The total flavonoid content was calculated from the calibration curve,

and the results were expressed as quercetin equivalent in mg/g (Kumari et al., 2016).

2.6 Antioxidant Assay

2.6.1 DPPH Free Radical Scavenging Assay

Radical scavenging activity was measured by method (Ayoola et al., 2008) with slight modification. Different concentrations of methanolic extract of *Spondias mombin* leaves (MESM) ranging from 0.5 to 3 mg/ml were prepared. The reaction mixture (3 ml) consists of 1 ml of DPPH solution (0.3 mM), l ml of the extract and 1 ml of methanol. The mixture was incubated for 30 min in dark and then the absorbance was measured at 517 nm. Quercetin was used as standard. A blank solution was prepared with DPPH and methanol.

The percentage of inhibition was calculated by using following equation

Percent inhibition = (A0—A1/A0) x 100

Where;

A0 = the absorbance of the blank

A1 = the absorbance of the sample.

The experiment was carried out in triplicate. Antioxidant activity was expressed as IC_{50} which is the concentration (ug/ml) of extract that is required for 50% of DPPH scavenging activity.

2.7 Ferric Reducing Antioxidant Power Assay

FRAP assay was performed according to the method (Muller et al., 2011) with slight modification. Different concentration of methanolic extract of *Spondias mombin* leaves at 5, 2.5, 1.25, 0.625, 0.312, 0.156 mg/ml were prepared each at volume of 100 µL and was mixed with 4.5 ml of FRAP reagent in test tubes, thoroughly mixed by vortexing and were incubated in water bath for 30 min at 37°C. The blank was prepared with FRAP working reagent and methanol. The aqueous solution of $FeSO_4.7H_2O$ was used as standard. The absorbance of the samples was determined at 593 nm by UV spectrophotometer (UV-Vis Spectrophotometer, United States). The results were expressed as mg of ferrous equivalent per Gram of extract.

2.8 Experimental Animals

Adult female sprague dawley rats weight between 160 ± 20 g were purchased from (Universiti Sains Malaysia, Penang, Malaysia) and placed at 23 ± 2°C under 50 ± 5% humidity level for 12 h light and 12 h dark cycle. Rat pellet and water provided to the rats and acclimatised for 7 days before permitted to take part in the experiment. The study was approved by the AIMST University Human and Animal Ethics Committee (AUHAEC/FAS/2017/01) and the study was conducted according to the Animal Research Review Panel guidelines.

2.9 Acute Toxicity

Adult female sprague dawley rats were selected for the study. Acute toxicity study was conducted by applying fixed-dose procedure. Rats were subjected oral dose of *Spondias mombin* starting from 50, 100, 250, 500, 1,000, and 2000 mg/kg body weight (n = 3 per dose), respectively. Body weight, behaviour, autonomic profiles, and neurological changes were continuously monitored for 24 h (Parasuraman et al., 2015). Then, the animals were further monitored for 2 weeks for mortality accordance with the current guidelines of Organization for Economic Co-operation and Development (OECD) (Guideline, 2001).

2.10 Induction of Diabetes

Adult female sprague dawley rats were selected for the study. After acclimatization period of 1 week, single intraperitoneal injections of STZ (55 mg/kg) mixed with 0.05 M citrate buffer administered to experimentally induce diabetes mellitus (Lin et al., 2019). Twenty four hours later upon induction of diabetes mellitus, 5% w/v of glucose solution (2 ml/kg/BW) was orally given to the rats to avoid hypoglycemic mortality. Citrate buffer was intraperitoneally injected to the control rats. Few drops of blood sample was collected from tail vein 3 days after STZ injection to measure glucose levels using a glucometer (ACCU-CHEK® Active, Roche Diagnostics, Mannheim, Germany). Rats with glucose levels >11 mmol/L were recognised as having diabetes and were used for the study.

2.11 Experimental Design

Adult female sprague dawley rats were divided into six groups each of six animals as follows:

Group I: Normal control
Group II: Diabetic control
Group III: Diabetic + glibenclamide (20 mg/kg)
Group IV: Diabetic + MESM (125 mg/kg)
Group V: Diabetic + MESM (250 mg/kg) Group VI: Diabetic + MESM (500 mg/kg)

The dose of glibenclamide was selected from the literature and the dose of *Spondias mombin* were selected from acute toxicity study findings (Parasuraman et al., 2019). Glibenclamide and MESM were suspended in 0.5% w/v carboxymethyl cellulose and administered orally. The animals in group I and II and were administered with 0.5% w/v carboxymethyl cellulose. Rats in group III were treated with 20 mg/kg body weight (BW) of glibenclamide (Gobinath et al., 2020). MESM at the dose levels of 125, 250, 500 mg/kg BW were administered to rats in group IV, V, and VII. On day 0, 7, 14, 21, and 28th day, few drops of blood samples were collected from tail vein to check the glucose levels using glucometer. Body weight variations were monitored on pre-study day, 14th day and 28th day for all the experimental animals. At the end of the study, blood samples were collected from the all the experimental animals, plasma were separated and used for the biochemical analysis.

2.12 Biochemical Analysis

At the 28th day, few millilitres (ml) of the blood sample were collected from tail vein in sample tubes containing EDTA. Biochemical parameter such as total plasma, total protein, glutamic oxaloacetic transaminase (AST), glutamic-pyruvic

TABLE 1 | Effect of oral administration of methanolic extract of leaves of *Spondias mombin* (MESM) on sprague dawley rats.

A) Phytochemical parameters

Types of tests	Result
Anthraquinone	+
Tannin	+
Saponin	+
Steroid	+
Phenolic compounds	+
Flavonoids	+
Alkaloids	+
Reducing sugar	+

B) Total phenol and flavonoids content

Types of tests	Concentration
Total phenols	124.71 ± 0.61 mg GAE/g
Total flavonoids	89.37 ± 0.13 mg QE/g

C) Antioxidant assay

Antioxidant activity assay	Concentration
S.mombin DPPH activity IC_{50}	1.62 ± 0.05 mg/ml
Quercetin DPPH activity IC_{50}	34.52 ± 0.51 mg/ml
S.mombin FRAP assay	541.63 ± 1.53 mg $FeSO_4E/g$

D) Effect of MESM on body weight (g)

	Pre-study day	14th day	28th day
Group I	175.88 ± 2.26	182.27 ± 1.88	191.77 ± 1.05
Group II	172.70 ± 1.36	162.19 ± 1.35***	150.94 ± 1.28***
Group III	174.04 ± 2.03	176.72 ± 2.01	187.44 ± 1.66
Group IV	170.71 ± 1.38	176.27 ± 0.87	188.09 ± 0.70
Group V	170.04 ± 2.14	177.44 ± 1.90	188.59 ± 1.91
Group VI	169.88 ± 2.46	178.60 ± 1.99	188.94 ± 1.22

E) Effect of MESM on glucose level (mmol/L)

	Pre-study day	7th day	14th day	21st day	28th day
Group I	5.60 ± 0.30	5.57 ± 0.35	5.28 ± 0.12	5.13 ± 0.26	5.22 ± 0.29
Group II	17.45 ± 0.61***	17.12 ± 0.68	15.98 ± 0.54	16.02 ± 0.43	16.52 ± 0.45
Group III	17.50 ± 0.61***	11.46 ± 0.57###	10.68 ± 0.38###	6.13 ± 0.11###	5.10 ± 0.09###
Group IV	18.27 ± 0.49***	14.83 ± 0.85	14.32 ± 0.51	9.90 ± 0.30###	7.67 ± 0.21###
Group V	17.85 ± 0.65***	13.75 ± 0.66#	12.78 ± 0.42##	9.22 ± 0.23###	6.10 ± 0.20###
Group VI	17.82 ± 0.47***	12.02 ± 0.49##	12.00 ± 0.47###	8.88 ± 0.19###	5.38 ± 0.07###

F) Effect of MESM on biochemical parameters

	Plasma insulin (μU/ml)	Total protein (g/dl)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio (g/dl)	Total bilirubin (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Group I	19.08 ± 0.41	9.81 ± 0.35	46.15 ± 0.66	60.87 ± 0.37	121.18 ± 0.53	4.45 ± 0.17	3.46 ± 0.15	1.17 ± 0.02	0.35 ± 0.01	34.96 ± 0.57	0.79 ± 0.04
Group II	4.11 ± 0.17***	4.31 ± 0.38***	100.61 ± 1.05***	116.85 ± 0.95***	201.84 ± 0.54***	3.23 ± 0.18***	1.99 ± 0.10***	0.86 ± 0.02***	0.97 ± 0.02***	72.96 ± 0.61***	1.48 ± 0.09***
Group III	19.26 ± 0.36###	8.96 ± 0.41###	60.27 ± 0.66###	60.43 ± 0.54###	131.48 ± 0.70###	4.18 ± 0.18###	3.32 ± 0.08###	1.15 ± 0.028###	0.38 ± 0.02###	37.50 ± 0.48###	0.75 ± 0.06###

(Continued on following page)

TABLE 1 | (*Continued*) Effect of oral administration of methanolic extract of leaves of *Spondias mombin* (MESM) on sprague dawley rats.

Group IV	7.77 ± 0.36$^{\#\#\#}$	5.60 ± 0.13$^{\#}$	98.19 ± 0.32	100.82 ± 1.24$^{\#\#\#}$	194.67 ± 0.75$^{\#\#\#}$	3.27 ± 0.10	2.33 ± 0.04	0.90 ± 0.02	0.91 ± 0.02	67.51 ± 0.26$^{\#\#\#}$	1.19 ± 0.04$^{\#\#}$
Group V	10.46 ± 0.39$^{\#\#\#}$	6.43 ± 0.16$^{\#\#\#}$	90.45 ± 0.44$^{\#\#\#}$	94.30 ± 0.33$^{\#\#\#}$	189.48 ± 0.37$^{\#\#\#}$	3.63 ± 0.08	2.45 ± 0.09$^{\#}$	0.95 ± 0.03	0.79 ± 0.03$^{\#\#\#}$	58.10 ± 0.32$^{\#\#\#}$	0.98 ± 0.02$^{\#\#\#}$
Group VI	14.33 ± 0.48$^{\#\#\#}$	7.92 ± 0.14$^{\#\#\#}$	79.18 ± 0.42$^{\#\#\#}$	85.02 ± 0.31$^{\#\#\#}$	163.17 ± 1.74$^{\#\#\#}$	3.92 ± 0.17$^{\#}$	2.89 ± 0.08$^{\#\#\#}$	1.12 ± 0.048$^{\#\#\#}$	0.55 ± 0.03$^{\#\#\#}$	44.10 ± 0.79$^{\#\#\#}$	0.89 ± 0.03$^{\#\#\#}$

G) Effect of MESM on lipid profile (mg/dl)

	TC	TG	HDL	LDL	VLDL
Group I	81.43 ± 0.46	101.97 ± 0.69	34.43 ± 0.75	43.72 ± 0.70	13.94 ± 0.28
Group II	195.83 ± 0.65***	168.25 ± 0.99***	16.08 ± 0.45***	101.29 ± 2.07***	42.22 ± 0.49***
Group III	94.12 ± 0.76$^{\#\#\#}$	81.81 ± 0.58$^{\#\#\#}$	37.95 ± 0.57$^{\#\#\#}$	41.26 ± 0.52$^{\#\#\#}$	13.75 ± 0.27$^{\#\#\#}$
Group IV	185.19 ± 1.22$^{\#\#\#}$	163.26 ± 1.51	18.88 ± 0.31	79.91 ± 0.18$^{\#\#\#}$	37.91 ± 0.30$^{\#\#\#}$
Group V	168.20 ± 1.18$^{\#\#\#}$	152.69 ± 1.24$^{\#\#\#}$	21.91 ± 0.61$^{\#\#\#}$	61.67 ± 0.46$^{\#\#\#}$	22.90 ± 0.56$^{\#\#\#}$
Group VI	132.56 ± 0.82$^{\#\#\#}$	136.91 ± 1.42$^{\#\#\#}$	28.80 ± 2.06$^{\#\#\#}$	49.39 ± 0.40$^{\#\#\#}$	14.74 ± 0.25$^{\#\#\#}$

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TC, total serum cholesterol; TG, serum triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.
All the values are Mean ± SEM (n = 6).
$^{***}p < 0.001$ *compare with control,* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, *and* $^{\#\#\#}p < 0.001$, *compare with diabetic control (Repeated measure ANOVA, followed by Tukey* post hoc *test).*

transaminase (ALT), and alkaline phosphatase (ALP), albumin, globulin, albumin/globulin (A/G) ratio, total bilirubin, urea, and creatinine and lipid parameter such as total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), very low density lipoprotein (VLDL), high density lipoprotein (HDL), were estimated by "Cobas Integra 400 plus" analyzer.

2.13 Estimation of Plasma Insulin

Plasma insulin level was estimated using rat insulin enzyme linked immunosorbent assay (ELISA) kits (RayBiotech Inc., Norcross, GA, United States).

2.14 Histopathological Analysis

At the end of 28th day, experimental group rats were sacrificed and pancreas, liver, and kidney were isolated for hispathological analysis. Specimens sections were stored in formalin 10%. Tissue is cut at 5 µm section with Microtome (Thermo Electron Corporation, England) from paraffin-embedded tissue blocks. The sections was placed on a glass slide, de-paraffinized, rehydrated and stained with hematoxylin and eosin (H&E). After mounting with DPX and coverslip the slides were analysed under a light microscope and recorded.

2.15 Statistical Analysis

The mean ± standard error of the mean (SEM) values was calculated for each group. Statistical differences among the groups were determined using repeated measure ANOVA followed by Tukey's *post hoc* test using SPSS. $p < 0.05$ was considered to be significant.

3 RESULTS

3.1 Phytochemical Parameters on *Spondias mombin*

Phytochemical analysis on MESM showed the presence of anthraquinone, tannin, saponin, steroid, phenolic compounds, flavonoids, reducing sugar, and alkaloids as indicated in **Table 1A**.

3.2 Total Phenol and Flavonoid Content

Total phenolic and flavonoid analysis of MESM reported as 124.71 ± 0.61 mg GAE/g and 89.37 ± 0.13 mg QE/g respectively. Acute toxicity study in MESM treated rats did not exhibit any toxicological sign up to the dose of 2000 mg/kg. Hence, the antidiabetic effect of MESM was studied at the dose levels of 125, 250, and 500 mg/kg as indicated in **Table 1B**.

3.3 Antioxidant Activity

The IC_{50} of the MESM and quercetin in DPPH assay was 1.62 ± 0.05 mg/ml and 34.52 ± 0.51 mg/ml respectively and FRAP assay was 541.63 ± 1.53 mg $FeSO_4E$/g as indicated in **Table 1C**.

3.4 Body Weight Analysis on Sprague Dawley Rats

The diabetic animals showed a significant reduction in the body weight from 14th day onwards when compared with that of control.

Whereas, the animals treated with glibenclamide and MESM did not showed any reduction in body weight when compared with that of control (**Table 1D**).

3.5 Blood Glucose Analysis on Sprague Dawley Rats

Blood glucose levels after different oral doses of MESM presented in **Table 1E**. Rats in Group III treated with glibenclamide (20 mg/kg) showed significant decrease ($p < 0.05$) in blood glucose level compared with diabetic control rats from day 7 onwards. As expected in Group IV and Group V, mean blood glucose level significantly declined ($p < 0.05$) from day 21 onwards compared with diabetic control rats. Furthermore, Group VI rats had distinctly reduced blood glucose levels ($p < 0.05$) from day 14 onwards when compared with diabetic control.

3.6 Biochemical Parameters on Sprague Dawley Rats

Induction of diabetes caused significant increase in AST, ALT, ALP, total bilirubin, urea, and creatinine when compared with normal control. Furthermore, significant decrease in total protein, albumin, globulin and A/G ratio ($p < 0.05$) when compared with that of normal control (**Table 1F**). However, administration of different doses MESM to diabetic rats for 28 days recovered ALT, ALP, and urea from Group IV onwards, total protein, AST and total bilirubin from Group V onwards, globulin, A/G ratio, and albumin in Group VI compared with diabetic control rats.

3.7 Lipid Profile on Sprague Dawley Rats

The diabetes rats showed significant increase in TC, TG, LDL, and VLDL and decrease in HDL ($p < 0.001$) compared with normal control rats (**Table 1G**). However, administration of MESM caused significant decrease in TC, TG, LDL, VLDL, and

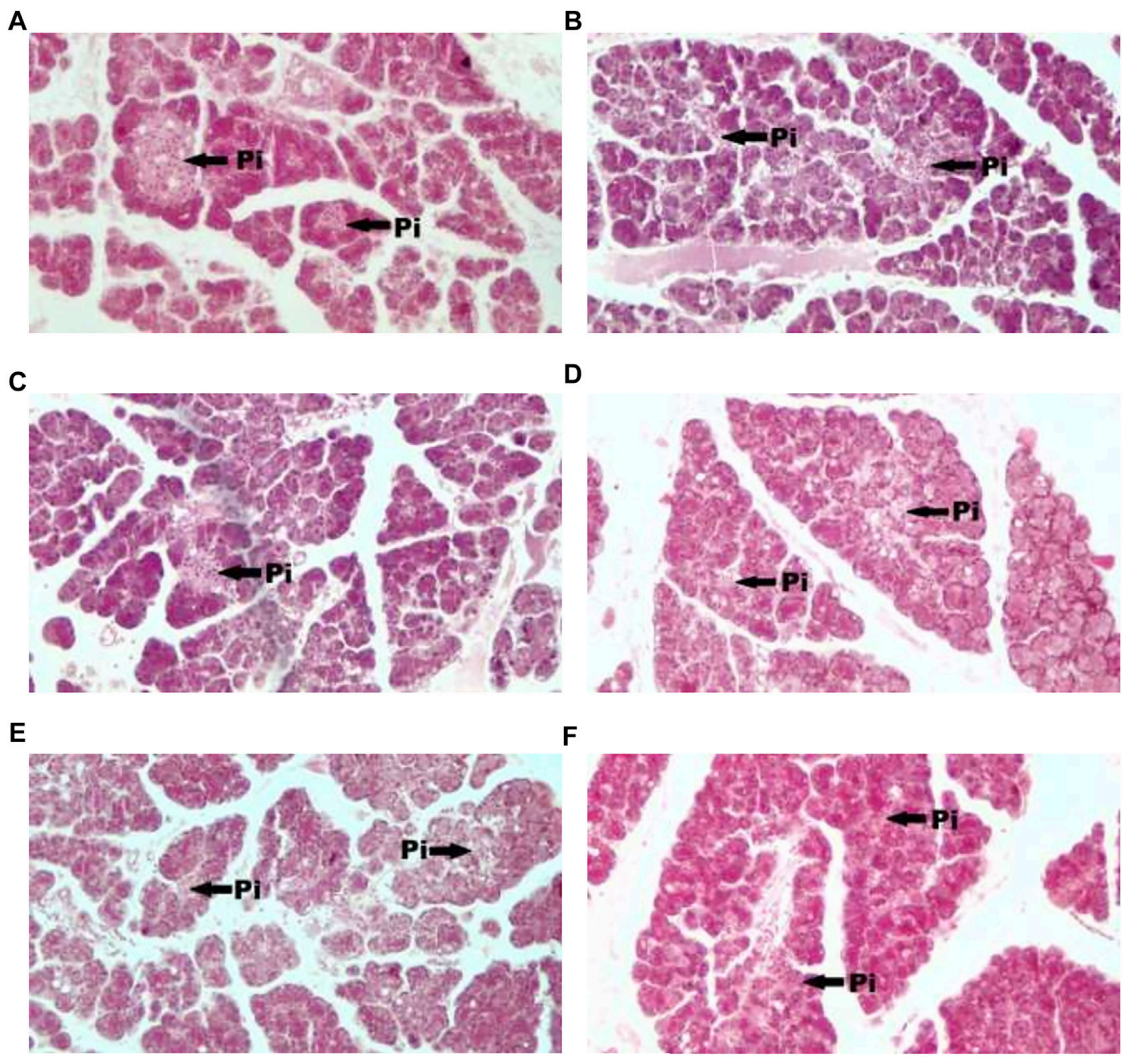

FIGURE 1 | Photomicrograph of a section of pancreas of normal control **(A)**, diabetic control **(B)**, glibenclamide 20 mg/kg **(C)**, Spondias mombin 125 mg/kg **(D)**, Spondias mombin 250 mg/kg **(E)**, Spondias mombin 500 mg/kg **(F)**. PI-Pancreatic Islet. H&E 100X.

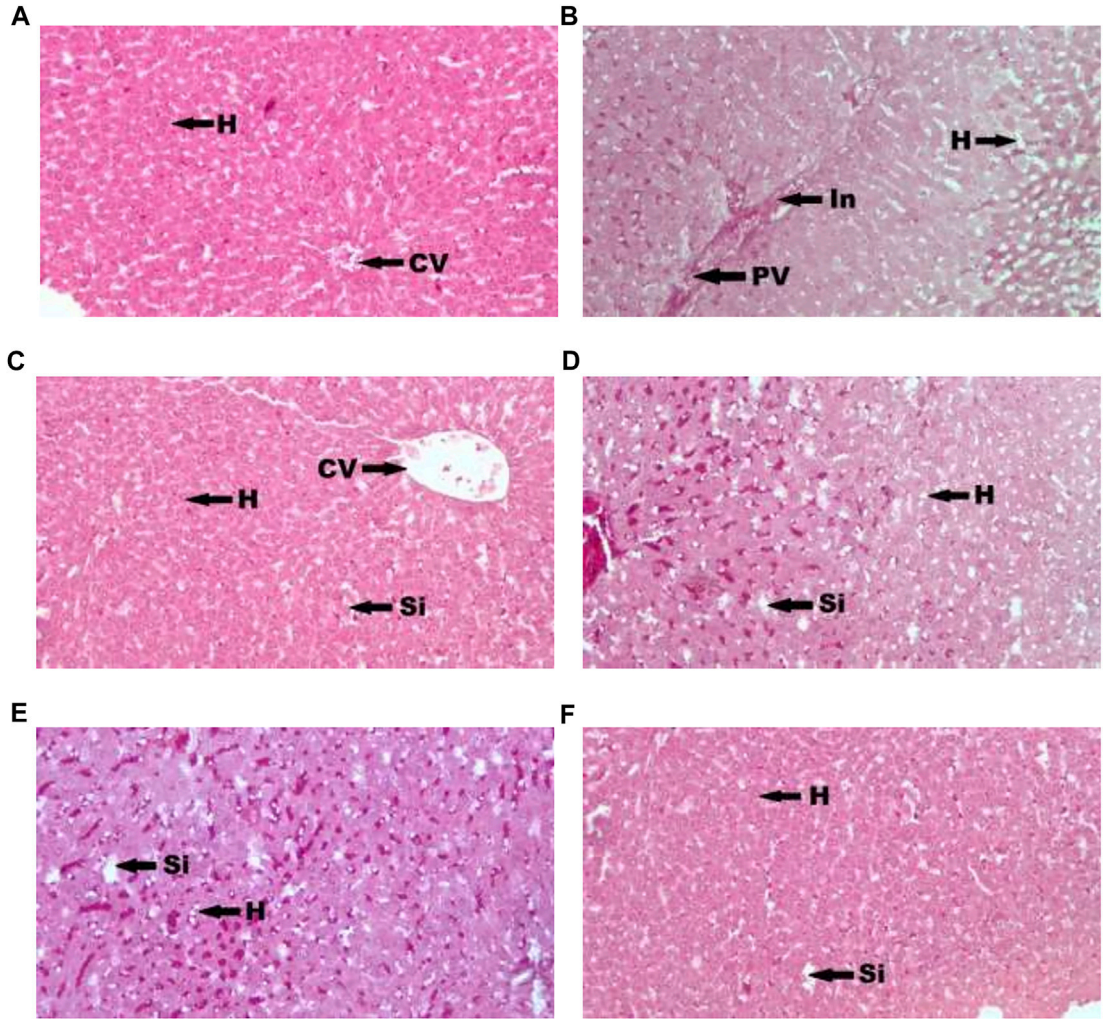

FIGURE 2 | Photomicrograph of a section of liver of normal control **(A)**, diabetic control **(B)**, glibenclamide 20 mg/kg **(C)**, Spondias mombin 125 mg/kg **(D)**, Spondias mombin 250 mg/kg **(E)**, Spondias mombin 500 mg/kg **(F)**. H-hepatocytes, CV-centrilobular vein, PV-Portal vein, In-Periportal Inflammation, and Si-sinusoids. H&E 100X.

increase in HDL (at 250 and 500 mg/kg) in comparison with control diabetic rats.

3.8 Plasma Insulin Level on Sprague Dawley Rats

Plasma insulin level was reduced significantly in diabetic rats while treatment with MESM increased plasma insulin level significantly ($p < 0.001$) at 125, 250, and 500 mg/kg dose (**Table 1F**).

3.9 Histopathological Analysis on Sprague Dawley Rats

Figure 1 showed photomicrograph of pancreas of normal control (A) rats showed normal architecture and the acinal cells are normal. Pancreas of diabetic control (B) rats showed degeneration of beta cells of Langerhans. Glibenclamide 20 mg/kg (C) treated rats showed regeneration of beta cells of Langerhans. Pancreas of *Spondias mombin* 125 mg/kg (D) treated rats showed minimal regeneration of beta cells of Langerhans. *Spondias mombin* 250 mg/kg (E) treated rats showed average regeneration of beta cells of Langerhans. However, *Spondias mombin* (500 mg/kg) treated rats showed pronounced regeneration of beta cells of Langerhans. **Figure 2** exhibited photomicrograph of liver section of normal control (A) showing normal lobular pattern with a centrilobular vein and radiating irregular anastomosing plates of hepatocytes. Section of liver of diabetic control (B) showing accumulation of droplets with distorted morphology of hepatocytes, portal vein, and periportal inflammation. Section of liver of glibenclamide 20 mg/kg (C) treated rats showing restored morphology of hepatocytes, centrilobular vein and minimal dilation of sinusoids. *Spondias mombin* 125 mg/kg (D) treated rats

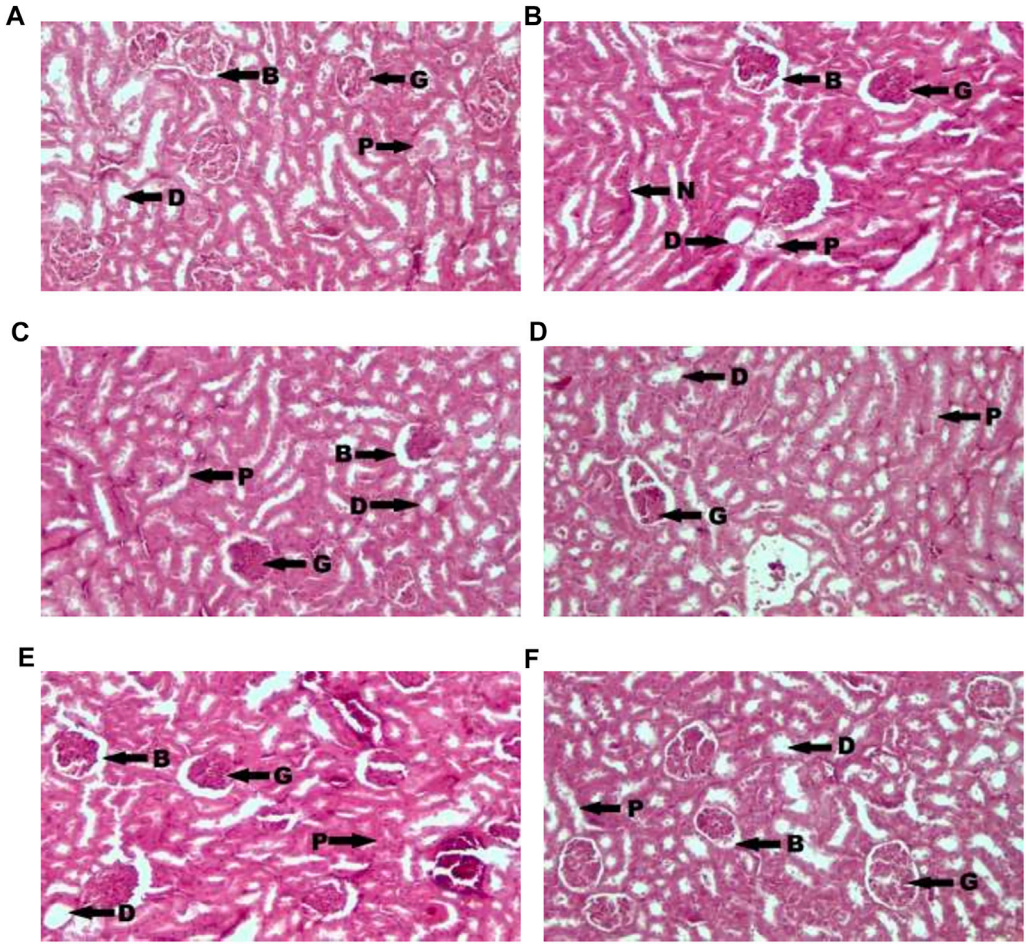

FIGURE 3 | Photomicrograph of a section of kidney of normal control **(A)**, diabetic control **(B)**, glibenclamide 20 mg/kg **(C)**, Spondias mombin 125 mg/kg **(D)**, Spondias mombin 250 mg/kg **(E)**, Spondias mombin 500 mg/kg **(F)**. B-Bowman's capsule, G-Glomerulus, P-Proximal convoluted tubule, D-Distal convoluted tubules, and N-Necrosis. H&E 100X.

showing moderated improvement in the slightly distorted hepatocytes and dilation of sinusoids. *Spondias mombin* 250 mg/kg (E) treated rats showing moderate improvement in the distorted hepatocytes and sinusoids. *Spondias mombin* 500 mg/kg (F) treated rats showing marked improvement in the distorted hepatocytes and sinusoids. **Figure 3** Showed photomicrograph of a section of kidney of normal control (A) rats showing normal bowman's capsule, glomerulus, and renal tubules. Kidney of diabetic control (B) rats showing distorted and shrunken glomerulus with proximal and distal convulated tubules and necrosis. Glibenclamide 20 mg/kg treated rats showing improved microarchitecture of bowman's capsule, glomerulus and proximal and distal convulated tubules. *Spondias mombin* 125 mg/kg (D) treated rats showing minimum improvement in microarchitecture of bowman's capsule, glomerulus and proximal and distal convulated tubules. *Spondias mombin* 250 mg/kg (E) treated rats showing moderate improvement in microarchitecture of bowman's capsule, glomerulus and proximal and distal convulated tubules. Mild also necrosis observed. *Spondias mombin* 500 mg/kg (F) showed restored microarchitecture of bowman's capsule, glomerulus, and proximal and distal convulated tubules.

4 DISCUSSION

MESM showed no significant changes in the body weight throughout the study comparison to normal control rats. Oral administration of different doses of MESM caused significant decrease in glucose levels in STZ induced diabetic rats. Furthermore, our results revealed that diabetic rats treated with MESM for 28 days significantly recovered lipid and biochemical parameters within normal levels.

Administration of STZ was found capable of developing peripheral insulin resistance or impairing insulin secretion from pancreatic β cells and it is sufficient to induce noninsulin-dependent diabetes mellitus (type 2) in animals (Qinna and Badwan, 2015). STZ also causes diabetes by damaging beta-cells of the Islets of Langerhans in the pancreas

due to uncontrolled production of reactive oxygen species (ROS) leads to decrease in insulin synthesis and release (Cerf, 2013). The purpose of STZ induced diabetic rat model used in this due to lineaments such as stable hyperglycemia without insulin requirement to survive and response to glibenclamide (Rabbani et al., 2010). Glibenclamide act as insulin secretogogues by closing K-ATP channels bypassing the β cell metabolism. Insulin secretion stimulated by increased blood glucose, free fatty acids, amino acids, gastrointestinal hormones (gastrin, cholecystokinin, secretin), gastric inhibitory peptide, sulfonylurea drugs (glyburide, tolbutamide), parasympathetic, β -adrenergic stimulation (Vaz et al., 2016).

Significant loss in body weight was observed in diabetic rats due to loss or damage of protein structure and muscle wasting (Fakhruddin et al., 2017). However, administration of MESM alleviated this condition almost comparable with glibenclamide treated rats agrees the idea of antidiabetic effect of MESM by recovering from muscle wasting.

MESM reduced blood glucose levels in diabetic rats which emphasizes extracts hypoglycaemic effect. Its antidiabetic effect could be due to the presence of free radical scavengers such as flavonoids, tannins and polyphenols reduce glucose absorption in small intestine, increase glucose update by peripheral tissues, enhance regulation of glycolysis process, and glycogen synthase (Adisakwattana, 2017). The possible mechanism of MESM on hypoglycemic action may be through potentiation of pancreatic secretion of insulin from β-cell of islets and/or due to enhanced transport of blood glucose to the peripheral tissue or by inhibition of endogenous glucose production or activation of gluconeogenesis in liver and muscles (Burcelin et al., 1995).

The declined plasma insulin in diabetic induced rats was reversed by oral administration of extract. This could be due to MESM's ability to regenerate beta cells (Patel et al., 2012). Decrease in total protein, albumin, and glubulin levels in diabetic rats caused by clinical markers in diabetic nephrophaty such as elevated protein catabolism, proteinuria and albuminurea (Kaleem et al., 2008). Diabetic condition also lowered levels of albumin, globulin and A/G suggesting chronic liver infection. However, treatment with MESM elevated total protein, albumin, globulin, and A/G into normal by restoring liver function. This improved protein synthesis, prevented protein degradation and elevated insulin's alanine, arginine, and glutamine uptake (Ramachandran et al., 2012). Furthermore, Serum levels like AST, ALT, and ALP increased in diabetic rats indicates liver damage and stimulated gluconeogenesis and ketogenesis. Toxicity action of STZ on liver showed secretion of liver cytosolic enzymes into bloodstream (Ghimire et al., 2018). Increase in levels of urea and creatinine indicated renal damage in diabetic rats due to insulin and glucose failed to improve gluconeogenesis process. Antioxidant nephroprotective role of flavonoids and polyphenol in MESM protected renal function during diabetes. Glucosides in extract might converted bilirubin into glucorunic, activated Constitutive Andostane Receptor (CAR) for bilirubin excretion and lowered total bilirubin levels to normalcy (Arthur et al., 2012). Our finding was supported by hitopathological analysis. STZ induced rats expressed cellular damage in pancreatic islets, liver histology, and renal glomeruli and tubules (Petchi et al., 2014). Rats treated with glibenclamide and MESM exhibited reduction in the pathological changes induced by STZ expressed protective effect of both glibenclamide and MESM.

Liver is important for detoxification of xenobiotics and liver damage related to exposure due excessive drugs leads to depletion in glutathione (GSH) levels plasma membrane damage, cellular necrosis and changes in serum lipids (Singh et al., 2016). Induction of diabetes altered lipoprotein and lipid profile in rats, increase in blood glucose levels was concomitant with changes in serum lipid indices in diabetic rats. These abnormalities caused by inactivated activity of lipolytic hormones on fat depots due to insulin action (Andallu et al., 2009). Inactivated lipoprotein lipase enzyme due to insulin deficiency during diabetic condition caused secondary complications from hypertryglyceridemia and hypercholesterolemia. HMG-CoA reductase is a rate limiting enzyme that metabolise high cholesterol LDL. However, insulin deficiency caused dyslipidemia due to inhibitory action of insulin on HMG-CoA reductase (Grice and Elmendorf, 2017). Diabetes-induced hyperlipidemia is responsible for excess movement of fat from adipose due to limited usage of glucose. Treatment with MESM reduced TC, TG, LDL, VLDL levels, and increased HDL levels suggesting that MESM possess possible hypolipidemic activity.

Flavonoids and tannins are important polyphenolic compounds which exhibited pharmacological activities during health problems especially oxidative stress, cardiovascular diseases, hyperglycemia cancers and inflammations (Zhang et al., 2015). Furthermore, flavonoids, tannins, saponins, and anthraquinone demonstrated primary antioxidant activity and useful in managing diabetic condition. Many research reports indicated that the natural antioxidants could inhibit biological enzymes such as Dipeptidyl Peptidase IV (DPP-4), α-amylase, and α- glycosidase which involved in diabetes mellitus type 2 (Deacon et al., 2000; Semighini et al., 2011). Hypoglycemic activity of MESM could be due to the presence of various phytochemical compounds acted individually or synergistically in reducing blood glucose by enhancing insulin production from pancreatic islets and glucose breakdown parallel with glibenclamide. Antioxiday assay indicates potent efficiency to DPPH, suggesting that polyphenolic compounds may be the main contributor to scavenging DPPH free radicals and polyphenolic compounds are the most efficient in reducing power in MESM.

5 CONCLUSION

Antidiabetic and antihyperlipidemic effect of methanolic extracts of *S. mombin* leaves is comparable with glibenclamide in term of its effect in reducing the elevated blood glucose levels and improved biochemical and lipid profile. Further studies, are required to investigate the phytoconstituents that responsible for antidiabetic effect *S. mombin* leaves.

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 AIMST University Human and Animal Ethical Committee.

AUTHOR CONTRIBUTIONS

RG, SP, SS, BE and SC have equally contributed to design the study, conceived the study, carried out the experiments, conducted the data analysis, and interpreted the data. RG, SP, SS, BE and SC have drafted, edited, and reviewed the manuscript. RG has procured the Institutional Ethical Approval for the study. All the authors have given their consent for submission.

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SYSTEMATIC REVIEW
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Effectiveness of Interventions on Improving Balance in Children and Adolescents With Hearing Impairment: A Systematic Review

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Although children and adolescents with hearing impairment are at risks of falls from balance problems, reliable information on effects of interventions are scare. Therefore, the purpose of this review is to systematically summarize studies on the evidence of interventions to improve balance ability in children and adolescents with hearing impairment. A systematic literature search was conducted on five major electronic databases. Studies were included if: 1) interventions or trials focusing on improving balance in children and adolescents with hearing impairment; 2) research targeting children with hearing impairment (samples with a mean age below 18 years); 3) studies were published in English peer-reviewed journals due to language barriers and resource limitations; and 4) study designs were randomized controlled trial or quasi-experiment. A nine-item tool adapted from the Consolidated Standards of Reporting Trials Statement was used to assess the quality of the studies. Through the search strategy, 373 articles were identified, and 15 studies published between 1981 and 2021 met the inclusion criteria. Most of the studies reviewed were categorized as medium or low quality, and only three were identified as high quality. Exercise interventions were adopted in 80% of the included studies, whereas studies that employed music + vibration, motor, and game as the intervention modalities accounted for the remaining 20.0%. The results of this review showed that the included trials with exercise interventions had a positive influence on the balance among children and adolescents with hearing impairment (the post-intervention scores were significantly higher than the pre-intervention or the control group scores). In addition, the interventions with duration of 8–16 weeks were more effective than those with less than 8 weeks. However, due to most of the reviewed studies were of low methodological quality, the trials results analyzed by this systematic review should be interpreted with caution. Further investigations of high-quality studies are therefore needed to prove the effectiveness of interventions on improving balance performance in children and adolescents with hearing impairment.

Systematic Review Registration: [https://www.crd.york.ac.uk/PROSPERO/], PROSPERO [308803].

Keywords: hearing impairment, balance, physical exercise, effectiveness, systematic review

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INTRODUCTION

Balance is an essential prerequisite for most daily life activities in children (Melo et al., 2020). It is the complex ability to maintain, achieve, or restore the state of balance of the body while a child stands still, prepares to move in movement, or prepares to stop moving (Pollock et al., 2000). Balance requires the integration of several sensory, motor, and biomechanical inputs (Nashner et al., 1982). However, changes in some of these sensory systems (e.g., visual, somatosensory, and vestibular) can trigger disturbances in the body balance (Melo et al., 2020; Paillard, 2017). The capability of balance can be examined in static (the body remains motionless) or dynamic (the body can react to perturbations or is in movement) conditions, as well as in both conditions (Paillard, 2017). For children and adolescents, maintaining balance is an essential prerequisite to competently perform most activities of daily living and is important for the proficient performance of fundamental movement skills (Mickle et al., 2011). Studies have shown that the most significant transitions in motor development occur in the first decade of life with balance control usually established at 7–10 years old (Ferdjallah et al., 2002; Roncesvalles et al., 2001). Therefore, addressing characteristics in the balance ability of children and adolescents is important for interventions in many practical fields.

Hearing impairment refers to the complete and partial loss of the ability to hear (Mathers et al., 2000). According to the World Health Organization, over 5% of the world's population are suffering from some form of hearing impairment and it is estimated that by 2050, over 700 million people (one in every 10 people) will have hearing impairment.[1] Childhood hearing impairment is a significant public health problem, which is associated with long term academic and communicative difficulties (Davis et al., 2001) and other physical deficits (e.g., vestibular-related impairments) (Pajor and Jozefowicz-Korcxynska, 2008; Siegel et al., 1991). The previous studies have proved that children and adolescents with hearing impairment are at increased risks of motor and balance problems due to their balance and/or motor deficits related to damage to the vestibular system (Fellinger et al., 2015; McPhillips, 2015). Furthermore, studies have shown that hearing impairment is associated with an increased risk of all-cause mortality (Karpa et al., 2010), possibly *via* physical activity-related parameters, such as mobility function and balance (Lin and Ferrucci, 2012; Loprinzi, 2015).

Over the decades, a few intervention studies have been conducted to improvise balance in children and adolescents with hearing impairment on different outcome measures (Ebrahimi et al., 2017; Effgen, 1981; Rine et al., 2004). Only one review conducted by Fernandes et al. (2015) analyzed three trials on the management of vestibular and balance functions in children with hearing impairment and concluded that vestibular rehabilitation has a positive influence on balance outcomes. Numerous works examined effects of interventions on balance improvements in this population since 2015 and have not been summarized and reviewed. Therefore, the purpose of our review is to systematically summarize studies on the evidence of interventions to improve balance ability in children and adolescents with hearing impairment until 2021. Our findings can provide insights for clinical practice and future studies on balance improvement among children and adolescents with hearing impairment.

[1]https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss.

METHODS

This systematic review was conducted while adhering to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement (Moher et al., 2009).

Search Strategy

The researchers systematically searched studies with the following databases from inception to December 2021: PubMed, PsycINFO, Scopus, Education Resources Information Centre (ERIC), and Web of Science (WoS) Core Collection. The search strategy included four groups of keywords: 1) hearing impairment* OR hearing disability* OR deaf OR hard of hearing; 2) child* OR adolescent* OR teenager* OR youth* OR youngster* OR student* OR pupil*; 3) motor ability* OR motor skill* OR balance OR posture control OR postural stability; 4) intervention OR trial OR experiment. In addition, the snowballing technique was used to identify potential studies by scanning the references of all the included articles.

Inclusion and Exclusion Criteria

Studies meeting the following criteria were included in this review: 1) interventions or trials focusing on improving balance in children and adolescents with hearing impairment; 2) research targeting children with hearing impairment (samples with a mean age below 18 years); 3) studies were published in English peer-reviewed journals due to language barriers and resource limitations; and 4) study designs were randomized controlled trial (RCT) or quasi-experiment (QE). Studies were excluded if they 1) were pertaining to other topics; 2) participants' mean age was above 18; 3) were unpublished articles, comments, conference proceedings, reviews, and dissertations; and 4) failed to report the detailed outcomes of trials. Two authors independently screened the returned articles according to the inclusion and exclusion criteria. Any disagreements between authors were resolved through discussion.

Quality Assessment

To determine the methodological quality of the included studies, the researchers used a nine-item tool adapted from the Consolidated Standards of Reporting Trials Statement (Schulz et al., 2010), which has been used to assess the methodological

TABLE 1 | Risk of bias checklist.

Item	Description
1	Randomization (generation of allocation sequence, allocation concealment and implementation) clearly described and adequately completed
2	Valid measures (validation data were provided by the author)
3	Blinded outcome assessment (assessor blinding)
4	Participants analyzed in group they were originally allocated to, and participants not excluded from analyses because of noncompliance to treatment or because of missing data
5	Covariates accounted for in analyses (e.g., baseline score and other relevant covariates when appropriate such as age or sex)
6	Power calculation reported for main outcome
7	Presentation of baseline characteristics separately for treatment groups
8	Dropout was described, with a ≤20% dropout for studies with follow-up of≤6 months and ≤30% dropout for studies with follow-up>6 months
9	Summary results for each group + estimated effect size (difference between groups) + its precision (e.g., 95% CI)

quality of previous systematic reviews in similar areas (Healy et al., 2021; Morgan et al., 2013) (**Table 1**). Two authors independently assessed all studies. Each item was scored as 1 (the assessed item was explicitly described and presented) or 0 (the assessed item was inadequately described or absent). If consensus could not be reached, then agreement was obtained through discussion between the authors. The score for each study was summed, and the median score for all included study scores was calculated. Articles were determined of high quality when they scored above the median score, medium quality when they scored equal to the median score, and low quality when they scored below the median score (Zeng et al., 2017).

Data Extraction and Analysis

The first author completed the data extraction and then the second author verified the data. Discrepancies were resolved through a consensus discussion. The researchers extracted the following information: the first author, publication year, geographic location, and intervention detail.

Given the heterogeneity of the included studies, meta-analysis was not conducted. Instead, the intervention characteristics of each study were first summarized and analyzed and then recorded on a standardized form created by the authors. The effective rate of the interventions was calculated using the following equation: effective trials (the post-intervention scores were significantly higher than the pre-intervention or the control group scores)/(total number of trials). Similarly, data analysis was performed by the first author and then verified by the second author.

RESULTS

Search Results

The initial search identified 373 studies (ERIC, n = 15; WoS Core Collection, n = 179; PubMed, n = 106; PsycINFO, n = 55; Scopus, n = 373). Of these articles, 186 duplicates were removed. An additional article was identified from a citation search of relevant published literature reviews. After screening the titles and abstracts, 126 studies were excluded. The remaining full-text articles were read, and 45 others were excluded. Therefore, a total of 15 studies were included in this review. The PRISMA flowchart is shown in **Figure 1**.

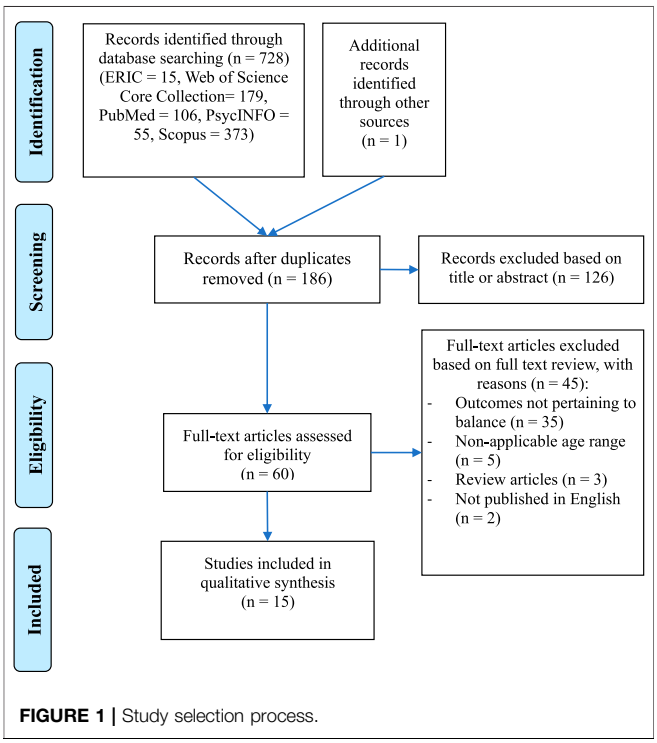

FIGURE 1 | Study selection process.

Methodological Quality

Table 2 outlines the results of the assessment of methodological quality. Overall, three out of the 15 included studies (20%) were categorized as high quality, and three (20%) and nine (60%) other studies were of medium and low quality, respectively. The weak components among the included studies were randomization, assessor blinding and result precision. Specifically, only 20% of the included studies described participant randomization, the blinded outcome assessment, and the outcome precision (e.g., 95% Confidence Interval).

TABLE 2 | Results of study quality evaluation of included studies.

First Author (year)	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Item 7	Item 8	Item 9	Score	Quality
Effgen (1981)	0	1	0	0	0	0	0	1	0	2	low
Fotiadou et al. (2002)	0	1	0	1	0	0	1	1	0	4	low
Rine et al. (2004)	0	1	1	1	0	0	1	1	0	5	medium
Borowiec (2011)	0	1	0	1	1	0	0	1	0	4	low
Rajendran et al. (2013)	1	1	0	1	0	1	1	1	1	7	high
Shah et al. (2013)	0	1	0	1	0	0	1	1	0	4	low
Majlesi et al. (2014)	0	1	0	1	0	1	1	1	0	5	medium
Karbunarova (2016)	0	1	0	0	0	0	1	1	0	3	low
Ebrahimi et al. (2017)	0	1	0	1	0	0	0	1	0	3	low
Vernadakis et al. (2018)	0	1	0	1	0	1	1	1	0	5	medium
Ilkim and Akyol (2018)	0	1	0	1	0	0	1	1	0	4	low
Demirel (2018)	0	1	0	1	0	0	0	1	0	3	low
Soori et al. (2019)	0	1	0	1	1	0	0	1	0	4	low
Zarei and Norasteh (2020)	1	1	1	0	1	1	1	0	1	7	high
Zarei et al. (2021)	1	1	1	0	1	1	1	0	1	7	high

Study Characteristics

Table 3 presents the study characteristics. The included studies were published between 1981 and 2021; those published before 2000, 2001–2010, and 2011–2021 accounted for 6.7, 13.3, and 80.0%, respectively. These studies were from seven countries and most of which were from Asian countries, including Iran (5, 33.3%), India (2, 13.3%), and Turkey (2, 13.3%). Eleven studies (73.3%) were quasi-randomized trials, and the remaining (26.7%) were RCT designs. The samples included children (73.3%) and adolescents (26.7%), majority of which reported gender composition. Ten studies (66.7%) involved sample sizes between 20 and 40, whereas 20.0 and 13.3% had sample sizes less than 20 and more than 40, respectively. In addition, 66.7% of the included studies failed to report specific types of hearing impairment. Among the trials that reported the degree of hearing loss, 72.7% had moderate to severe hearing loss (with a hearing loss of 56–90 dB) and 27.3% had profound loss (hearing loss>90 dB).

Intervention Characteristics

Table 4 presents the intervention characteristics.

Intervention Modality

Exercise interventions were adopted in most studies (12, 80.0%), whereas studies that employed music + vibration, motor, and game as the intervention modalities accounted for the remaining 20.0%. Exercise intervention mainly used a series of structured course or training, such as vestibular-specific neuromuscular training (Rajendran et al., 2013), exercise balance program based on proprioception (Majlesi et al., 2014) and so on. High-frequency music and vibration equipment were used in music + vibration intervention. In motor intervention, the 15-min of standing movements in different positions were chosen (Effgen, 1981). While Nintendo Wii-Fit Plus interactive games were selected for game intervention (Vernadakis et al., 2018).

Duration and Frequency

The total duration of interventions ranged from 10 days to 16 weeks, 80% of which opted for a duration of 8–16 weeks. In terms of intervention frequency, 66.7% of the included studies performed their experiments three times a week, whereas 20.0 and 6.7% conducted their experiments twice and once a week, respectively. Sessions of 30–45 min per time were selected by 46.7% of the trials, whereas sessions of more than 45 min and less than 15 min per time accounted for 26.7 and 13.3%, respectively. However, 13.3% of the trials failed to report the duration of each session.

Outcome Measure

A total of 10 studies evaluated the static balance, among which the most common assessment was one-leg standing test, such as One-leg Standing Balance Test, Stork Balance Test, Flamingo Balance Test, and Single-limb Standing Test. Six studies tested dynamic balance, two of which used Y Balance Test. Five other studies tested subjects' postural stability, and another one measured overall balance using the Pediatric Balance Scale.

Intervention Effect

Overall, 13 out of 15 trials (86.7%) showed a positive effect on improving balance among children and adolescents with hearing impairment (the post-intervention scores were significantly higher than the pre-intervention or the control group scores). Interventions with a total duration of 8–16 weeks were more effective than those with less than 8 weeks (91.7 *vs.* 75.0%). And trials in children with hearing impairment were more effective than adolescents with hearing impairment (91.7 *vs.* 75.0%). Besides, among 10 studies that measured static balance, the effective rate was 90%, whereas the efficiency of six studies that assessed dynamic balance was 100%. Five studies that evaluated postural stability had an effective rate of 80%, whereas the effective rate of one study that measured overall balance was 100%.

Method of Statistics

Nearly half of the studies (46.7%) conducted independent t-test or paired t-test, and 20.0% of which performed U-test. Repeated-measures ANOVA and its combination with t-test accounted for

TABLE 3 | Characteristics of included studies.

First Author (year, country)	Design/Sample Characteristic	Modality	Intervention	Instruments (outcomes)	Method of Statistics	Effect
Effgen (1981, United States)	QE *n* = 49 (29 male) M age: 9.1 hearing impairment type: NR Hearing loss >75dB	Motor	10 days. ① 15 min/day, Standing activities of static balance; ② Normal classroom activity (free play).	Force platform (Static balance and stability)	independent *t*-test	① > ② (Time of standing on one leg) ① ≈ ② (Sway degree)
Fotiadou (2002, Greece)	QE *n* = 29 (15 male) M age: 7.9/8.0 hearing impairment type: SNHL Hearing loss >70dB	Exercise	16 weeks. ① 3 × 40 min/week, Rhythmic gymnastics activities; ② 3 × 40 min/week, Physical education activities.	Stabilometer (Dynamic balance)	independent *t*-test; paired *t*-test	① > ②
Rine (2004, United States)	RCT *n* = 21 (9 male) M age: 5.6/5.7 hearing impairment type: SNHL + VI Hearing loss: moderate to profound	Exercise	12 weeks. ① 3 × 30 min/week, Exercise intervention focused on substitution, including eye hand coordination, general coordination activities, visual motor training, and balance training; ② 3 × 30 min/week, Language development training.	PSCT (Stability)	independent *t*-test; Wilcoxon signed rank tests	① > ②
Borowiec (2011, Poland)	QE *n* = 25 (12 male) age range: 10–13 hearing impairment type: NR Hearing loss: NR	Music + vibration	16 weeks. ① 1 × 45 min/week, Physical exercise performed to the music with enhanced high frequencies and vibration devices; ② 1 × 45 min/week, Traditional dancing classes using every day hearing aids.	Balancing Backward Test (Dynamic balance)	Mann–Whitney *U*-test	① > ②
Rajendran (2013, India)	RCT *n* = 21 (14 male) M age: 7.5/8.1 hearing impairment type: NR Hearing loss >90dB	Exercise	6 weeks. ① 3 × 45 min/week, Vestibular-specific neuromuscular training, including training of balance retraining, Eye-hand coordination and FMS; ② Regular school activities.	One Leg Standing Balance Test/ Postural sway meter and Pediatric Reach Test (Static balance and stability)	Mann–Whitney *U*-test	① > ②
Shah (2013, India)	QE (Pre-post test) *n* = 10 (6 male) M age: 7.6 hearing impairment type: SNHL Hearing loss: NR	Exercise	12 weeks. 3 × 45 min/week, exercise sessions including eye-hand and general coordination exercises, visual motor training, balance training.	Pediatric Balance Scale (Overall balance)	paired *t*-test	① > ②
Majlesi (2014, Iran)	QE *n* = 20 (20 male) M age: 10.4/11.3 hearing impairment type: NR Hearing loss >75dB	Exercise	4 weeks. ① 3 × 45 min/week, Exercise balance program based on proprioception training; ② NR.	Force platform (Static balance and stability)	independent *t*-test; repeated-measures ANOVA	① > ②
Karbunarova (2016, Ukraine)	QE *n* = 20 (16 male) age range: 6–10 hearing impairment type: NR Hearing loss: NR	Exercise	10 weeks. ① 2 times/week, Swimming classes; ② 2 times/week, Soccer, volleyball and basketball classes.	Romberg and Bondarevsky's difficult test/Walking on balance beam test (Static and dynamic balance)	independent *t*-test; paired *t*-test	① > ②
Ebrahimi (2017, Iran)	QE *n* = 24 (18 male) M age: 9.8/10.1 hearing impairment type: SNHL + VI Hearing loss>90dB	Exercise	8 weeks. ① 3 × 45 min/week, Progressive exercise program including adaption, eye-head coordination and substitution exercises; ② NR.	PSOT (Static balance and stability)	independent *t*-test; paired *t*-test	① > ②
Demirel (2018, Turkey)	QE *n* = 18 (12 male) M age: 7.4 hearing impairment type: NR Hearing loss: NR	Exercise	10 weeks. ① 2 × 50–75 min/week, Special movement training program; ② NR.	Gross motor development tests (Dynamic balance)	Mann–Whitney *U*-test	① > ②
Vernadakis (2018, Greece)	QE *n* = 20 (10 male) M age: 18.3 hearing impairment type: SNHL Hearing loss >70dB	Game	8 weeks. ① 2 × 15 min/week, Interactive games Wii-Fit Plus of the Nintendo Wii console; ② Traditional adapted physical education balance training program.	Flamingo Balance Test (Static balance)	independent *t*-test; repeated-measures ANOVA	① ≈ ②

(Continued on following page)

TABLE 3 | (*Continued*) Characteristics of included studies.

First Author (year, country)	Design/Sample Characteristic	Modality	Intervention	Instruments (outcomes)	Method of Statistics	Effect
Ilkim (2018, Turkey)	QE *n* = 60 (NR) M age: 12.4/12.8 hearing impairment type: NR Hearing loss: 90–110 dB	Exercise	14 weeks. ① 3 times/week, Athletic exercises; ② 3 times/ week, Gymnastic exercises.	Flamingo Balance Test (Static balance)	independent *t*-test; paired *t*-test	① > ②
Soori (2019, Iran)	QE *n* = 20 (20 female) M age: 9.35 hearing impairment type: NR Hearing loss >61dB	Exercise	8 weeks. ① 3 × 60 min/week, Perceptual-motor training (such as balance training, running between obstacles); ② Daily routine works.	Stork balance test/Y Balance Test (Static and dynamic balance)	independent *t*-test; paired *t*-test	① > ②
Zarei (2020, Iran)	RCT *n* = 20 (20 male) M age: 16.4/16.9 hearing impairment type: NR Hearing loss >75dB	Exercise	8 weeks. ① 3 × 60 min/week, Proprioception training without visual input; ② Daily activities.	Single-limb standing test (Static balance)	repeated-measures ANOVA	① > ②
Zarei (2021, Iran)	RCT *n* = 19 (19 female) M age: 16.7 hearing impairment type: NR Hearing loss >75dB	Exercise	8 weeks. ① 3 × 60 min/week, Pilates training program; ② Daily activities.	Balance Errors Test/Y Balance Test (Static and dynamic balance)	repeated-measures ANOVA	① > ②

Note. QE, quasi-experiment; RCT, randomized controlled trial; M age, mean age; dB, decibel; SNHL, sensorineural hearing loss; ①, intervention group; ②, control group; In the Pre-post test, ① post-test evaluation, ② pre-test evaluation; VI, vestibular impairment; PSCT, Posturography sensory conditions testing; PSOT, posturography sensory organization testing; FMS, fundamental motor skill; NR, not reported.

TABLE 4 | Summary of intervention characteristics of included studies.

Description	Category	n (%)	Effective Rate (%)	Description	Category	n (%)	Effective Rate (%)
Year of publication	<2000	1 (6.7%)	50.0%	Duration	<8 weeks	3 (20.0%)	75.0%
	2001–2010	2 (13.3%)	100.0%		8–16 weeks	12 (80.0%)	91.7%
	2011–2021	12 (80.0%)	91.7%		1 time/day	1 (6.7%)	50%
Country	Iran	5 (33.3%)	100.0%	Frequency	1 time/week	1 (6.7%)	100%
	India	2 (13.3%)	100.0%		2 times/week	3 (20.0%)	66.7%
	Turkey	2 (13.3%)	100.0%		3 times/week	10 (66.7%)	100%
	United States	2 (13.3%)	66.7%	Session (min)	≤15	2 (13.3%)	33.3%
	Greece	2 (13.3%)	50.0%		30–45	7 (46.7%)	100%
	Poland	1 (6.7%)	100.0%		>45	4 (26.7%)	100%
	Ukraine	1 (6.7%)	100.0%		NR	2 (13.3%)	100%
Design	RCT	4 (26.7%)	100.0%	Instruments	One-Leg Standing Balance Test	5 (26.3%)	80%
	QE	11 (73.3%)	83.3%		Y Balance Test	2 (10.5%)	100%
Sample size	<20	3 (20.0%)	100.0%	—	Pediatric Balance Scale	1 (5.3%)	100.0%
	20–40	10 (66.7%)	90.0%		Force Platform	2 (10.5%)	66.7%
	>40	2 (13.3%)	66.7%		Other instrument	9 (47.4%)	100.0%
Mean age (year)	6–12	11 (73.3%)	91.7%	Outcomes	Dynamic Balance	6 (27.3%)	100%
	13–18	4 (26.7%)	75.0%		Static Balance	10 (45.5%)	90%
hearing impairment type	SNHL	5 (33.3%)	80%	—	Overall Balance	1 (4.5%)	100%
	NR	10 (66.7%)	90.9		Stability	5 (22.7%)	80%
Hearing loss (dB)	56–90	8 (53.3%)	77.8	—	—	—	—
	>90	3 (20.0%)	100.0		—	—	—
	NR	4 (26.7%)	100.0		—	—	—
Modality	Exercise training	12 (80.0%)	100.0	—	—	—	—
	Music + vibration	1 (6.7%)	100.0		—	—	—
	Motor intervention	1 (6.7%)	50.0		—	—	—
	Game intervention	1 (6.7%)	0		—	—	—

Note. NR, not reported.

13.3% of the total, whereas t-test combined with Wilcoxon signed rank tests accounted for 6.7% of the studies.

DISCUSSION

For this review, we identified 15 studies that used the intervention program to improve the balance of children and adolescents with hearing impairment. The results of methodological quality assessment showed that most of the studies reviewed were categorized as medium or low quality, and only three were identified as high quality. Most research used QE designs, being one of the reasons that reduce the methodology quality. Even in the RCT designs reviewed, the random assignment, allocation concealment, and assessor blinding procedures were not fully described. RCT designs pose methodical, practical, and ethical challenges to researchers (Hein & Weeland, 2019), especially in populations with social or cognitive impairments, such as children and adolescents with hearing impairment (Mulhall et al., 2018). Nevertheless, randomized trials and their systematic reviews can provide the most reliable evidence about the effects of healthcare interventions (Higgins et al., 2011).

One area that must be pointed in the reviewed trials is the relatively small sample sizes. Most studies (86.7%) had sample sizes less than 40, and none of the authors specified if they used calculations to establish such sample sizes. Theoretically, sample size depends on three aspects: the main measurement variable, the variance in the primary variable, and the acceptable error. Bartlett et al. (2001) described the procedures for determining the appropriate sample sizes for different types of variables, which may provide some methodological references for researchers. Moreover, budget and experiment feasibility are other aspects that must be considered in determining sample sizes.

Another shortcoming of some trials analyzed in this review is not controlling the types of hearing impairment. According to the American Speech–Language–Hearing Association, hearing loss has three basic types: conductive hearing loss, sensorineural hearing loss (SNHL), and mixed hearing loss (Fernandes et al., 2015). However, only 33.3% of the included studies reported a type of hearing impairment, that is, SNHL. The remaining works failed to report a specific type. SNHL is the most common type of permanent hearing loss, which is caused by functional problems in the cochlea or the auditory pathway to the brain (McPhillips, 2015). It has been reported that children with SNHL as opposed to conductive hearing loss, have progressive developmental delay, and is related to concomitant damage to vestibular structure (Rine et al., 2004). Thus if the trials included children with and without SNHL and the results were not reported separately or did not control the vestibular dysfunction among the subjects with SNHL, this may underestimate the effect size of the interventions. Controlling the type of hearing impairment in future trials may guide rehabilitation strategies specifically, help easily obtain comparison results, and synthesize pieces of trial evidence.

In terms of the instruments for the evaluation of the outcomes, future studies should fully consider the applicability of measurement tools to children and adolescents with hearing impairment. The use of unvalidated instruments would reduce the reliability of the evidence (Melo et al., 2020). Romberg test, for example, which was proposed for the evaluation of the elderly balance, may not be suitable for balance assessment in children and adolescents with hearing impairment.

The results from this review showed that the included trials with exercise interventions had a positive influence on the balance among children and adolescents with hearing impairment (the post-intervention scores were significantly higher than the pre-intervention or the control group scores). Given that most studies employed structured exercise training with an emphasis on balance and coordination that present wide displacements of the gravity center, the experimental group therefore may obtain further practice on the balance ability (Melo et al., 2020). Moreover, some exercise interventions focused on improving substitution (Ebrahimi et al., 2017; Rajendran et al., 2013; Rine et al., 2004; Shah et al., 2013). The neuromuscular control on balance was improved as a result of enhancing substitution through the development of visual and somatosensory awareness and incorporation of fundamental motor skills. Even so, we still need to look further into the neurological basis of balance and to design programs to improve the psychomotor integration of all factors that affecting the balance ability of children and adolescents with hearing impairment.

Another important finding of this review showed that the balance interventions were more effective in participants with an average age of 6–12 years than those with an average age of 13–18 years. This finding expanded, as well as affirmed, previous studies, that the most critical period in motor development occur in the first decade of life with balance control usually established at 7–10 years old (Ferdjallah et al., 2002; Roncesvalles et al., 2001). However, due to the processes that are responsible for resolving multi modal sensory conflict of postural stability are not fully developed before the age of 7 years old (Shumway-Cook and Woollacott, 1985), the future studies should categorize age groups when include children under 7 years old, in order to observe the results of the interventions in children with postural stability mature or in development (Melo et al., 2019).

Regarding the duration of training, interventions with duration of 8–16 weeks were more effective than those with less than 8 weeks. Short-duration programs may not suffice to support the physical and cognitive integration of new skills to achieve the long-term modification of balance ability (Maiano et al., 2019). Planning an exercise program of appropriate duration to promote full realization of each child's balance potential therefore may be more helpful. Meanwhile, it should also be noted that the experimenter or assistant can maintain effective non-verbal communication with the subjects.

Studies that employed music + vibration, motor, and game as the intervention modalities had an effective rate of 100, 50, and

0%, respectively. Considering that each of these three modalities was adopted by only one study, drawing a conclusion on the effectiveness of these interventions on enhancing balance among children and adolescents with hearing impairment is difficult. Additional studies are needed to confirm the results of interventions adopting these modalities.

CONCLUSION

Based on our findings, we conclude that the exercise interventions were effective on improving balance in children and adolescents with hearing impairment. In addition, the interventions with duration of 8–16 weeks were more effective than those with less than 8 weeks. However, most of the reviewed studies were of low methodological quality; thus, the trial results analyzed in this systematic review should be interpreted with caution. Further investigations of high-quality studies are therefore needed to prove the effectiveness of interventions on improving balance performance in children and adolescents with hearing impairment.

LIMITATIONS

Two limitations inherent within the current review should be noted. First, although we conducted an extensive literature search on five major databases to identify potential studies, a few published studies were possibly missed because our search was limited to English journal articles. Certain studies, which could have added relevant information to the field, might have been discarded. Second, due to the heterogeneity of reviewed studies, such as participant characteristics and outcome measures, a meta-analysis could not be conducted.

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

YZ performed the methodological search on the research topic and helped write the draft manuscript. JQ contributed to conception and design of manuscript. JQ also critically wrote and revise the manuscript.

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Isolated Soy Protein Supplementation Combined With Resistance Training Improves Muscle Strength, Mass, and Physical Performance of Aging Female Mice

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Background/Purpose: In recent years, the aging population has gradually increased, and the aging process is accompanied by health-associated problems, such as loss of muscle mass and weakness. Therefore, it is important to explore alternative strategies for improving the health status and physical fitness of the aged population. In this study, we investigated the effect of soy protein supplementation combined with resistance training on changes in the muscle mass, muscle strength, and functional activity performance of aging mice.

Methods: Female Institute of Cancer Research (ICR) mice were divided into four groups (n = 8 per group): sedentary control (SC), isolated soy protein (ISP) supplementation, resistance training (RT), and a combination of ISP and RT (ISP + RT). The mice in designated groups received oral ISP supplementation (0.123 g/kg/day), RT (5 days/week for a period of 4 weeks), or a combination of both ISP plus RT for 4 weeks. Afterward, we assessed muscle strength, endurance, and anaerobic endurance performance and analyzed blood biochemical and pathological tissue sections to investigate whether there were adverse effects or not in mice.

Results: ISP supplementation effectively improved the muscle mass, muscle endurance, and endurance performance of aging female mice. The RT group not only showed similar results with ISP but also increased muscle strength and glycogen content. Nevertheless, the combination of ISP supplementation and RT had greater beneficial effects on muscle strength, physical performance, and glycogen levels ($p < 0.05$). In addition, the combination of ISP supplementation and RT had significantly increased type II muscle percentage and cross-sectional area ($p < 0.05$).

Conclusion: Although ISP or RT alone improved muscle mass and performance, the combination of ISP with RT showed greater beneficial effects in aging mice. Our findings suggest that regular exercise along with protein supplementation could be an effective strategy to improve overall health and physical fitness among the elderly.

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INTRODUCTION

The significant increase in life expectancy in the past 60 years is due to the decline in mortality among people in their 60s and 70s (Davis and Bailey, 1997). According to United Nations estimates, by 2050, one in six people in the world will be 65 years of age or older (He et al., 2016). The increase in life expectancy is itself a positive human development. However, aging is related to a variety of adverse reactions, leading to a decline in the ability to live independently, and many people are living with poor health and impaired physical fitness. Such changes in the elderly make them susceptible to age-related diseases, such as weakness, sarcopenia, cardiovascular disease, cancer, neurodegenerative diseases, and metabolic disorders (United Nations, 2020). Skeletal muscle is not only the largest organ of the human body but also directly affects a person's bodily functions, is the largest amino acid library, providing essential amino acids for other key tissues/organs to synthesize new proteins for various purposes, and has the most glycogen storage (Franceschi et al., 2018). With age, skeletal muscles will undergo structural and functional changes. The reduction in muscle mass, function, fiber number, and fiber cross-sectional area may be affected by the loss of protein homeostasis, mitochondrial dysfunction, and changes in cell-to-cell communication (Jang et al., 2021), especially the pathways related to inflammation, protein turnover, and mitochondrial function (Larsson et al., 2019). In addition, muscle mass is controlled by a complex balance of muscle protein synthesis and muscle protein degradation. The ability of aging skeletal muscle to stimulate muscle protein synthesis in response to anabolic stimulation is weakened, mainly due to the impaired activation of the PI3K/Akt/mTOR/p70S6K signal axis or PI3K-Akt pathway, which is called anabolic resistance (Phillips et al., 2009; Lenk et al., 2010). The gradual decrease in muscle mass will lead to muscle atrophy. In addition to causing dysfunction, falls, and fractures, it will also increase the risk of cancer and metabolic-related diseases, such as insulin resistance, diabetes, and obesity (Fry et al., 2011). Although there is no clear treatment mechanism to improve the muscle loss caused by aging, finding alternative strategies to slow or prevent the aging-induced sarcopenia is important for the elderly.

Exercise is an effective therapy for accelerating the production of various cytokines and growth factors or regulating homeostasis and can maintain the functions of the elderly (Dalle and Koppo, 2021). Many studies demonstrated exercise as one of the strategies to improve muscle strength, muscle quality, and skeletal muscle dysfunction (Demontis et al., 2013). In addition, regular exercise helps maintain muscle mass and reduces susceptibility to age-related chronic diseases and cancer (Consitt et al., 2019). Among them, resistance training (a gradually overloaded strength training exercise in which the muscles are loaded from the outside) (Cartee et al., 2016) has been shown to have a positive effect on functional improvement and disease prevention, including muscle growth, body composition, body function, the elderly, and chronic metabolism (American College of Sports Medicine et al., 2009). Theoretically, the amount of training performed in the RT round (determined here by the formula: number of repetitions/×/group) plays an important role in chronic muscle adaptation (such as muscle size and strength) (Westcott, 2012). Compared with single-group training, acute studies have shown that multi-group training can enhance the phosphorylation of p70S6 kinase and muscle protein synthesis (Dankel et al., 2017). However, resistance exercise stimulates the rate of muscle protein breakdown to a lesser degree but stimulates the rate of muscle protein synthesis to a greater degree. When performing resistance exercise before ingesting protein, the two stimuli will synergistically combine to make the stimulation rate of muscle protein synthesis exceed the rate of muscle protein breakdown. Therefore, when combined with protein intake, repeated resistance exercises can lead to an increase in skeletal muscle protein (Terzis et al., 2010).

In addition to resistance exercise training, diet, a modifiable lifestyle factor, plays an important role in the prevention and treatment of sarcopenia (Stokes et al., 2018). Nutritional supplements, including protein omega-3, and vitamin D are considered to alleviate age-associated physical impairments and health issues (Fry et al., 2011). At present, more protein intake is considered to help in improving muscle mass and strength (Cruz-Jentoft et al., 2020). Protein supplements are rich in branched-chain amino acids (BCAAs), which can change the net balance of protein metabolism from catabolism. In addition, BCAAs composed of leucine, isoleucine, and valine have been shown to increase the level of protein synthesis and metabolism, and the synthesis of skeletal muscle protein (Jackman et al., 2017). Therefore, compared with resistance exercise alone, the combination of BCAA intake with resistance exercise can induce a higher protein synthesis rate of muscle myofibrils (Phillips and Martinson, 2019). Previous studies have shown that essential amino acids, in middle-aged mice, mainly increase mitochondrial biosynthesis and muscle function through their BCAAs and help to improve the stability of the neuromuscular junction to change nerve passage which influence muscle strength (Negro et al., 2008; D'Antona et al., 2010). In addition, it was confirmed in a long-term study that the combined treatment of RT and essential amino acids improved human muscle mass and muscle strength (Manini and Clark, 2012). Because of the potential health benefits of vegetarian and vegan diets, clinical and consumer markets are increasingly interested in them (Willoughby et al., 2007) However, plant protein is of lower quality than animal protein, so vegetarians need to consume more protein than non-vegetarians to meet their biological requirements for essential amino acids (IAAs), especially vegetarian elderly who need some high-quality protein sources (Domić et al., 2022). Soy protein has always been the preferred plant protein because it has almost complete essential amino acids. In addition, the BCAA content in the isolated soy protein (ISP) accounts for about 35% of the amino acids required for skeletal muscle formation (Melina et al., 2016) and contains isoflavones, a type of phytoestrogen that past

research has shown to benefit muscle and bone in older women (Brandi et al., 1993). In addition, a higher intake of total dietary protein may overcome the different characteristics of animal protein and plant protein and their effect on muscle results (Shimomura et al., 2006).

In our previous research, we have shown that ISP combined with high-intensity interval training (HIIT) had no significant effect on the grip strength, endurance performance, and muscle mass of ovariectomized mice that simulate menopausal women, but could effectively increase bone strength and attenuate exercise-induced fatigue (Lin et al., 2018). Another study conducted on postpartum mice showed that ISP combined with HITT increased lean muscle mass, prevented weight/fat gain, improved grip and endurance performance, and promoted fatty acid oxidation in brown adipose tissue (Wei et al., 2019). However, the combined effect of ISP and resistance training on fitness variables has not yet been investigated. In this study, we aimed to explore the effect of ISP supplementation and resistance exercise training on muscle mass, strength, exercise performance, and physical fitness of 19-month-old aging female mice. We further performed histological and immunohistochemistry analyses to identify the tissue architectural changes and muscle fiber types of aging mice after the intervention.

MATERIALS AND METHODS

Animal Care and Study Design

Female ICR mice were purchased from BioLASCO (Charles River Licensee Corp., Yi-Lan, Taiwan) and bred until 19 months of age. All mice were housed in the animal facility of the Graduate Institute of Sport Science at National Taiwan Sport University, and maintained under a stable photoperiod, temperature, and humidity conditions (12-h light/12-h dark cycle, 22 ± 2°C, and 60%–70%, respectively). During the experiment, we were provided with a standard laboratory diet (No. 5001; PMI Nutrition International, Brentwood, MO, United States) and water *ad libitum*. The Institutional Animal Care and Use Committee (IACUC) of National Taiwan Sport University and IACUC ethics committee (IACUC no. 10720) approved the animal experimentation and procedures. Thirty-two aging female mice were randomly divided into four groups (8 mice/group) for ISP supplementation and/or resistance training (RT) as follows: 1) sedentary control with vehicle (SC), 2) sedentary control with ISP supplementation (SC + ISP, 0.123 g/kg/mice/day), 3) resistance training with vehicle (RT), and 4) resistance training with ISP supplementation (RT + ISP, 0.123 g/kg/mice/day). All groups were administered with the same volume of distilled water or ISP by oral gavage. Water consumption, food intake, and animal weights were recorded twice a week.

Isolated Soy Protein

The isolated soy protein (ISP) was purchased from Bestjet Biotechnology Co. Ltd. (New Taipei City, Taiwan). The nutrients and amino acids present in the ISP were analyzed by SGS Taiwan, Ltd. (New Taipei City, Taiwan). The nutritional information of the ISP, including hydrolyzed amino acid profiles and total branched-chain amino acids (BCAAs), is shown in **Table 1**.

TABLE 1 | Nutrients, hydrolyzed amino acid profiles, and total branched-chain amino acids (BCAAs).

Nutrition facts	/100 g ISP	/100 g chow 5001
Total calories	373.6 kcal	336 kcal
Protein	83.4	23.9
Fat	3.6	5
Saturated fat	0.91	1.56
Trans fat	0	0
Carbohydrate	1.9	48.7
Sugar	0	0
Sodium	746 mg	400 mg
Hydrolyzed amino acid profiles	g/100 g ISP	/100 g chow 5001
Leucine	6.61	1.83
Valine	4.29	1.17
Isoleucine	4.26	1.14
Cystine	0.72	0.31
Tryptophan	1.07	0.29
Methionine	1.21	0.67
Threonine	3.03	0.91
Histidine	2.35	0.57
Tyrosine	2.91	0.71
Alanine	3.42	1.43
Glycine	3.44	1.21
Serine	4.15	1.19
Proline	4.46	1.49
Phenylalanine	4.62	1.04
Lysine	5.21	1.41
Arginine	6.24	1.41
Aspartic Acid	9.94	2.81
Glutamic Acid	16.99	4.37

Resistance Training and Anaerobic Exercise Capacity Test

The resistance training protocol was performed 5 days/week for a period of 4 weeks. The equipment was set in water (5 cm depth) to provide negative stimulation to motivate climbing (**Figure 1A**) and the indicated intensity load was adjusted by individual animal weight using the protocol, as shown in **Figure 1B**. In resistance training, the climbing procedures were performed with four repetitions/set and three sets/day, with 1 min of rest provided between the sets (Kan et al., 2018). Performance was evaluated as the climbing time, and the number of climbs until exhaustion was used to evaluate the anaerobic performance. The criterion for exhaustion is that the mouse stagnates three times in a single crawl, and each stagnation does not climb up after five nudges or taps.

Forelimb Grip Strength

A low-force testing system (Model-RX-5, Aikoh Engineering, Nagoya, Japan) was used to measure the grip strength of the forelimb as described previously (Lee et al., 2021).

Endurance Exercise Performance Test

We used a motor-driven treadmill for rodents (model MK-680, Muromachi Kikai, Tokyo, Japan) to evaluate the aerobic endurance performance, and an electric shock grid was used to increase test motivation through veterinary monitoring. Before the exhaustive exercise test, all mice were initially adapted to run

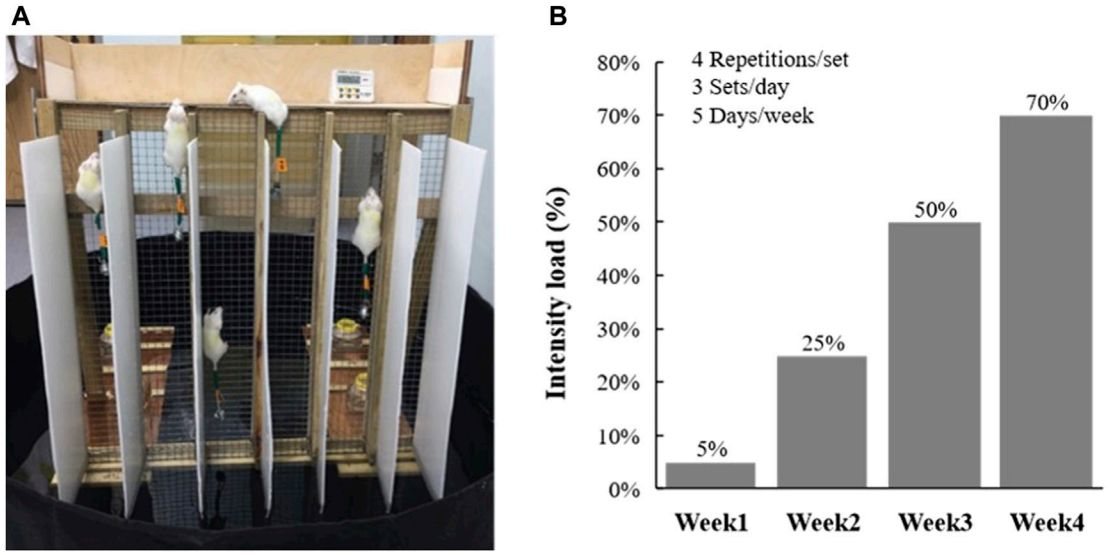

FIGURE 1 | (A) Climbing device for resistance training. **(B)** Incremental loading intensity applied to current training protocol.

on a motorized treadmill at 10 m/min, 5% grade, for 5 min/day for a week. On the test, we set a fixed slope of grade 15° and an initial speed of 15 m/min for mice running on the treadmill, then, every subsequent 2 min, the speed was increased by 3 m/min until the mice maintained continuous contact with the shock grid for 5 s, that we defined it to exhaustion (Kan et al., 2018).

Clinical Biochemical Profiles

A series of blood biochemical assessments were performed to evaluate the functional ability of essential body organs. At the end of the experiments, all mice were sacrificed by 95% CO_2 asphyxiation, and the blood sample was collected immediately at rest. Serum was collected by centrifugation, and the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total cholesterol (TC), triacylglycerol (TG), blood urea nitrogen (BUN), creatinine (CREA), uric acid (UA), total protein (TP), creatine kinase (CK), and lactate dehydrogenase (LDH) were assessed by an auto-analyzer (Hitachi 717, Hitachi, Tokyo, Japan).

Body Composition and Glycogen Content Analysis

After the mice were euthanized, the important body organs, including the liver, kidney, heart, lung, muscle (gastrocnemius), MT (thigh muscle), ovary fat pad (OFP), and brown adipocyte tissue (BAT), were accurately excised and weighed. Among them, the muscle and liver tissues were kept at −80°C for a subsequent glycogen content analysis. For the assay, 100 μg of liver and muscle tissue were homogenized in 500 μl cold perchloric acid and then centrifuged at 15,000 × g for 15 min at 4°C. The resultant supernatant was collected before determining the glycogen concentration. We used a commercial assay kit (Sigma-Aldrich, St. Louis, MO, United States) according to the manufacturer's instructions to determine the levels of glycogen in the liver and muscle (mg/g).

Histopathological and Immunohistochemical Staining

The liver, kidney, muscle, MT, heart, lung, OFP, and BAT tissues were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm-thick sections for morphological and pathological evaluation. Tissue sections were stained with hematoxylin and eosin (H & E) and examined by light microscopy with a CCD camera (BX-51, Olympus, Tokyo, Japan) by a clinical pathologist.

The muscle tissues (gastrocnemius) were further analyzed to see the effects of training and ISP supplementation on type I and type II fiber types. Primary antibodies of myosin-heavy chain fast (WB-MHCf) and myosin-heavy chain slow (WB-MHCs) were purchased from Novocastra (Leica Biosystem, Wetzlar, Germany) and applied to distinguish the fiber types. ER2 repair solution (AR9640, Leica Biosystem, Wetzlar, Germany) was used to repair the epitopes of MHCf and MHC, and then performed the initial incubation. The detection kits (Bond Polymer Refine Detection & Bond Polymer Refine Red Detection) used an automated BondMax double staining system, as previously described (Kan et al., 2018). The cross-sectional area (CSA, μm^2) of a muscle, and type I muscle observed per high-power-field (HPF), were measured and analyzed using ImageJ software (NIH, MD, United States).

Statistical Analysis

Data were expressed as mean ± SD. Statistical analyses were performed using SAS v9.0 (SAS, Cary, NC, United States). Two-way ANOVA was performed to assess the effect of exercise training and ISP supplementation on all the experimental data. $p < 0.05$ was considered statistically significant.

TABLE 2 | General characteristics of the experimental groups.

Characteristics	SC	ISP	RT	RT + ISP	Main factor *p* value		
					ISP	RT	RT + ISP
Initial BW (g)	42.4 ± 2.80[a]	42.2 ± 3.50[a]	42.2 ± 4.50[a]	42.6 ± 4.10[a]	0.8406	0.9483	0.8140
Final BW (g)	43.4 ± 2.70[a]	42.7 ± 3.70[a]	42.7 ± 4.60[a]	43.1 ± 4.10[a]	0.3338	0.0879	0.7832
Food intake (g/day)	6.24 ± 0.80 [b]	5.19 ± 1.51[a]	6.10 ± 0.86 [b]	5.21 ± 1.14[a]	<0.0001	0.3003	0.6840
Water intake (ml/day)	11.18 ± 1.42[c]	10.15 ± 1.54[b]	11.78 ± 1.75[c]	9.05 ± 1.42[a]	<0.0001	0.6840	0.6628
Liver (g)	2.14 ± 0.31[a]	2.12 ± 0.29[a]	2.18 ± 0.18[a]	2.12 ± 0.34[a]	0.6880	0.8479	0.8479
Kidney (g)	0.67 ± 0.12[a]	0.69 ± 0.13[a]	0.64 ± 0.09[a]	0.65 ± 0.13[a]	0.3993	0.7324	0.9881
OFP (g)	0.35 ± 0.12[a]	0.34 ± 0.12[a]	0.34 ± 0.10[a]	0.34 ± 0.08[a]	0.8687	0.7164	0.9473
Heart (g)	0.23 ± 0.02[a]	0.24 ± 0.03[a]	0.23 ± 0.01[a]	0.24 ± 0.02[a]	0.1865	0.8129	0.8129
Lung (g)	0.32 ± 0.04[a]	0.32 ± 0.05[a]	0.33 ± 0.06[a]	0.33 ± 0.04[a]	0.7234	0.5252	1.0000
Muscle (g)	0.26 ± 0.02[a]	0.30 ± 0.03 [b]	0.30 ± 0.02 [b]	0.30 ± 0.03 [b]	0.0745	0.0424	0.0973
MT (g)	0.41 ± 0.05[a]	0.41 ± 0.06[a]	0.41 ± 0.03[a]	0.41 ± 0.03[a]	0.9691	0.9691	0.9691
BAT (g)	0.10 ± 0.02[a]	0.09 ± 0.03[a]	0.09 ± 0.02[a]	0.11 ± 0.02[a]	0.9421	0.6122	0.1353
Relative liver weight (%)	5.11 ± 0.70[a]	5.26 ± 1.24[a]	5.48 ± 0.54[a]	5.53 ± 1.33[a]	0.7808	0.3782	0.8941
Relative kidney weight (%)	1.61 ± 0.29[a]	1.70 ± 0.43[a]	1.59 ± 0.14[a]	1.68 ± 0.31[a]	0.4244	0.8529	0.9955
Relative OFP weight (%)	0.83 ± 0.26[a]	0.84 ± 0.32[a]	0.85 ± 0.26[a]	0.87 ± 0.24[a]	0.8820	0.8516	0.9434
Relative heart weight (%)	0.55 ± 0.06[a]	0.58 ± 0.09[a]	0.57 ± 0.04[a]	0.62 ± 0.10[a]	0.1365	0.3518	0.7887
Relative lung weight (%)	0.76 ± 0.10[a]	0.80 ± 0.21[a]	0.82 ± 0.13[a]	0.87 ± 0.14[a]	0.3811	0.2515	0.9815
Relative muscle weight (%)	0.61 ± 0.06[a]	0.70 ± 0.09 [b]	0.70 ± 0.05 [b]	0.70 ± 0.07 [b]	0.1037	0.0629	0.0772
Relative MT weight (%)	0.98 ± 0.14[a]	1.02 ± 0.26[a]	1.03 ± 0.11[a]	1.06 ± 0.17[a]	0.5466	0.4724	0.9378
Relative BAT weight (%)	0.24 ± 0.06[a]	0.22 ± 0.10[a]	0.23 ± 0.05[a]	0.28 ± 0.08[a]	0.6338	0.3933	0.2574

Data are expressed as mean ± SD for n = 10 mice in each group. (1) Sedentary control with vehicle (SC), (2) sedentary control with ISP supplementation (SC + ISP, 0.123 g/kg/mice/day), (3) resistance training with vehicle (RT), and (4) resistance training with ISP supplementation (RT + ISP, 0.123 g/kg/mice/day). Data in the same row with different letters (a, b) differ significantly at $p < 0.05$ *by two-way ANOVA; OFP: ovary fat pad; BAT: brown adipose tissue.*

RESULTS

General Characteristics of Aging Mice With ISP Supplementation and RT

As shown in **Table 2**, the body weights of mice were not significantly changed after 4 weeks of ISP supplementation or in combination with RT. However, water ($p < 0.0001$) and diet ($p < 0.0001$) intake were significantly lower in ISP and ISP plus RT groups compared with SC and RT groups (**Table 2**).

On the body composition, no significant differences were observed in absolute or relative weights of the liver, kidney, heart, lung, MT, OFP and BAT tissues among the groups. However, muscle weights in ISP, RT, and ISP plus RT groups were significantly greater than in SC by 1.13-fold ($p = 0.0112$), 1.12-fold ($p = 0.0176$), and 1.14-fold ($p = 0.0089$), respectively, and only supplementation had a significant effect ($p = 0.0424$). Similarly, relative muscle weights in ISP, RT, and ISP plus RT groups were significantly greater than in SC by 1.15-fold ($p = 0.0126$), 1.15-fold ($p = 0.0191$), and 1.15-fold ($p = 0.0062$), respectively, but had no significant main effect of exercise or ISP (**Table 2**).

ISP Supplementation and RT Improves Grip Strength of Aging Mice

The forelimb grip strength of mice in SC, ISP, RT, and ISP + RT groups were, 124 ± 8, 127 ± 4, 138 ± 5, and 143 ± 7 (g), respectively. The grip strength of mice in RT and ISP + RT groups were significantly higher than in SC by 1.11-fold ($p = 0.0002$) and 1.15-fold ($p < 0.0001$), respectively, with only exercise as the main effects ($p < 0.0001$) (**Figure 2A**). The relative grip strength (%), normalized to body weight was found to be significantly higher in RT and ISP + RT groups than in SC by 1.21-fold ($p = 0.0021$) and 1.28-fold ($p = 0.0001$), respectively, with only exercise as the main effects ($p = 0.0001$) (**Figure 2B**).

Effect of ISP Supplementation and RT on the Anaerobic Exercise Performance of Aging Mice

We used speed and the maximum number of repetitions to measure the anaerobic exercise performance. In terms of climbing speed, except SC mice (13.49 ± 2.13 s), all other groups (ISP, RT, and ISP + RT) spent less time (9.87 ± 3.08, 10.43 ± 1.13, and 8.47 ± 2.03 s, respectively) to complete the climbing (**Figure 3A**). The decreased climbing time of aging mice in ISP + RT was prominent (37.21%; $p < 0.0001$) than in ISP (26.79%; $p = 0.0027$) and RT (22.69%; $p = 0.0098$) groups. The main effect of ISP ($p = 0.0081$) and RT ($p = 0.0012$) was the significantly increased climbing speed, but there was no interaction effect (**Figure 4A**). The repetition maximum (RM), an index of muscular endurance performance of mice in SC, ISP, RT, and ISP + RT groups was 4.00 ± 1.41, 11.25 ± 1.49, 9.50 ± 1.60, and 12.50 ± 1.51 (times), respectively (**Figure 3A**). Compared with SC, the exhaustive time in ISP, RT, and ISP + RT groups was significantly longer by 2.81-fold ($p = 0.0027$), 2.38-fold ($p = 0.0098$), and 3.13-fold ($p < 0.0001$), respectively. In

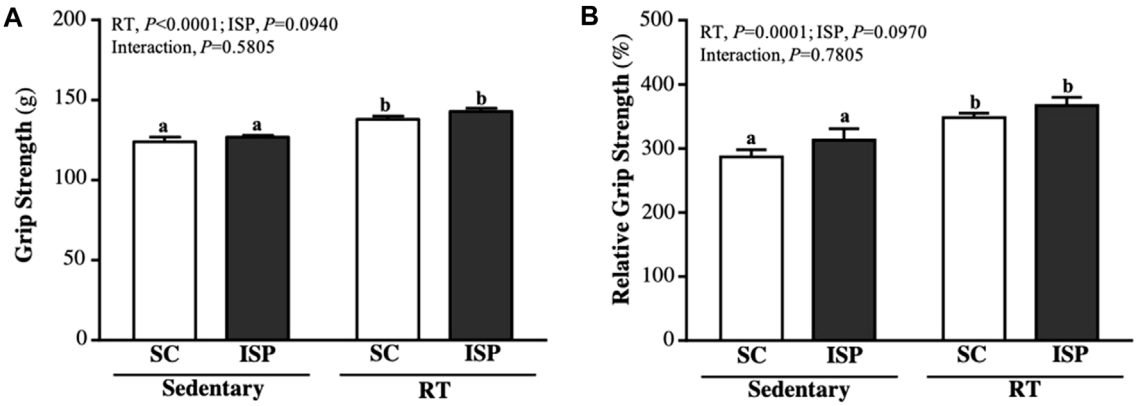

FIGURE 2 | Effect of ISP supplementation and RT of aging mice on **(A)** absolute forelimb grip strength and **(B)** forelimb grip strength (%) relative to body weight. Data are expressed as mean ± SD for n = 8 mice per group. Different superscript letters (a, b, and c) indicate significant difference at $p < 0.05$.

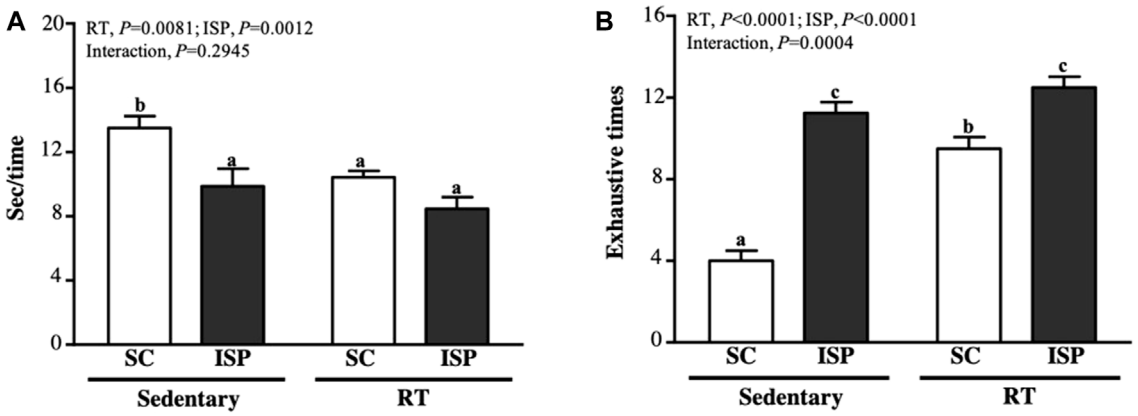

FIGURE 3 | Effect of ISP supplementation and RT of aging mice on **(A)** time for each climbing and **(B)** exhaustion times for climbing. Data are expressed as mean ± SD for n = 8 mice per group. Different superscript letters (a, b, and c) indicate significant difference at $p < 0.05$.

addition, the exhaustive time in ISP and ISP + RT groups was significantly longer than in RT group by 1.18-fold ($p = 0.0276$) and 1.32-fold ($p = 0.0004$), respectively. The main effect of ISP ($p < 0.0001$) and RT ($p < 0.0001$) was significantly increased muscular endurance performance, and had a significantly interactive effect ($p = 0.0004$) (**Figure 3B**).

ISP Supplementation With RT Promotes Exercise Performance in Aging Mice

As seen in **Figure 4**, the time to exhaustion in SC, ISP, RT, and ISP + RT groups was 5.21 ± 1.80, 9.46 ± 2.83, 10.02 ± 2.38, and 12.90 ± 1.92 (min), respectively. The climbing time of mice in ISP, RT, and ISP + RT groups was significantly longer than the SC group by 1.81-fold ($p = 0.0008$), 1.92-fold ($p = 0.0002$), and 2.74-fold ($p < 0.0001$), respectively. Among them, the ISP + RT group represented with the most effective improvement. The main effect of ISP ($p < 0.0001$) and RT ($p = 0.0001$) was the significantly increased endurance exercise performance, but there was no significant interactive effect.

Combination of ISP and RT Preserves Liver and Muscle Glycogen Levels in Aging Mice

Glycogen is mainly present in the liver and skeletal muscle and is used for energy demand and homeostasis. Aging mice liver glycogen levels were found to be higher in RT (22.38 ± 3.67 mg/g) and ISP + RT groups (25.20 ± 3.86 mg/g). Compared with SC, liver glycogen levels in RT and ISP + RT groups were significantly higher by 1.57-fold ($p = 0.0002$) and 1.76-fold ($p < 0.0001$), respectively (**Figure 5A**). The muscle glycogen content in SC, ISP, RT, and ISP + RT groups were 1.06 ± 0.18, 1.21 ± 0.26, 1.23 ± 0.20, and 1.29 ± 0.15 (mg/g), respectively. In contrast to the liver, higher muscle glycogen levels were maintained only in the ISP + RT group (1.22-fold; $p = 0.0277$) (**Figure 5B**).

Effect of ISP Supplementation and RT on Blood Biochemical Parameters in Aging Mice

At the end of the experiment, we further performed a blood biochemical analysis to explore the effect of ISP and RT on

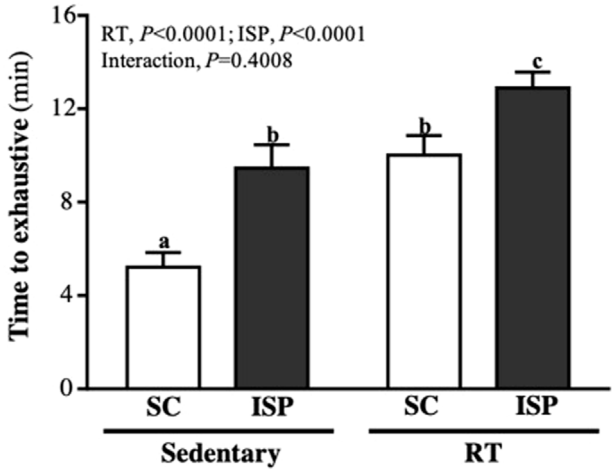

FIGURE 4 | Effect of ISP supplementation and RT of aging mice on endurance exercise performance. Data are expressed as mean ± SD for $n = 8$ mice per group. Different superscript letters (a, b, and c) indicate significant difference at $p < 0.05$.

various key clinical outcomes. In the results, we found that there were no significant differences in the levels of AST, ALT, ALB, TC, TG, CREA, UA, TP, CK, LDH, and glucose among the groups (**Table 3**). However, serum BUN levels were significantly increased with ISP supplementation and also with a combination of RT. The elevated BUN levels in ISP and ISP + RT groups were 1.10-fold ($p = 0.0235$) and 1.12-fold ($p = 0.0087$), respectively (**Table 3**).

Effect of ISP Supplementation and RT on the Histological Observations of Aging Mice

At the end of the study, histological examinations of the liver, MT, muscle, heart, kidney, lung, OFP, and BAT of aging mice were performed. As shown in the images, no abnormalities were observed in aging tissues of all experimental groups (**Figure 6**). After the prescribed treatment, the arrangement of hepatic sinusoids and hepatic cords in the liver showed no change. Only large senile liver nucleoli appeared in each group, which is a normal senescence phenomenon. In addition, Zenker degeneration and hyperplasia were not observed in the skeletal muscle or cardiomyocytes. The structures of renal tubules and glomeruli were also not different among the treatment groups.

Effect of ISP Supplementation and RT on the Muscle Fiber Types and Morphology of Aging Mice

We further analyzed the ratio of type I and type II fibers and the cross-sectional area (CSA) of the thigh muscles to verify the effect of resistance exercise and ISP supplementation on aging mice. Type I and type IIa fibers were reddish in color, while type II was brownish in color (**Figure 7A**). **Figure 7B** showed the percentage of type II muscle in total muscle. The type II fiber percentage in SC, ISP, RT, and ISP + RT groups were 48.13 ± 2.75, 51.12 ± 2.65, 52.05 ± 2.13, and 53.97 ± 1.98 (%), respectively. Here, we noticed that ISP, RT, and ISP + RT interventions significantly increased type II fiber percentage by 1.06-fold ($p = 0.0188$), 1.08-fold ($p = 0.0027$), and 1.12-fold ($p < 0.0001$), respectively compared with SC. The main effects were ISP ($p = 0.0004$) and RT ($p = 0.0073$), but there was no significant interactive effect. In addition, the CSA of the SC muscle was 543 ± 24 μm^2, while the CSA of ISP, RT, and ISP + RT groups was 635 ± 24, 703 ± 41, and 705 ± 30 (μm^2), respectively. The CSA scores in ISP, RT, and ISP + RT groups were 1.17-fold ($p < 0.0001$), 1.30-fold ($p < 0.0001$), and 1.30-fold ($p < 0.0001$), respectively, greater than in SC. The main effects of ISP ($p < 0.0001$) and RT ($p = 0.0002$) were the significantly increased percentage of type I muscle, and had a significant interactive effect ($p = 0.0003$) (**Figure 7B**).

DISCUSSION

In this study, we demonstrated the influential role of ISP supplementation alone and also in combination with

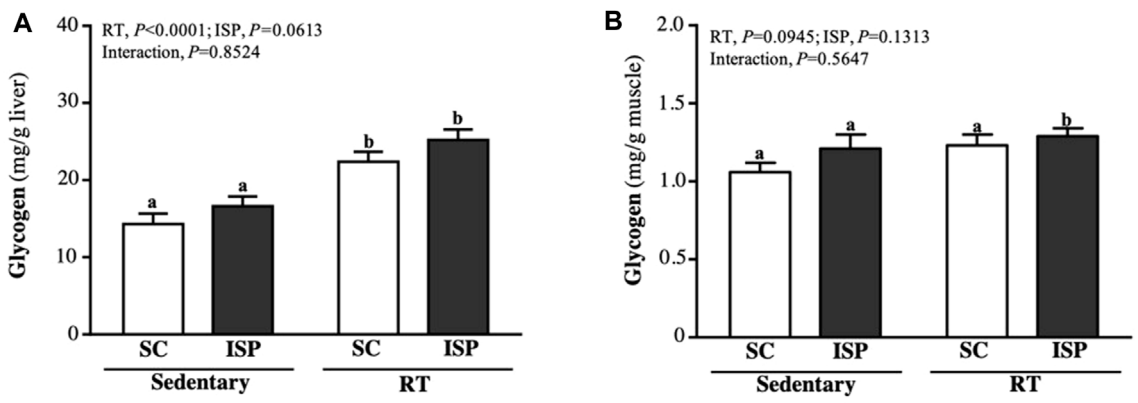

FIGURE 5 | Effect of ISP supplementation and RT of aging mice on **(A)** liver glycogen and **(B)** muscle glycogen. Data are expressed as mean ± SD for $n = 8$ mice per group. Different superscript letters (a and b) indicate significant difference at $p < 0.05$.

TABLE 3 | Effect of ISP supplementation and RT on biochemical assessments of serum at the end of the experiment.

Parameter	SC	ISP	RT	RT + ISP	Main factor *p* value		
					ISP	RT	RT + ISP
AST (U/L)	88 ± 10[a]	85 ± 9[a]	86 ± 7[a]	88 ± 7[a]	0.8972	0.9656	0.3921
ALT (U/L)	52 ± 5[a]	51 ± 9[a]	50 ± 4[a]	52 ± 9[a]	0.7884	0.7512	0.6090
TC (mg/dl)	131 ± 13[a]	124 ± 11[a]	128 ± 20[a]	129 ± 16[a]	0.5679	0.9195	0.4675
TG (mg/dl)	87 ± 17[a]	84 ± 11[a]	86 ± 13[a]	86 ± 14[a]	0.8404	0.9799	0.7626
LDH (mg/dl)	447 ± 92[a]	434 ± 119[a]	491 ± 83[a]	472 ± 65[a]	0.6289	0.2120	0.9301
ALB (g/dl)	3.09 ± 0.2[a]	3.12 ± 0.61[a]	3.18 ± 0.35[a]	3.05 ± 0.21[a]	0.7464	0.9631	0.5490
CPK (U/L)	259 ± 70[a]	273 ± 83[a]	259 ± 67[a]	268 ± 81[a]	0.6764	0.9172	0.9319
TP (g/dl)	5.18 ± 0.36[a]	5.24 ± 0.32[a]	0.38 ± 0.12[a]	5.21 ± 0.46[a]	0.6797	0.4714	0.3559
BUN (mg/dl)	21.5 ± 1.7[a]	23.7 ± 1.8 [b]	22.4 ± 1.7[a]	24.1 ± 2.1[b]	0.0048	0.3631	0.4758
CREA (mg/dl)	0.38 ± 0.03[a]	0.38 ± 0.03[a]	0.39 ± 0.02[a]	0.39 ± 0.03[a]	0.6282	0.3710	0.9447
UA (mg/dl)	2.25 ± 1.07[a]	2.30 ± 0.68[a]	2.23 ± 0.38[a]	2.36 ± 1.05[a]	0.7720	0.9537	0.8924
Glucose (mg/dl)	189 ± 24[a]	195 ± 20[a]	186 ± 19[a]	193 ± 18[a]	0.3863	0.6853	0.9656

Data are expressed as mean ± SD for n = 10 mice in each group. (1) Sedentary control with vehicle (SC), (2) sedentary control with ISP supplementation (SC + ISP, 0.123 g/kg/mice/day), (3) resistance training with vehicle (RT), and (4) resistance training with ISP supplementation (RT + ISP, 0.123 g/kg/mice/day). Data in the same row with different letters (a, b) differ significantly at $p < 0.05$ *by two-way ANOVA. AST, aspartate aminotransferase; ALT, alanine aminotransferase; TC, total cholesterol; TG, triglycerides; LDH, lactate dehydrogenase; ALB, albumin; CK, creatine kinase; TP, total protein; BUN, blood urea nitrogen; CREA, creatinine; UA, urea acid.*

resistance training on aging mice's muscle strength, endurance performance, muscle mass, and muscle histological changes. Our findings revealed that ISP supplementation alone significantly improved the muscle mass, muscle endurance, and endurance performance of aging mice. The RT not only had similar effects as ISP, but also increased the muscle strength and liver glycogen content. Nevertheless, the combination of ISP supplementation plus RT had greater beneficial effects, and this was evidenced by improved muscle strength, glycogen storage, and physical performance in aging mice.

First, we found decreased food and water intake after ISP supplementation; however, this did not result in decreased bodyweights of the mice. We assume that ISP intake might increase the feeling of satiety in mice, and thereby decreased food intake. In this study, aging female mice were used (19-month), so the body weight might be maintained at a high but stable state. Previous studies have shown that weight gain is not directly related to muscle mass and strength. However, muscle mass is the main determinant of muscle strength (Reid et al., 2008). The muscles after exercise are more sensitive to nutrition and can synthesize more available amino acids into skeletal muscle protein (Schoufour et al., 2019). RT and essential amino acids have independent effects. Both of these interventions can stimulate muscle proteins to replace old, non-functional proteins with new functional proteins, and increase muscle protein synthesis faster than muscle protein breakdown. Therefore, under the two synergistic effects, it has the benefit of increasing net muscle protein synthesis (Tipton et al., 1999; Franceschi et al., 2018). In addition, acute exercise combined with the ingestion of protein or amino acids can enhance the muscle protein anabolic response by activating the mTORC1 pathway, which is beneficial for promoting recovery following exercise and may improve muscle mass and quality over the long term (Burd et al., 2009). However, resistance training and protein supplementation are not as effective for the elderly as for the young, which is called the chronic slow response of the elderly (Kumar et al., 2009). In most sedentary elderly subjects, the sensitivity of skeletal muscle tissue to anabolic stimulation by physical activity or protein intake may be reduced. A previous study had shown that after 14 days of reduced physical activity in the elderly, the postprandial muscle protein synthesis rate is significantly reduced by 26% (Breen et al., 2013). Nevertheless, the combination of exercise and increasing protein intake is still one of the best strategies. A previous study showed that dietary protein supplementation after resistance exercise training increased muscle protein synthesis in the elderly by 28% (Pennings et al., 2011). In another long-term trial, 24 weeks of protein supplements combined with resistance training increased the muscle mass, strength, and physical function of frail elderly participants (Tieland et al., 2012). Therefore, in this study, we used 19-month-old aging female mice and supplemented them with ISP in combination with RT for 4 weeks. Our findings showed improved muscle mass with ISP and RT alone, and also with a combination of both ISP plus RT. Although RT and ISP + RT effectively improved the maximum muscle performance, ISP, RT, and ISP + RT intervention effectively increased the muscle endurance and anaerobic exercise performance of aging mice. Furthermore, time to exhaustion (climbing) and relative grip strength was significantly higher with the combination of ISP plus RT. These findings emphasize the benefits of ISP plus RT synergistic effect on improving the muscle mass, strength, and physiology of aging mice.

As age leads to a decrease in muscle mass, the type II muscle fiber atrophy may appear in muscle fibers (Nilwik et al., 2013; McCormick and Vasilaki, 2018), accompanied by a type-specific decrease in the number and function of skeletal muscle stem cells or satellite cells (Dreyer et al., 2006). However, resistance exercise training more than three times a week has been shown as an effective strategy to increase the quality and strength of skeletal muscles in the elderly (Peterson et al., 2011). In addition, a previous study has pointed out that long-term resistance

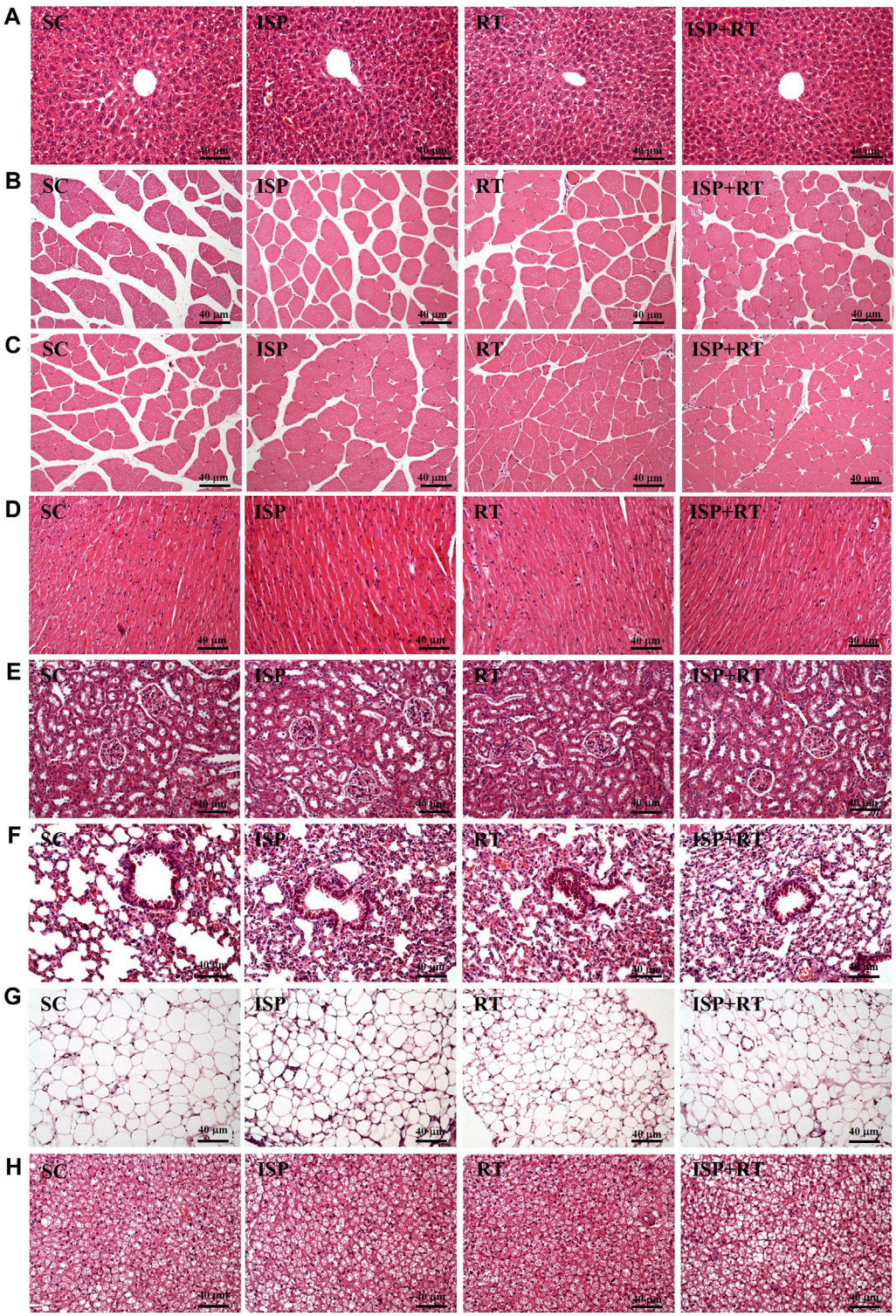

FIGURE 6 | Effect of ISP supplementation and RT of aging mice on **(A)** liver, **(B)** thigh muscle, **(C)** muscles, **(D)** heart, **(E)** kidney, **(F)** lung, **(G)** OFP, and **(H)** BAT tissue in mice (H&E stain, magnification: ×200; bar, 40 μm; BAT magnification: ×100; bar, 80 μm).

exercise training in the elderly can restore the content of type II muscle fiber satellite cells to the level of untrained healthy young people, and improve the response of acute type II muscle fiber satellite cells. In this study, we found that ISP supplementation and RT alone, and a combination of both (ISP + RT) significantly increased the percentage of type II muscle fiber types in aging

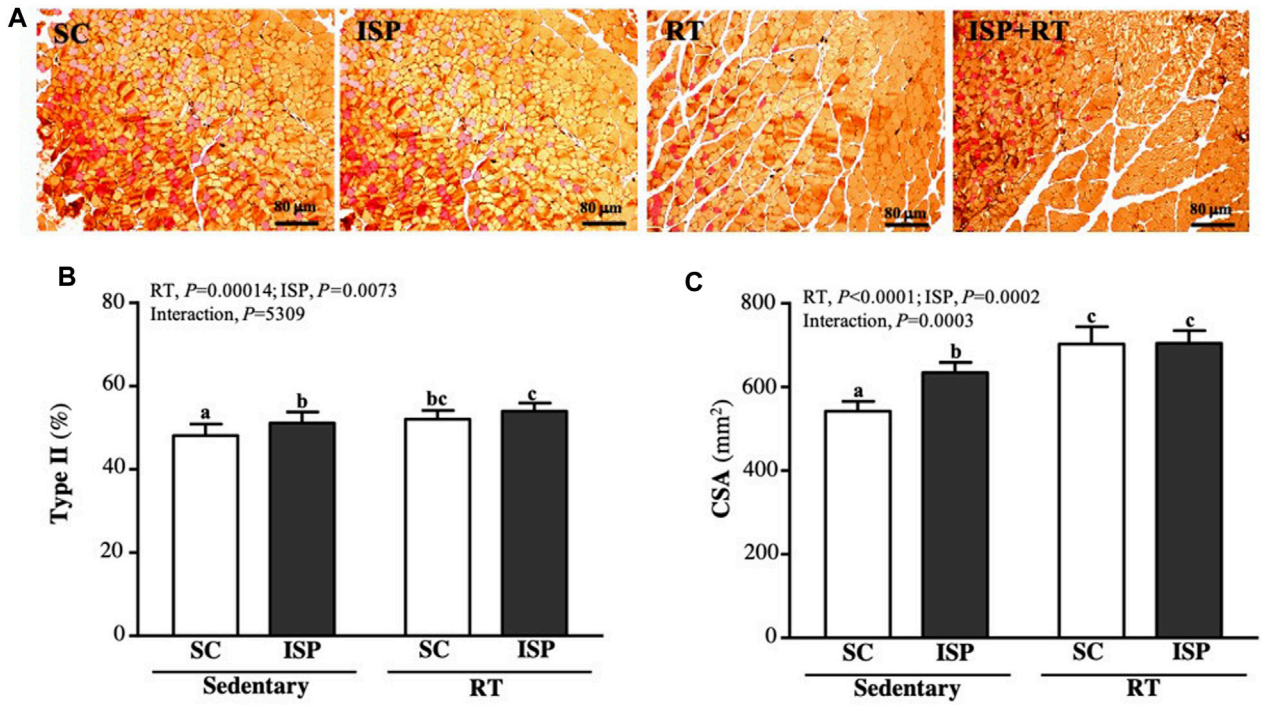

FIGURE 7 | Effect of ISP supplementation and RT of aging mice on **(A)** muscle of thigh with IHC staining, **(B)** muscular type proportions, and **(C)** cross section area (CSA). Specimens were photographed under a light microscope (Hematoxylin and eosin stain, magnification: ×200; scale bar, 40 μm). Data are expressed as mean ± SD for n = 8 mice per group. Different superscript letters (a and b) indicate significant difference at $p < 0.05$.

mice. This was further witnessed by the increased size of the muscle fiber types CSA. A previous study demonstrated that when RT is performed, animals show significant muscle remodeling, which is characterized by a decrease in the bulk density of slow and medium-speed fibers, with a significant increase in the bulk density of fast fibers (Leenders et al., 2013). In particular, RT related to a plant protein diet can be an auxiliary factor in inhibiting or reversing the sarcopenia by promoting the slight recovery of rapid glycolytic fiber atrophy, and accompanied by an increase in collagen (Figueiredo Braggion et al., 2016).

Muscular glycogen content affects the quantity and quality of muscle fibers. In addition to stable glycogen reserves, our study further confirmed that ISP, RT, and ISP + RT effectively increased skeletal muscle mass, muscle strength, and muscle fiber CSA. It has been claimed that the skeletal muscle glucose transporter type 4 (GLUT4) protein would increase with RT, but this may depend on the body composition and metabolic status (diabetes) before training. Six weeks of single-leg strength training (three times a week) increased skeletal muscle GLUT4 (~40%) of the exercised leg in the elderly and non-obese type 2 diabetic patients, which helps to improve the insulin sensitivity. Higher levels of glycogen in the skeletal muscle may be due to the increased insulin sensitivity (Holten et al., 2004). Another study conducted on men aged 50–63 found that resistance training for 16 weeks increased insulin-stimulated non-oxidized glucose processing (40%). This phenomenon may help in improving systemic insulin sensitivity (22%), which revealed that RT can improve skeletal muscle glycogen metabolism (Miller et al., 1994). In addition, after 6 weeks of RT, both healthy and diabetic elderly had increased skeletal muscle glycogen content (~16%) and significantly increased basal glycogen synthase activity (~9% and 20%, respectively) (Holten et al., 2004). Glycogen is the storage form of glucose (energy) in mammals. Most glycogen is produced and stored by liver (~100 g) and muscle (~350–700 g) cells, and glycogen content depends on the training status, diet, muscle fiber type composition, gender. and weight of an individual. Glucose output in the liver is the main source of glucose available to increase muscle exercise. Increased liver glycogen storage helps in maintaining the constant blood sugar and it is a key factor in improving endurance (Knuiman et al., 2015). Knuiman et al. (2015) conducted experiments on mice with high concentrations of liver glycogen to explore the correlation between energy reserves and performance. Under a low-intensity running program, these mice can run farther, indicating that liver glycogen is closely related to the endurance ability (López-Soldado et al., 2021). It is well known that glycogen is mainly derived from carbohydrate intake and converted into glucose storage. However, supplementation of BCAAs also increases glycogen storage. In a previous study, young rats were supplemented with 45 mg BCAA/body weight per day to perform 5-weight swimming training. The results showed that supplementation significantly increased the glycogen content in the liver (de Campos-Ferraz et al., 2011). Similarly, we reported that supplementation of ISP combined with RT effectively improved glycogen reserves in

aging mice, and this was accompanied by improved muscle strength, muscle mass, and performance. Increased muscle fiber CSA and fiber type transformation after the combination treatment further supports the beneficial effects of ISP in aging mice. Taken together, the combination treatment promotes glycogen reserves and exercise performance in aging mice without adverse effects.

In addition to physical performance and functional testing of aging female mice supplemented with ISP and RT, we also performed blood analysis and histopathological interpretation to confirm that the health status of aging mice and interventional substances did not cause the risk of injury. In terms of histopathology, except for the appearance of aging under normal conditions, no other damages were observed. In the blood analyses, the liver function biomarkers and lipid profile did not significantly differ with any of the treatments in aging mice. For the kidney function assessments, only BUN was found to be significantly increased in the ISP supplement group, but this increase was still in the normal range. This is a normal phenomenon, because BUN is a serum by-product of protein metabolism, formed by the liver and carried by the blood to the kidneys for excretion (Wang et al., 2021). Therefore, higher protein intake will result in higher BUN concentration under normal metabolism.

In recent years, the demand for the use of plant protein has increased, and a growing number of studies have compared the effects of various plant and animal protein sources in stimulating muscle protein synthesis, improving exercise training fitness, and enhancing physique (Kerksick et al., 2021). Past studies have compared net protein utilization values using the protein digestibility corrected amino acid score (PDCAAS), a similar dichotomy. For example, on a scale of 100, plant sources range from 53–67, while animal sources range from 73–94 (Schaafsma, 2000). Nonetheless, one study noted that 48 untrained men and women were randomized over 12 weeks to either 19 g of whey protein isolate or 26 g of soy protein isolate, both containing a protein dose of 2 g of leucine. Results showed significant increases in body weight, lean body mass, maximal extension, and flexion torque in both groups before and after supplementation, while muscle thickness tended to increase after 12 weeks of resistance. However, no significant differences were observed between the groups (Lynch et al., 2020). Another study also showed that habitual (over 12 months) vegetarians were given soy protein and omnivorous groups were given whey protein, with continuous supplementation at a protein intake of 1.6 g/kg/day combined with resistance training twice a week. After 12 weeks, strength, muscle mass, and cross-sectional area improved in both groups, but there were no differences in protein between the two groups (Hevia-Larraín et al., 2021). Although animal-based protein sources have long been considered to have higher absorption and utilization than plant-based protein sources, there appears to be little difference in muscle mass and functional performance. Therefore, vegetarians need more plant-based protein nutritional supplements to supplement their daily protein needs, especially elderly vegetarians. This study confirms that ISP supplementation in combination with resistance exercise training promotes and improves muscle mass and functional performance in the elderly and can serve as the basis for future applications and research in humans to help prevent and improve sarcopenia in elderly vegetarians.

CONCLUSION

For the first time, we demonstrated that ISP supplementation in combination with RT effectively improved skeletal muscle mass, muscle endurance, and endurance performance of aging female mice. The RT group not only showed similar effects as ISP, but also increased muscle strength and glycogen content. Most importantly, the combination of ISP plus RT intervention in aging rats had greater beneficial effects than ISP and RT alone on various muscle strength and physical performance parameters. Therefore, age-induced muscle loss could be maintained and/or delayed through ISP supplementation and resistance exercise training strategies.

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 Care and Use Committee (IACUC) of National Taiwan Sport University and IACUC ethics committee (IACUC no. 10720).

AUTHOR CONTRIBUTIONS

W-CC, C-CH, and H-YL conceived the study and participated in its design. M-CL, Y-JH, and F-YW carried out the laboratory experiments. M-CL and Y-JH analyzed the data, interpreted the results, prepared figures, and wrote the manuscript. W-CC revised the manuscript. W-CC contributed the study funding.

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Effect of vitamin D supplementation on the incidence and prognosis of depression: An updated meta-analysis based on randomized controlled trials

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Background: There have been several controversies about the correlation between vitamin D and depression. This study aimed to investigate the relationship between vitamin D supplementation and the incidence and prognosis of depression and to analyze the latent effects of subgroups including population and supplement strategy.

Methods: A systematic search for articles before July 2021 in databases (PubMed, EMBASE, Web of Science, and the Cochrane Library) was conducted to investigate the effect of vitamin D supplementation on the incidence and prognosis of depression.

Results: This meta-analysis included 29 studies with 4,504 participants, indicating that the use of vitamin D was beneficial to a decline in the incidence of depression (SMD: −0.23) and improvement of depression treatment (SMD: −0.92). Subgroup analysis revealed that people with low vitamin D levels (<50 nmol/L) and females could notably benefit from vitamin D in both prevention and treatment of depression. The effects of vitamin D with a daily supplementary dose of >2,800 IU and intervention duration of ≥8 weeks were considered significant in both prevention and treatment analyses. Intervention duration ≤8 weeks was recognized as effective in the treatment group.

Conclusion: Our results demonstrate that vitamin D has a beneficial impact on both the incidence and the prognosis of depression. Whether suffering from depression or not, individuals with low vitamin D levels, dose >2,800 IU, intervention duration ≥8 weeks, and all females are most likely to benefit from vitamin D supplementation.

KEYWORDS

depression, vitamin D supplementation, incidence, prognosis, meta-analysis

Introduction

Depression is a worldwide health issue for people that commonly coexists with other debilitating chronic illnesses. According to the World Health Organization, approximately 350 million people worldwide suffer from depression. It has been regarded as the leading cause of significant disability and mortality (1–3). Despite decades of intensive neurobiologically focused psychiatric study, the etiology of depression still remains a mystery. Classical antidepressants mainly increase the concentration of monoamines in the synaptic space by blocking the reuptake of serotonin and norepinephrine at presynaptic nerve terminals (4). However, approximately half of the patients fail to respond to the first-line treatment of antidepressants (5). Besides, the side effects of antidepressants are hard to be ignored. Hence, finding other clinical programs to optimize the treatment of depression appears extremely urgent.

Vitamin D has been investigated to be vital in the normal development and functionalization of the brain. The insufficient and deficiency of vitamin D are related to neurological disorders (6, 7). Also, the correlation between low-level vitamin D and depression has been proved (8). Epidemiological surveys demonstrated that the incidence of depression was 8–14% higher in people with vitamin D deficiency (9–11). Therefore, the implementation of vitamin D in anti-depression treatment has raised awareness among healthcare professionals. Compared with antidepressants, vitamin D is still believed by some to have more favorable safety and better patient compliance despite the adverse effect caused by excessive vitamin D. Nevertheless, the related results still remain controversial.

Previously, a meta-analysis exploring the effects of vitamin D supplements in the management of depression showed that vitamin D supplementation was therapeutically effective in relieving the symptoms of depression (2). However, in two subsequent meta-analyses, there was no evidence that vitamin D supplementation was always desirable in relieving depressive symptoms in people with different health problems (12). A recent meta-analysis demonstrated that vitamin D supplementation could reduce the occurrence of negative emotions, including depression and anxiety, to some extent (13). However, due to the small number of studies, absence of clear distinction between the prevention and treatment consequences of vitamin D for depression, and no clear requirements for the placebo control of the trial, these findings should be interpreted with caution. Based on the shortcomings above and in the hope of providing some guiding significance for clinical application, we intend to review the relevant randomized controlled trials (RCTs) and conduct an updated meta-analysis to investigate the effect of vitamin D supplementation on the incidence and prognosis of depression.

Methods

Search strategy

According to the preferred reporting items for Systematic Review and Meta-Analysis (PRISMA) 2015 (14), two authors independently ran a systematic search of PubMed, EMBASE, Web of Science, and the Cochrane Library, and covered all potentially relevant articles to appraise the effect of vitamin D supplementation on the incidence and prognosis of depression. And the time scope of the literature search was limited from the establishment of the database to July 2021. Literature retrieval was carried out through the random combinations of the following search terms: "vitamin D" OR "vitamin D supplementation" OR "25-hydroxyvitamin D" OR "25-hydroxyvitamin D supplementation" OR "25(OH)D" OR "cholecalciferol" AND "depression" OR "depressive" OR "negative emotion" OR "major depressive disorder" AND "placebo." The references in the primary articles and relevant reviews were also reviewed manually to avoid the omission of any potentially relevant research.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) RCTs were included in the study; (2) studies in which the experimental groups were supplied with vitamin D supplementation, and the control groups were in absence of vitamin D or only supplemented with placebo in both at baseline and terminal of the intervention; (3) no psychological symptoms in depression or a diagnosis of depression was in the population (two groups of the population could not be mixed into the same study); (4) the participants in each group were categorized under the depression assessment scale in both at baseline and terminal of the intervention, based on their psychological symptoms, to assess the impact of vitamin D on the incidence and prognosis of depression.

The exclusion criteria were as follows: (1) study that was not published in English; (2) study in which the effects of vitamin D supplementation on the baseline and terminal of were only compared in the experimental groups without a control group; (3) study that was just a study protocol or without outcome reported; (4) the intervention contained nutritional supplements/medications; (5) study that unable to obtain the full text or extract available data; (6) study that was a duplicate publication (the latest published or most complete studies would be selected for inclusion).

Data extraction

The retrieved articles were independently screened by two authors, and the data were extracted following a pre-designed data extraction table. Any divergence was resolved through a third-party discussion. Data extracted from each study included: author; year of publication; country; follow up duration; participants' characteristics (e.g., mean age, sample size, body mass index (BMI), serum level of 25(OH)D at baseline and terminal); the dose of vitamin D supplementation; trial registration number; the mean and standard deviation in participants' depression assessment scale scores at baseline, terminal, and the difference between. Different depression assessment scales that were used for the evaluation of the psychological symptoms in different studies include the Beck Depression Inventory (BDI), BDI-II, Hospital Anxiety and Depression Scale (HADS), Hamilton Depression Rating Scale (HAM-D), etc. When multiple depression assessment scales were used in the same study, priority was given to the more well-known and the more commonly used ones (13).

Quality evaluation and outcome measures

The Cochrane Collaborative Risk of Bias Assessment Tool was used to assess the potential risk of bias in RCTs (15). It contains six domains: sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome reporting, and other sources of bias. The risk was classified into three levels: high risk, unclear risk, and low risk.

The efficacy outcome measure of this meta-analysis was the differences in the depression scores from baseline to terminal both in the experimental groups and the control groups, which were assessed by multiple depression assessment scales.

Statistical analysis

By using Revman 5.3 software, each study on the total differences in changes in depressive symptoms between vitamin D groups and control groups was all combined to evaluate the overall effect of vitamin D supplementation on the incidence and prognosis of depression. Through the data extracted from each study, for continuous variables, the effect size was calculated as standardized mean differences with a 95% confidence interval (CI). For the entire sample, the heterogeneity test was estimated using the Cochran chi-square test and quantified using the inconsistency test (I^2) (16). Considering the differences in the type and dose of vitamin D supplements, a random-effects model was applied. All probabilities (p-values) of data were two-sided, with $p < 0.05$ regarded as being statistically significant.

The potential variables leading to sources of heterogeneity were investigated *via* the subgroup analysis. The potential variables included: participants' level of serum 25(OH)D, the dose of vitamin D supplementation, the depression assessment scale that was well-known and commonly used, BMI of participants, mean age of participants, participant's gender, duration of intervention. When included studies $\geq$10, the publication bias would be assessed using a funnel plot (17). In addition, the software was also used for the sensitivity analysis, which was conducted to estimate whether it would affect the pooled effect by excluding each study successively.

Results

Study selection

A total of 3,432 relevant studies were reviewed after the database search. In addition, one study was identified through other origins. After removing 1,977 duplicate studies, 1,398 studies were ruled out through the screening of their title and abstract. Additionally, at the back of the full-text review and evaluation of the remaining 58 studies, one study protocol was excluded, and 20 were excluded due to unavailable data. Furthermore, two studies were excluded because of non-RCT, four articles were excluded because they failed to report relevant outcomes, and two articles containing medications or nutritional supplements were also excluded. Ultimately, in accordance with inclusion and exclusion criteria, 29 eligible studies (7, 18–45) were included (Figure 1), 18 studies (18–35) for the correlation between vitamin D and the incidence of depression, and 11 studies (7, 36–45) for the correlation between vitamin D and the development of depression.

Study characteristics

The basic characteristics of 18 studies illustrating the correlation between vitamin D and the occurrence of depression are displayed in Table 1 and Supplementary Table 1. The locations of the studies were classified as North America, Europe, Asia, and Oceania. Participants in these studies ranged in age from 13 to 85 years old with 2,111 cases in the experimental groups and 2,147 cases in the control groups. Dosage of vitamin D ranged from 200 IU per day to about 10,714 IU per day. The follow-up duration ranged from 6 to 144 weeks. The basic characteristics of 11 studies demonstrating the correlation between vitamin D and the development of depression are shown in Table 2 and Supplementary Table 2. The locations of the studies were classified as Europe and Asia.

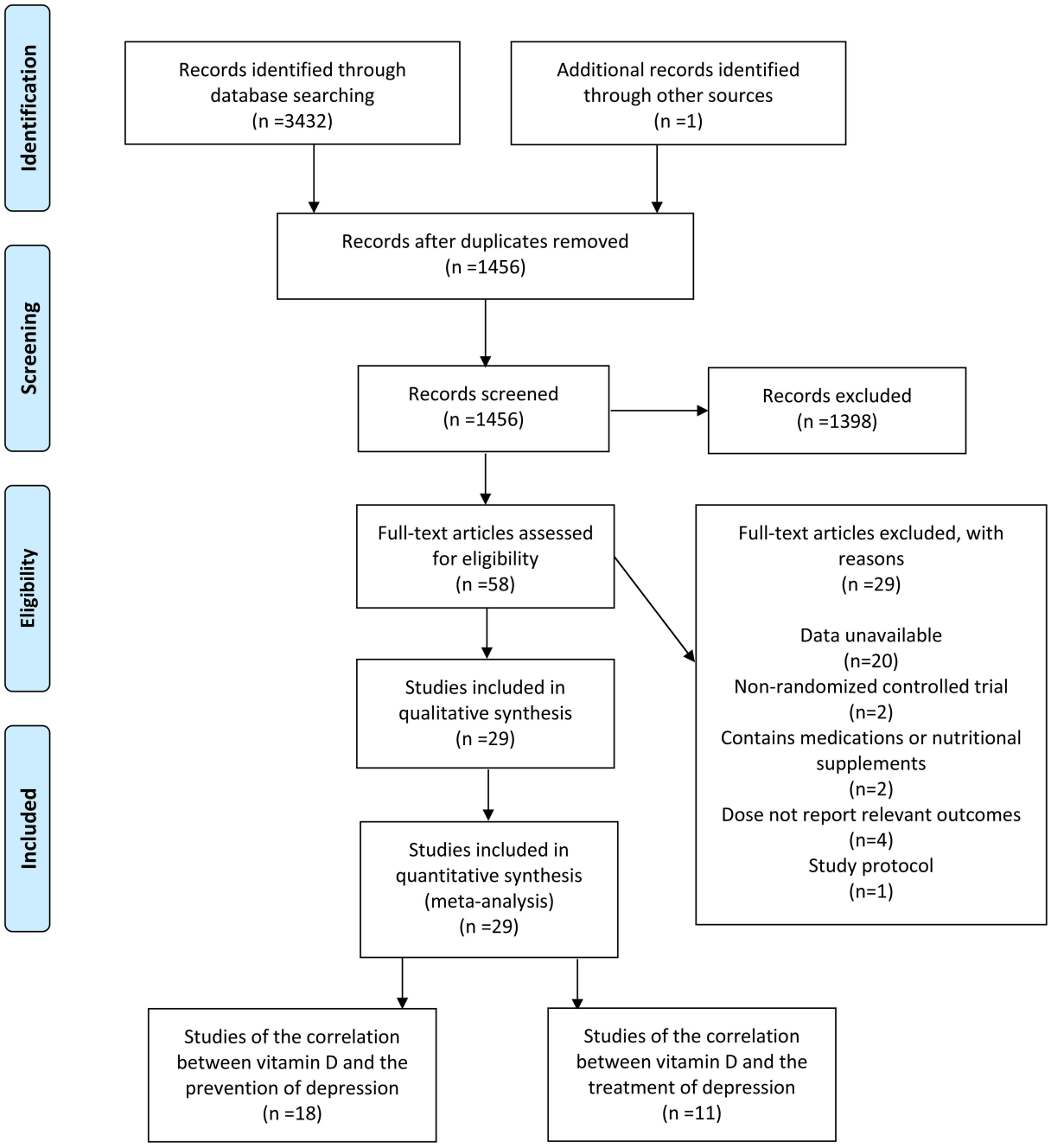

FIGURE 1
Flow diagram describing inclusion and exclusion criteria.

Participants in these studies varied in age from 18 to 79 years old with 878 cases in both the experimental groups and the control groups. Dosage of vitamin D ranged from 1,500 IU per day to about 7,143 IU per day. The follow-up duration varied between 4 and 52 weeks. On the basis of the Cochrane Collaboration tool, particulars concerning the quality assessment of 29 studies are shown in Supplementary Figures 1, 2.

Overall effects and subgroup analysis of vitamin D and the incidence of depression

A pooled analysis of 18 studies demonstrated the overall effects of the correlation between vitamin D supplementation and the occurrence of depression. Despite

TABLE 1 The characteristics of studies included in this meta-analysis (the correlation between vitamin D and the incidence of depression).

Author, year	Country	Follow-up (weeks)	Dose of experimental group	Sample size		Participants inclusion criteria	Depression assessment scale
				Experiment	Control		
Jalali-Chimeh et al. (24)	Iran	8	300,000 IU/4 weeks	38	38	BMI: 18.5–24.9 kg/m^2, Age: 18–45 years for women with sexual dysfunction	BDI
Jorde et al. (27)	Norway	48	40,000 IU/week	116	112	Obese adults	BDI
			20,000 IU/week	106			
Dean et al. (20)	Australia	6	5,000 IU/day	63	65	Age ≥ 18 years for healthy adults	BDI
Kjærgaard et al. (28)	Norway	24	40,000 IU/week	120	110	Age: 30 to 75 years with low 25(OH)D levels	BDI-II, HADS, MADRS
Bertone-Johnson et al. (18)	USA	96	400 IU/day	1109	1143	Age: 50–79 years for postmenopausal women	Burnam scale
Frandsen et al. (21)	Denmark	12	2,800 IU/day	22	21	Healthy adults	SIGH-SAD
Mason et al. (30)	USA	48	2,000 IU/day	109	109	BMI ≥ 25 kg/m^2, Age: 50–75 years for postmenopausal womenwith low 25(OH)D levels	BSI-18
Vaziri et al. (34)	Iran	12	2,000 IU/day	75	78	Age ≥ 18 years for healthy pregnant women	EPDS
Rolf et al. (33)	the Netherlands	48	14,000 IU/day	20	20	Age: 18–55 years with multiple sclerosis	HADS-D
Grung et al. (23)	Norway	21-28	1,600 IU/day	23	23	Age: 13–14 years for healthy adolescents	YSR-CBCL
Ghaderi et al. (22)	Iran	12	50,000 IU/fortnight	34	34	Age: 25–70 years with methadone treatment	BDI
Jorde and Kubiak (26)	Norway	16	100,000 IU bolus and 20,000 IU/week	192	193	Age > 40 years with low 25(OH) D levels	BDI-II
			100,000 IU bolus and 20,000 IU/week (+psychopharmaca)	14	9		
Mousa et al. (31)	Australia	16	100,000 IU bolus and 4,000 IU/day	26	22	BMI ≥ 25 kg/m^2 or BMI≥ 29 kg/m^2, Age: 18–60 years with low 25(OH) D levels	BDI-II
Raygan et al. (32)	Iran	12	50,000 IU/fortnight	30	30	Age: 45–85 years for diabetics with CHD	BDI
Jamilian et al. (25)	Iran	12	50,000 IU/fortnight	30	30	Age: 18–40 years for women with polycystic ovary syndrom	BDI-II, DASS
Choukri et al. (19)	New Zealand	24	50,000 IU/months	76	74	Age: 18–40 years for healthy adult women	CES-D
Krivoy et al. (29)	Israel	8	14,000 IU/week	24	23	Age: 18–65 years for schizophrenic patients with clozapine treatment and low 25(OH)D levels	CDS
Fazelian et al. (35)	Iran	16	50,000 IU/fortnight	26	25	Age: 20–60 years for T2DM women with low 25(OH) D levels	DASS-21

BMI, body mass index; BDI, Beck Depression Inventory; HADS, Hospital Anxiety and Depression Scale; MADRS, Montgomery-Asberg Depression Rating Scale; GDS, Geriatric Depression Scale; SIGH-SAD, The Structured Interview Guide for the Hamilton Rating Scale for Depression-Seasonal Affective Disorder version; BSI, Brief Symptom Inventory Depression Subscale; EPDS, Edinburgh Postnatal Depression Scale; YSR-CBCL, Youth Self-report version of the Child Behavior Checklist; DASS, Depression Anxiety and Stress Scales; CES-D, Center for Epidemiological Studies Depression Scale; CDS, Calgary Depression Scale; CHD, coronary heart disease; T2DM, type 2 diabetes; HT, hormone therapy.

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TABLE 2 The characteristics of studies included in this meta-analysis (the correlation between vitamin D and the prognosis of depression).

Author, year	Country	Follow-up (weeks)	Dose of experimental group	Sample size		Participants inclusion criteria	Depression assessment scale
				Experiment	Control		
Yalamanchili and Gallagher (36)	USA	144	200 IU/day (+HT)	122	120	Age: 65–77 years for postmenopausal women	GDS-30
			200 IU/day	123	123		
Khoraminya et al. (40)	Iran	8	1,500 IU/day	20	20	Age: 18–65 years with MDD and deficiency of vitamin D	BDI-II, HAM-D
Mozaffari-Khosravi et al. (42)	Iran	12	300,000 IU/3 months	39	34	Age: 18–65 years with MDD, Free from other psychiatric diagnoses, any antidepressant drugs or dietary supplements	BDI-II
			150,000 IU/ 3 months	36			
Wang et al. (44)	China	52	50,000 IU/week	362	364	Age: 20 to 60 years for dialysis patients with MDD and deficiency of vitamin D, Free from other psychiatric diagnoses,any antidepressant drugs or dietary supplements	BDI-II
Sepehrmanesh et al. (43)	Iran	8	50,000 IU/week	18	18	Age: 18–65 years with MDD	BDI-II
Hansen et al. (39)	Denmark	24	2,800 IU/day	28	34	Age: 50–79 years with MDD	HAM-D17, MDI
Alavi et al. (37)	Iran	8	50,000 IU/week	39	39	Age > 60 years with MDD and deficiency of vitamin D, Free from other psychiatric diagnoses	GDS-15
Zhang et al. (45)	China	8	100,000 IU/week	58	65	Age ≥ 18 years for recurrent pulmonary tuberculosis patients with MDD	BDI-II
Amini et al. (38)	Iran	8	50,000 IU/fortnight	26	24	BMI ≤ 35 kg/m^2 for women with PPD	EPDS
			50,000 IU/fortnight(+Ca)	26			
Libuda et al. (41)	Germany	4	2,640 IU/day	56	57	Patients with MDD and low 25(OH)D levels	BDI-II, DISYPS-II
Abiri et al. (7)	Iran	8	50,000 IU/week (+Mg)	25	26	BMI: 35 kg/m^2, Age > 20 years for women with MDD and low 25(OH)D levels	BDI-II
			50,000 IU/week	26	25		

MDD, Major depressive disorder; PPD, Postpartum depression; BMI, body mass index; NA, no available; BDI, Beck Depression Inventory; HAM-D, Hamilton Depression Rating Scale; MDI, Major depression inventory; GDS, Geriatric Depression Scale; EPDS, Edinburgh Postnatal Depression Scale; DISYPS-II, Diagnostic System for Mental Disorders in Children and Adolescents according to ICD-10 and DSM-IV.

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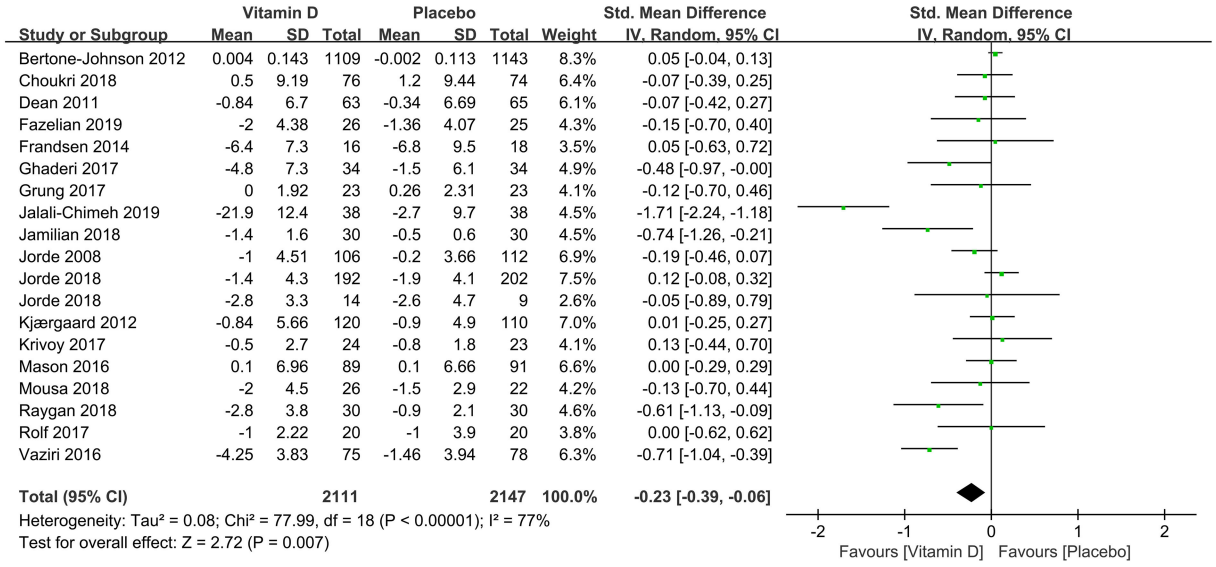

FIGURE 2
Forest plot of the correlation between vitamin D and the incidence of depression.

the high heterogeneity, the depression assessment scale scores from baseline to terminal in the experimental groups have decreased compared with those in the control groups (SMD: −0.23) (Figure 2), which revealed that the usage of vitamin D supplements might reduce the incidence of depression.

In subgroup analysis, the correlation between vitamin D supplementation and the occurrence of depression based on 1,436 participants with low levels of serum 25(OH)D at baseline were conducted in 12 studies. Compared to the control group, the experimental group had a significant effect on reducing the incidence of depression [SMD: −0.33; 95%CI: (−0.60, −0.07); $p = 0.01$] (Figure 3). To compare the effects of low doses (≤2,800 IU/day) and high doses (>2,800 IU/day) of vitamin D supplementation, seven and 10 studies were examined, respectively. The pooled effects (SMD) for low doses and high doses of vitamin D supplements were −0.11 (Figure 4A) and −0.33 (Figure 4B), respectively. The results revealed that vitamin D supplementation in high doses might promote a decrease in the incidence of depression, while low doses of vitamin D supplements were futile.

In nine studies where the BDI or BDI-II was used as the depression assessment scale, the use of vitamin D might also be helpful to the reduction of the incidence of depression compared with the control group (SMD: −0.36). Vitamin D supplements were shown in 13 RCTs to have effectively decreased the incidence of depression among people with normal weight (SMD: −0.28). On the contrary, it was useless for the overweight people in 4 RCTs (SMD: −0.11). Also, seven studies reported a significant diminution compared to the control group in the incidence of depression in females among 2,922 participants with the vitamin D supplementation (SMD: −0.44). Nevertheless, no apparent change relating to vitamin D supplementation was discovered neither in the elderly (SMD: 0.04) through two studies nor in the older female (SMD: 0.04) through other two studies. Finally, among those who received vitamin D supplements, there was no significant difference between the two groups for the intervention duration ≤8 weeks (SMD: −0.55). However, it was worth noting that when the intervention duration is ≥8 weeks, vitamin D supplementation might be conducive to the decrease in the occurrence of depression compared with the control group (SMD: −0.15) (Table 3).

Overall effects and subgroup analysis of vitamin D and the prognosis of depression

To probe the overall effects of the correlation between vitamin D supplementation and the prognosis of depression, 11 studies were included in a pooled analysis. From baseline to terminal, although there was also high heterogeneity, depression assessment scale scores showed a significant decline (SMD: −0.92) (Figure 5), indicating that the interventions with vitamin D supplements might be beneficial to the treatment of depression.

In subgroup analysis, eight studies compared the effects among 1,325 participants with a low level of serum 25(OH)D

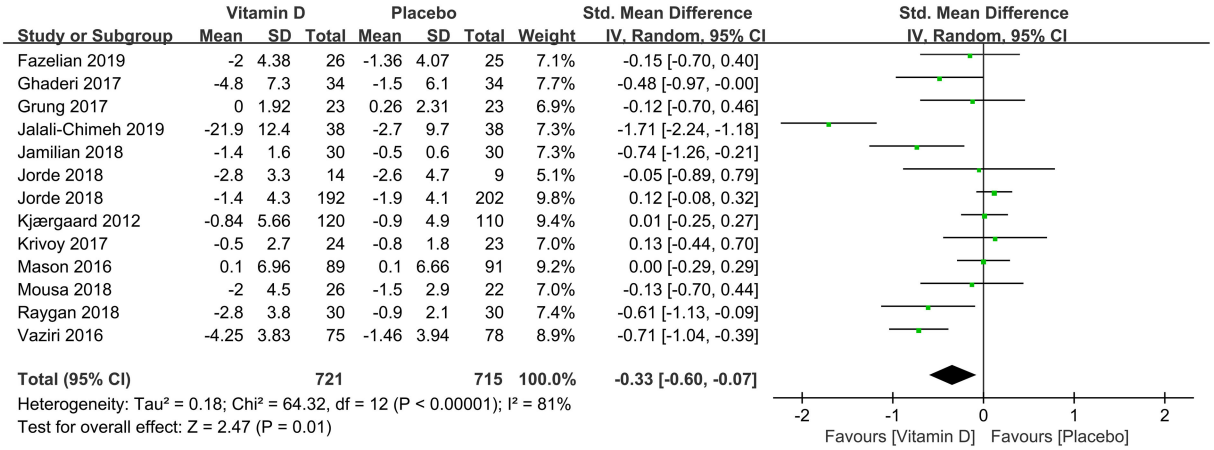

FIGURE 3
Forest plot of the correlation between vitamin D and the incidence of depression for population with low vitamin D levels (<50 nmol/L).

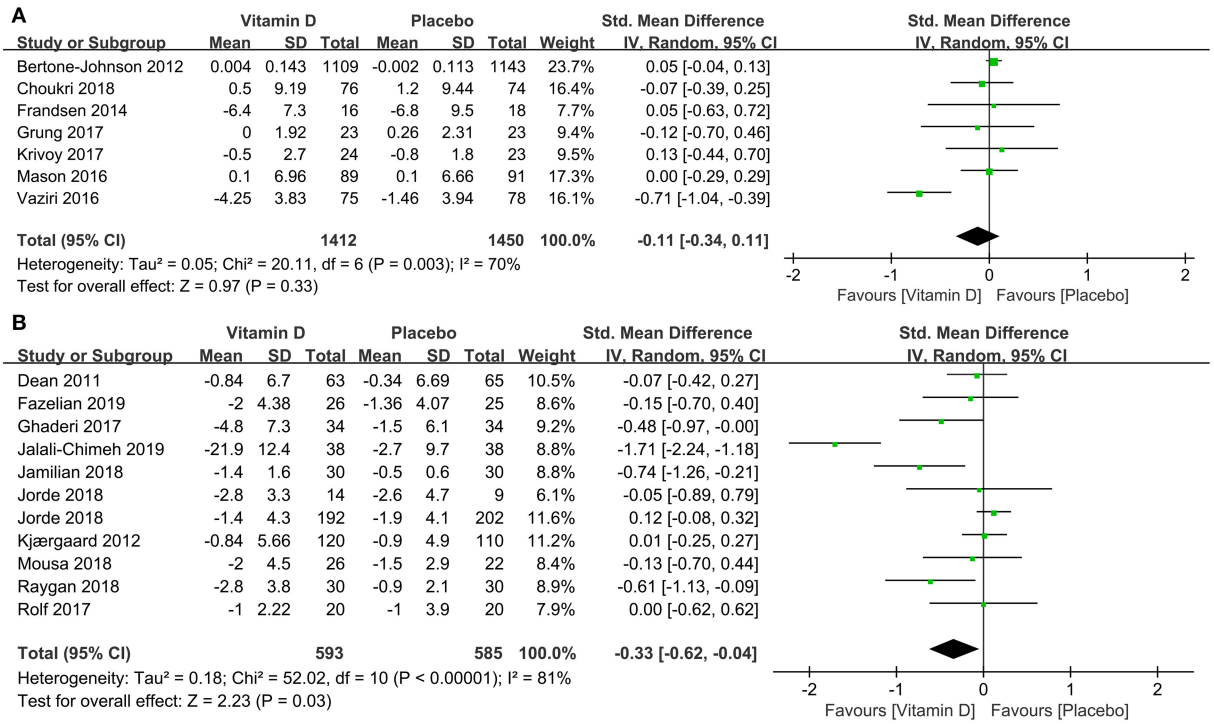

FIGURE 4
Forest plot of the correlation between supplementary doses of vitamin D and the incidence of depression. **(A)** low doses (≤2,800 IU/day); **(B)** high doses (>2,800 IU/day).

at baseline between the two groups. The results illustrated that compared to the control group, those with depression and low serum vitamin D levels might benefit from vitamin D supplementation (SMD: −1.10) (Figure 6). The correlation between vitamin D usage and the development of depression among low doses (≤2,800 IU/day) and high doses (>2,800 IU/day) of vitamin D supplementation were examined in five and seven studies, separately. The results demonstrated that high-dose vitamin D supplementation might be more favorable in the depression therapy, whereas low-dose vitamin D supplementation was relatively ineffective by comparing parameters with the control group, the

TABLE 3 Subgroup analysis of the correlation between vitamin D and the incidence/prognosis of depression.

Subgroup		No. of studies	Participants	Changes in depression	SMD	95%CI	*p*	Heterogeneity(I^2) (%)
Occurrence								
BDI		9	1,305	↓	−0.36	−0.65–(−0.07)	0.02	83
Overweight (BMI >24 kg/m^2)		4	497	-	−0.11	−0.29–0.06	0.21	0
Nomal weight (BMI: 18–24kg/m^2)		13	3,721	↓	−0.28	−0.49–(−0.07)	0.01	83
The elderly (≥50 years old)		2	2,432	-	0.04	−0.04–0.12	0.29	0
Female		7	2,922	↓	−0.44	−0.81–(−0.06)	0.02	91
Older female		2	2,432	-	0.04	−0.04–0.12	0.29	0
Intervention duration	≤8 weeks	3	251	-	−0.55	−1.61–0.52	0.32	93
	≥8 weeks	16	4,054	↓	−0.15	−0.28–(−0.01)	0.03	60
Development								
BDI		7	1,236	-	−1.04	−2.29–0.21	0.10	99
Overweight (BMI >24 kg/m^2)		2	178	↓	−0.79	−1.34–(−0.24)	0.005	72
Nomal weight (BMI: 18–24 kg/m^2)		4	445	↓	−0.34	−0.68–0.00	0.05	60
Female		3	448	↓	−0.61	−1.16–(−0.06)	0.03	84
Intervention duration	≤8 weeks	7	555	↓	−0.69	−1.08–(−0.30)	<0.001	80
	≥8 weeks	10	1,656	↓	−1.00	−1.90–(−0.09)	0.03	98

SMD, standard mean difference; BMI, body mass index; BDI, Beck Depression Inventory.
-, There was no statistical difference in the change of depression scores between the experimental and control groups.
↓, The depression scores of the experimental group decreased compared with control groups.

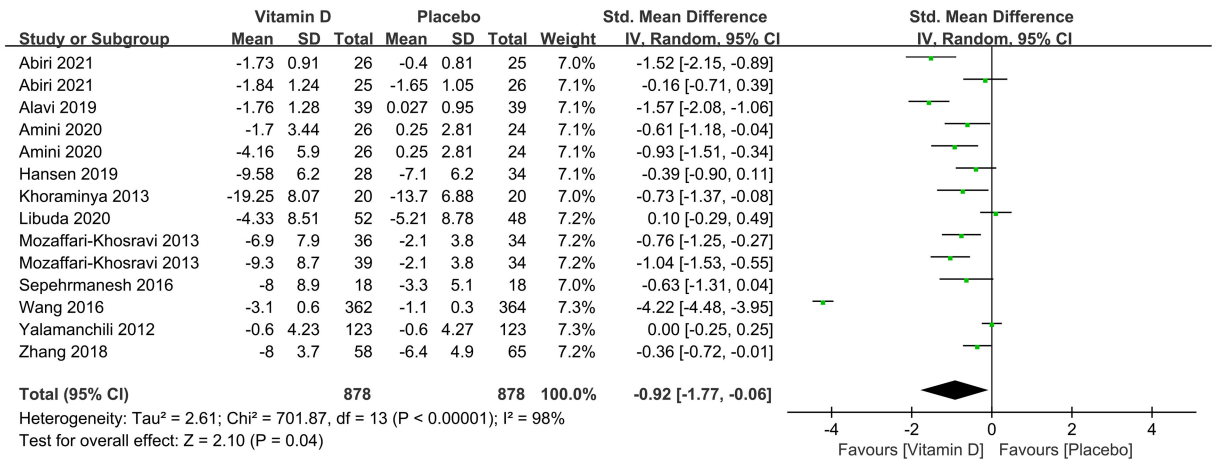

FIGURE 5
Forest plot of the correlation between vitamin D and the prognosis of depression.

pooled effects (SMD) for low-dose analysis was −0.30 (Figure 7A) and high-dose supplementation was −1.23 (Figure 7B), respectively.

However, in the subset of seven studies where the BDI or BDI-II was used as the depression assessment scale, no significant difference was found (SMD: −1.04). With additional consideration of patients' BMI, vitamin D supplementation was effective in the treatment of depression both in people with normal weight (SMD: −0.34) in four studies and those who were overweight (SMD: −0.79) in another two studies. In three RCTs where the vitamin D was supplied to 448 females, compared to the control group, a significant therapeutic effect on depression was also observed (SMD: −0.61). Last but not least, vitamin D supplementation was beneficial as an adjunct to the treatment of depression, compared with the control group, not only for the intervention duration ≥8 weeks (SMD: −1.00) but also for the

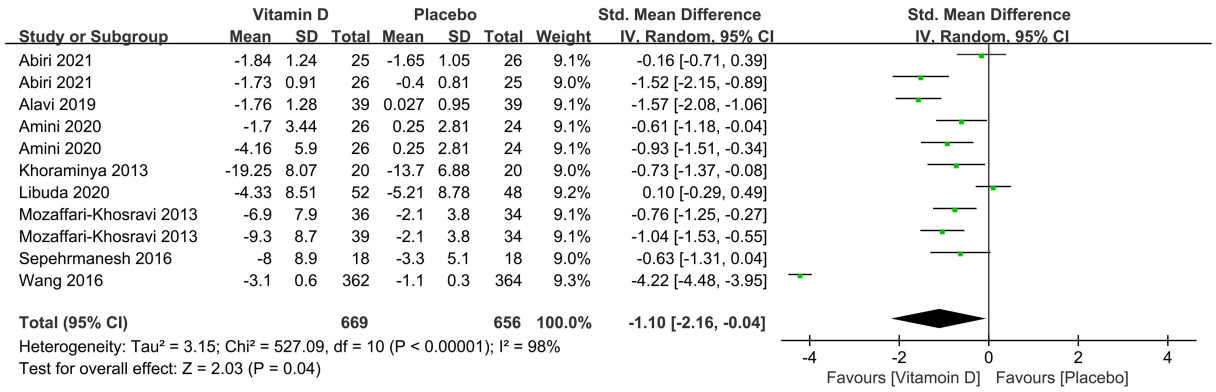

FIGURE 6
Forest plot of the correlation between vitamin D and the prognosis of depression for patients with low vitamin D levels (<50 nmol/L).

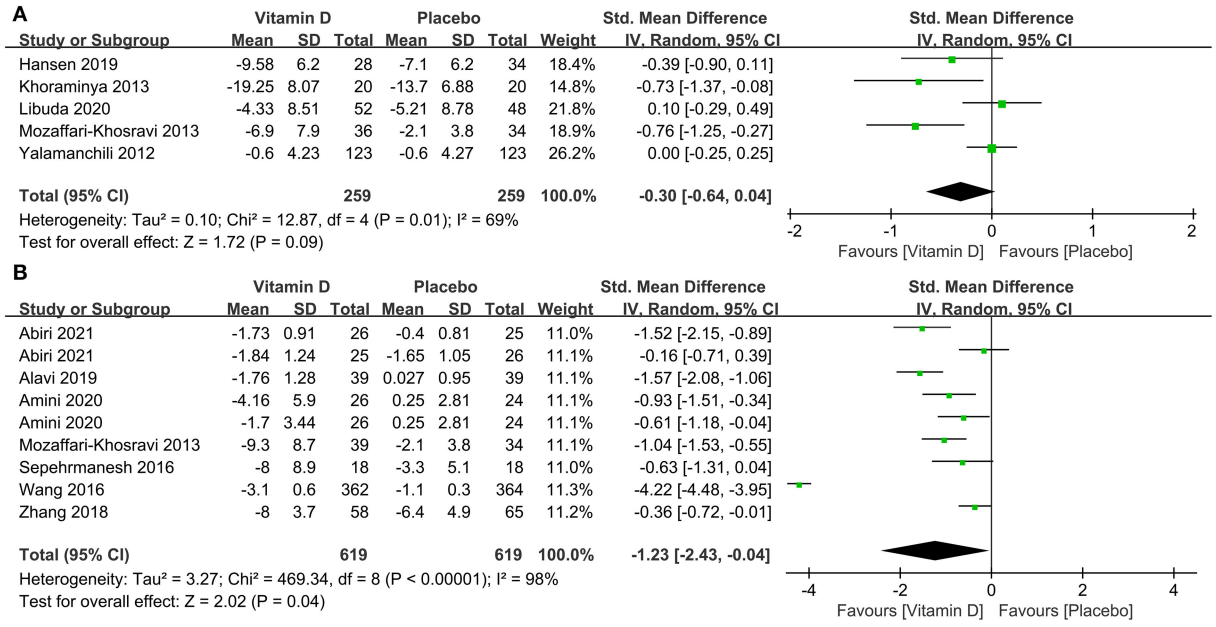

FIGURE 7
Forest plot of the correlation between supplementary doses of vitamin D and the prognosis of depression. **(A)** low doses (≤2,800 IU/day); **(B)** high doses (>2,800 IU/day).

intervention duration ≤8 weeks (SMD: −0.69; 95%CI: [−1.08, −0.30]; $p < 0.001$) (Table 3).

Publication bias and sensitivity analysis

To evaluate publication bias, the funnel plots of the publication bias of vitamin D and the incidence/prognosis of depression was derived (Supplementary Figures 3, 4). All the funnel plots in our results seem to be symmetrical with the effect estimates, which indicated that there was no significant publication bias in our studies. Sensitivity analysis was also conducted to estimate the stability of the pooled effects by excluding each study successively, and the results were supportive to prove the stability of our results.

Discussion

In the present systematic review, we summarized the published outcomes related to the effect of vitamin D supplements on depression. A meta-analysis based on 18 studies was conducted to evaluate the correlation between vitamin D supplementation and the incidence of depression

whereas the correlation between vitamin D supplementation and the prognosis of depression from 11 studies was estimated. Congruent with the previous systematic reviews and meta-analysis (13, 46, 47), in our main study, vitamin D supplementation was found to be associated with a decrease in the occurrence of depression as well as an improvement in the treatment of depression. The impact was also observed in subgroup analyses of serum 25(OH)D levels, doses of vitamin D supplementation, BMI, gender, and intervention duration. Consequently, our results may be supportive to consider vitamin D supplementation to be effective in lowering the risk and improving the treatment of depression.

So far, the correlation between vitamin D and mental health has been investigated mostly in the context of depression or depressive symptoms (2, 12, 46–50), with several putative mechanisms for the effect of vitamin D being proposed (51). Vitamin D has been discovered to have effects on the central nervous system. Some studies have shown that vitamin D receptors are widely distributed in areas of the brain, such as the amygdala and substantia nigra (52). Vitamin D is able to cross the blood-brain barrier, activate vitamin D receptors, and play a role in human behavior control (53).

In addition, in recent years, the cyclo-AMP responsive element-binding protein (CREB) cascade and its effect on the brain-derived neurotrophic factor (BDNF) have made some rationales for the pathogenesis and treatment of depression (54). The hypothesis of catecholamine and serotonin deficiency suggests that depression may be largely caused by the lack of monoamines (norepinephrine and serotonin) in the synaptic space (55). Vitamin D, however, weighs on the synthesis of monoamines and may regulate the activity of GABA-A receptors to some extent (52, 56, 57). By regulating the release of neurotransmitters and the synthesis of neurotrophic factors, vitamin D is relevant in ameliorating mood and behavior in humans (58). Meanwhile, the function of vitamin D may be attributed to its neuroprotective effect on the brain, as it has been proved that vitamin D is able to lower plasma C-reactive protein in patients with psychiatric disorders and modulate inflammation by suppressing proinflammatory cytokines (59, 60).

Although the relationship between vitamin D and depression has been extensively discussed, the evidence in terms of data and mechanism appears to be logical. Several studies (12, 49) have found that taking vitamin D supplements does not result in a substantial reduction in depression. These might stem from the biological defects of the preliminary studies, two studies (13, 50) revealed that those unflawed trials contributed to a statistically prominent improvement in depression. Similarly, a significant association between the use of vitamin D and depression by Anglin agreed with the previous finding (2). Moreover, in the meta-analysis by Shaffer (46), vitamin D was shown to be beneficial in relieving depressive symptoms in patients with clinically significant depression as well.

As for the assessment scale that was used to measure symptoms of depression, in this study, when taking into account the most frequently used assessment scale BDI and BDI-II only, vitamin D supplementation was only effective in the prevention of depression, not in the treatment of depression. This issue might be owing to the fact that the level of depression was incapable of being estimated with meta-regression when under different measurements (13). It served as a reminder to pay attention to the impact of various measurement tools.

In line with prior meta-analyses that have been reported (2, 61), in terms of the impact of the serum vitamin D level on depression, the subgroup analysis showed that patients with low vitamin D levels (<50 nmol/L) benefit from supplementation in both prevention and treatment of depression. Vitamin D deficiency is defined as serum 25(OH)D levels of <50 nmol/L, with levels of 50–75 nmol/L being deemed insufficiency (62). The prevalence of neurodegenerative, neuroinflammatory, and neuropsychological disorders has been linked to hypo-vitamin D in studies based on humans. In a large cohort study (63), higher serum vitamin D levels were authenticated to be associated with a lower incidence of depression at follow-up, suggesting that vitamin D deficiency may signify a latent biological vulnerability for depressive disorder. Hence, it comes as no surprise that depressive patients with hypovitaminosis D are more prone to be relieved from adequate vitamin D supplementation. Furthermore, patients with hypovitaminosis D are supposed to be more alert to the incidence of depression to some extent. It is advised that individuals with vitamin D deficiency should take suitable vitamin D supplements, including dietary sources or pills.

Concerning the BMI, our results revealed that supplementing with vitamin D worked well in both prevention and treatment in people with normal weight, whereas only treatment and not prevention in those who were overweight. According to previous studies (27, 64), BMI appeared to have an essential mediating role in the relationship between vitamin D and depression, and vitamin D supplements might improve depressive symptoms in obese patients. As mentioned before, in the overweight population, no significant difference was observed in the prevention group. The different findings might be ascribed to the truth that only a small proportion of the studies in subgroup analysis included assessed the effect of vitamin D supplementation on overweight patients in the aspect of prevention. Moreover, another contributing factor might contain differences in the study population, variable vitamin D supplementation dosages, and differences in baseline serum 25 (OH) D concentrations levels. A recent comparative observational study showed that the decrease in serum 25 (OH) D level in obese adults was related to incident depression, while the proportion of having vitamin D deficiency in depressed people was higher than that in non-depressed people (65). Vitamin D deficiency was also proved to be less common in healthy individuals (66). Due to the large body and adipose

mass, overweight people might have a low increase in serum vitamin D level and require more vitamin D than normal (67), a regular dose of vitamin D would possibly be insufficient for them. Besides, the adipose mass could also lead to higher systemic inflammation, so higher doses of vitamin supplements were also considered necessary. As a result, the regular dose of vitamin D supplementation might not be as effective in overweight people without a depressive disorder.

Intriguingly, the subgroup analysis of gender demonstrated that vitamin D supplements significantly alleviated depression scores in all females, regardless of whether they were depressed or not. A previous study (68) found that female patients with depressive symptoms improved significantly more following vitamin D administration than male patients, which was in accordance with our findings. The mechanism might involve that vitamin D has a bearing on the synthesis of serotonin and a moderate amount of vitamin D may elevate the level of extracellular serotonin (69), particularly in females, so as to improve the symptoms of depressive disorder. Concerning vitamin D supplementation was ineffective among the elderly population in our results, due to the small number of studies and the unavailability of research data, the outcomes were tough to explain. Additionally, according to a pilot study (70), even though there was a correlation between an increase in vitamin D levels and a decrease in depressive symptom scores, the association vanished as the research population over 65 years old. Furthermore, since most included studies defined depression as being above the dividing point of the depression scale with no adjustment for physical frailty, the outcome could be easily influenced by the confounding factors as well and should be treated with more caution.

When it comes to the strategy of vitamin D supplement, considering the supplementary dosage, the replenishment threshold was set at 2,800 IU each day. Our findings illustrated that high doses (>2,800 IU/day) were beneficial in the prevention and treatment of depression, while low doses (≤2,800 IU/day) were regarded as ineffective. The common dose of vitamin D supplements for adults is 800 IU per day, with a range from 400 to 2,000 IU per day depending on age, weight, illness status, and race (71). Besides, 50,000 IU is acknowledged to be the upper limit of the recommended daily intake (72–75), excessive intake might result in toxicity and some adverse reactions such as hypercalciuria (76). Yet owing to the limited number of studies included in the treatment group in terms of low-dose supplementation, the relevant results should be interpreted with caution either. As for the intervention duration, "8 weeks" was recognized as the time point that might trigger the response to vitamin D, whether in the prevention or treatment groups. Although a previous study showed that the response to vitamin D could not be observed even at 20 weeks, this was based on a status of relative vitamin D repletion (77). As a secosteroid hormone, vitamin D functions through transcription in the nucleus, which takes a long time to work. This might be a plausible explanation for why observing the response to vitamin D took so long. Unexpectedly, we also observed a vitamin D response in the treatment group under the circumstances that the intervention duration was <8 weeks. This phenomenon can also be interpreted by the fact that the mean level of vitamin D in depressed people is more likely to be lower than that in non-depressed people, making depressed ones more sensitive to vitamin D administration. Anyway, our analysis indicated the effects of vitamin D with daily supplementary doses >2,800 IU and an intervention duration of 8 weeks was significant. High heterogeneity was also observed in some subgroups in our meta-analysis, which could be attributed to several reasons. Initially, due to the differences in race, outdoor activity intensity, sunshine time, diet, etc., the baseline and terminal serum vitamin D levels between participants also exist lot of discrepancies. Besides, on account of the differences in the cognition of mental diseases among different populations, there was also a discrepancy in the results of the depression assessment scale to some extent.

Strengths and limitations

To our knowledge, this is the first comprehensive meta-analysis to assess the correlation between vitamin D and the incidence as well as the prognosis of depression. And there were several strengths to our study. First, more recent RCTs were incorporated into this study, thereby updating previous findings. Additionally, although the tool evaluation constructs that were used to measure the psychological symptoms of subjects varied slightly, compared with previous studies (13, 59), they were limited within the depression assessment scale, which decreased the impact of other emotional or physical symptoms on confounding factors of outcomes.

Nonetheless, some associated limitations of our study should be mentioned. First, we have not pre-registered a protocol for this meta-analysis, which might introduce potential bias to the review. Second, due to the race, the intensity of outdoor activity, sun exposure time, dietary differences, and cognition of mental diseases in different ethnic groups and other factors, the high heterogeneity in some subgroups of our meta-analysis was predictable. Third, detailed information such as simultaneous psychosocial interventions, use of calcium supplementation, and antidepressants were not provided in a few studies, our results might be disturbed by overlapping factors. As a consequence, the possibility that the observed amelioration of psychological state in respondents might be the result of overlapping elements acting together should be taken into account. Fourth, the relationship between serum vitamin D levels and the severity of depression was not examined in this study. Also, vitamin D supplementation cannot be fully equivalent to an increase in serum vitamin D levels. Hence, relevant findings should be treated with more care.

Conclusion

To conclude, this meta-analysis demonstrates that vitamin D has a positive impact on both the decreased incidence of depression and a better prognosis of depression. The impact is consistently observed in subgroup analyses of serum 25(OH)D levels, doses of vitamin D supplementation, BMI, gender, and intervention duration. Whether suffering from depression or not, individuals with low vitamin D levels and females are most likely to benefit from vitamin D supplementation. A daily supplementary dose of 2,800 IU and an intervention duration of 8 weeks are considered as the point that may cause the observational effect of vitamin D. Besides, our results also reveal that vitamin D supplementation works well in both prevention and treatment in normal-weight people, whereas only in treatment not prevention for those who are overweight.

Author contributions

LR designed the research process. FX and TH searched the database for corresponding articles and drafted the meta-analysis. DL extracted useful information from the articles above. RF used statistical software for analysis. CN and JH polished this article. All authors contributed to manuscript revision, read, and approved the submitted version.

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

Supplementary material

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SUPPLEMENTARY TABLE 1
The characteristics of studies included in this meta-analysis (the correlation between vitamin D and the incidence of depression).

SUPPLEMENTARY TABLE 2
The characteristics of studies included in this meta-analysis (the correlation between vitamin D and the prognosis of depression).

SUPPLEMENTARY FIGURE 1
Quality assessment of studies included in this meta-analysis (risk of bias graph).

SUPPLEMENTARY FIGURE 2
Quality assessment of studies included in this meta-analysis (Risk of bias summary).

SUPPLEMENTARY FIGURE 3
Funnel plot for publication bias (the correlation between vitamin D and the incidence of depression).

SUPPLEMENTARY FIGURE 4
Funnel plot for publication bias (the correlation between vitamin D and the prognosis of depression).

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Antidiabetic activity of *Solanum torvum* fruit extract in streptozotocin-induced diabetic rats

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Background: *Solanum torvum* Swartz, a medicinal plant belonging to the family Solanaceae, is an important medicinal plant widely distributed throughout the world and used as medicine to treat diabetes, hypertension, tooth decay, and reproductive problems in traditional systems of medicine around the world including Malaysia. The objective of this study was to investigate hypoglycemic, antilipidemic, and hepatoprotective activities, histopathology of the pancreas, and specific glucose regulating gene expression of the ethanolic extract of *S. torvum* fruit in streptozotocin-induced diabetic Sprague–Dawley rats.

Materials and methods: Acute toxicity study was done according to OECD-423 guidelines. Diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (55 mg/kg) in male Sprague–Dawley rats. Experimental diabetic rats were divided into six different groups; normal, diabetic control, and glibenclamide at 6 mg/kg body weight, and the other three groups of animals were treated with oral administration of ethanolic extract of *S. torvum* fruit at 120, 160, and 200 mg/kg for 28 days. The effect of ethanolic extract of *S. torvum* fruit on body weight, blood glucose, lipid profile, liver enzymes, histopathology of pancreas, and gene expression of glucose transporter 2 (slc2a2), and phosphoenolpyruvate carboxykinase (PCK1) was determined by RT-PCR.

Results: Acute toxicity studies showed LD_{50} of ethanolic extract of *S. torvum* fruit to be at the dose of 1600 mg/kg body weight. Blood glucose, total cholesterol, triglycerides, low-density lipoproteins, very low-density lipoproteins, serum alanine aminotransferase, and aspartate aminotransferase were significantly reduced, whereas high-density

lipoproteins were significantly increased in *S. torvum* fruit (200 mg/kg)-treated rats. Histopathological study of the pancreas showed an increase in number, size, and regeneration of β-cell of islets of Langerhans. Gene expression studies revealed the lower expression of *slc2a2* and *PCK1* in treated animals when compared to diabetic control.

Conclusion: Ethanolic extract of *S. torvum* fruits showed hypoglycemic, hypolipidemic, and hepatoprotective activity in streptozocin-induced diabetic rats. Histopathological studies revealed regeneration of β cells of islets of Langerhans. Gene expression studies indicated lower expression of *slc2a2* and *PCK1* in treated animals when compared to diabetic control, indicating that the treated animals prefer the gluconeogenesis pathway.

KEYWORDS

antidiabetic activity, streptozotocin, lipid profile, hepatoprotective activity, gene expression, *Solanum torvum*

Introduction

Diabetes mellitus is a chronic and non-communicable leading public health problem. Diabetes mellitus is a metabolic disorder of carbohydrate due to insulin deficiency resulting from dysfunction of pancreatic beta cells. Over the past decade, diabetes prevalence has risen faster in low- and middle-income countries compared to high-income countries. One of the risk factors being the overweight with possible complications include heart attack, stroke, kidney failure, leg amputation, vision loss, and nerve damage. In addition, during pregnancy, poorly controlled diabetes increases the risk of fetal death and other complications (1).

Diabetes is a potential public health problem in Malaysia and according to the National Health Survey the Ministry of Health, Malaysia reported that the prevalence of diabetes was 13.80% for men and 14.54% for women. In terms of the main ethnic groups, the most common is in the Indian's subpopulation (25.10%), followed by the Malays (15.25%), Chinese (12.87%), Bumiputera (8.62%), and others (6.91%) (2). About two to three decades ago, most of the drugs were obtained from natural sources. Herbal plants have been used for the treatment of various disorders with no sound scientific knowledge on its function, phyto-chemistry, and adverse effects (3). Thus, the focus of this study is to establish scientific basis of antidiabetic effect of ethanolic fruit extract of *Solanum torvum* fruit through biochemical, histopathological, and molecular evidence.

Medicinal plants play an important role in both preventive and curative medicinal preparations for human beings. Herbal medicines are the only affordable source of healthcare, especially for the poorest patients (4). Furthermore, herbal medicines are gaining popularity both in developing and developed countries due to their safety, efficacy, quality, very low adverse effects, and easy availability. Some of the currently available drugs such as aspirin, digitalis, quinine (anti-malarial), vincristine, and vinblastine (anti-cancerous) were derived from the plant sources. Plant-derived phytochemicals have beneficial effect against diabetes, microorganism, inflammation, cardiovascular diseases, blood disorders, cerebral disorders, immune system, oxidative stress, reproductive disorder, and cancer chemotherapy (5). According to the World Health Organization (WHO), more than 21,000 plants are used for medicinal purposes in the world (6). Ethnobotanical information reports about 800 plants which possess antidiabetic potential (7).

Despite the introduction of many new antidiabetic drugs from natural and synthetic sources, diabetes and its secondary complications continue to be a major medical problem. Many indigenous medicinal plants have been found to be useful to successfully manage diabetes. One of the great advantages of medicinal plants is that these are readily available and have very low adverse effects (8). Even though plant sources are potential antidiabetic drugs, they have not gained sufficient momentum among the scientific community. In recent times, it is understood that a proper nutritional regulation with appropriate herbs in our diet shall help to reduce the incidence of diabetes (9). *S. torvum* fruit is also infrequently used by a section of a population as a vegetable. From the literature, it is evident that it is used against various diseases because of its rich phytochemical contents. Furthermore, not much study has been directed toward its potential as antidiabetic activity particularly with the *S. torvum* found locally in Malaysia. Therefore, the aim of this study was to find out the scientific basis of the use of *S. torvum* fruits in the management of diabetes used by traditional practitioners using 70% ethanol extracts on **streptozotocin**-induced diabetic rats.

Collection of plant material

Solanum torvum fruits were collected from the local market, Bedong, Sungai Petani, Kedah, Malaysia. The fruits were authenticated by a plant biologist, AIMST University, Malaysia. The herbarium with voucher specimen (specimen no: 13455) was deposited with the Faculty of Applied Sciences, AIMST University, Malaysia. The fruits were washed with distilled water and dried under shade, powdered finely using heavy duty blender (Waring commercial, USA), and stored at 4°C until further use.

Chemicals and instruments

Streptozotocin (Sigma Chemical Company, St Louis, MO, United States) was used to induce diabetes in rats, and glibenclamide (Hoechst Pharmaceuticals, Mumbai, India) was used as a standard hypoglycemic drug. Diethyl ether was used as anesthetic, and ethanol (BDH Ltd., Mumbai, India) and distilled water were used for extraction of the plant materials. Glucometer, 3,5-dinitrosalicylic acid (DNSA) (Sigma-Aldrich Co., St. Louis, MO, USA), and Accu-Check® Active glucometer test strips (Hoffman-La Roche Ltd., Basel, Switzerland) were used to carry out the experiment. All other used reagents were of analytical grade.

Preparation of plant crude extract

The finely powdered *S. torvum* fruits (100 gm) were mixed with ethanol (500 ml) for the preparation of ethanolic extracts by cold maceration process for a period of 72 h (10). Ethanolic extracts were prepared by maceration process and concentrated using rotary evaporator (Eyela Rotary evaporator N-1000, Japan). Then, the extracts were dried in an oven (Sanyo Microwave Oven Electric Co. Ltd., Taipei, Taiwan) at 40°C. After drying, the amount of dry extract obtained was harvested, and the dried extract was transferred into airtight bottles and stored in a refrigerator at −4°C until used. The weight of the dry extract was expressed as percentage of the total mass of dry plant matter to determine the percentage yield.

Experimental animals

This study was carried on forty healthy adult male Sprague–Dawley (SD) rats, weighing 170–200 gm, which were obtained from Central Animal House, AIMST University, Bedong, Malaysia. The animals were housed in large spacious poly-acrylic cages at an ambient room temperature with 12-h light/12-h dark cycle under standard laboratory and environmental conditions. The animals were free access to water and fed with standard rat feed *ad libitum*. The study was approved by AIMST University Human and Animal Ethics Committee (AUHAEC8/FAS, 2012).

Acute oral toxicity study

The determination of LD_{50} for the extract was carried out as per the guidelines of OECD-423 (11). In this toxicity study, Sprague–Dawley (SD) male rats were weighed 170–200 gm g ($n = 3$) and selected by random sampling technique. The animals were fasted for 4 h prior to the experiment and maintained under standard conditions of temperature (22 ± 1°C) and humidity (55 ± 3°C). The rats were allowed to free access to water. The *S. torvum* fruit extract was dissolved in distilled water and administered orally by gavage with the initial doses. The general behavior of the experimental rats was observed continuously over a period of 24 h for any signs of toxicity and the latency of death (12).

Induction of diabetes in Sprague–Dawley rats

In this study, 16-week-old normal (fasting blood glucose level of 90–110 mg/dl) rats were used. A single dose of intraperitoneal injection of STZ (55 mg/kg/i.p) (Sigma, St. Louis, MO, USA) dissolved in 0.1M citrate buffer (pH 4.5) was used to induce diabetes in overnight fasted male SD rats weighing 170–200 gm. Rats were allowed to free access to 10% glucose water to prevent hypoglycemia. After 72 h, the rats were checked for the blood glucose level from the tail vein using glucometer (Accu-check, Roche Diagnostic, Indianapolis, IND, United states). Only the rats with fasting blood glucose levels ≥ 250 mg/dl were considered as diabetic-induced rats and included in this study.

Animal experimental design

No mortality was observed at the acute oral dose of 1600 mg/per kg body weight by the oral route. The medial lethal dose following oral administration was 1800 mg/per kg body weight. For the selection of doses, 7.5, 10, and 12.5% of 1600 mg/kg body weight were used as concentration of doses for *S. torvum* (13). Six normal healthy rats were chosen randomly for the control group. Thirty diabetic-induced rats were selected, and six rats were randomly assigned for each group for the study.

Group I: Control rats orally administered with distilled water.

Group II: Streptozotocin-induced diabetic rats administered orally with distilled water.

Group III: Streptozotocin-induced diabetic rats administered orally with *Glibenclamide* (5 mg/kg) dissolved in distilled water.
Group IV: Streptozotocin-induced diabetic rats administered orally with ethanolic extract of *S. torvum* fruit (120 mg/kg) dissolved in distilled water.
Group V: Streptozotocin-induced diabetic rats administered orally with ethanolic extract of *S. torvum* fruit (160 mg/kg) dissolved in distilled water.
Group VI: Streptozotocin-induced diabetic rats administered orally with ethanolic extract of *S. torvum* fruit (200 mg/kg) dissolved in distilled water.

All the treatments were started on the fourth day after STZ injection and once a day continued for 28 days.

Biochemical analysis

After 28 days of treatment, blood samples were collected from the retro-orbital plexus, and blood glucose levels were estimated using glucometer (Accu-check, Roche Diagnostic, Indianapolis, IND, United states) before sacrificing the rats (14). The remaining blood was centrifuged at 3000 rpm for 5 min. Serum was collected immediately and stored at −70°C until the analysis of biochemical parameters. The serum was used for the estimation of biochemical parameters such as lipid profile [total serum cholesterol, serum triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL)] and liver function tests [serum glutamate oxaloacetate transaminase (ALT) and serum glutamate pyruvate transaminase (AST)]. These biochemical parameters were measured using Reflectron plus (Roche, Germany) (15).

Histopathological study

The animals were sacrificed by anesthetized with diethyl ether and cervical dislocation; after the dissection of pancreas, it was quickly washed in ice-cold isotonic saline and blotted on ash-free filter paper. Then, the organ was processed immediately for the histopathological studies. The tissue processing procedure includes fixation, dehydration, clearing, and embedding of the materials in wax and block making, microtomy, and finally stained by hematoxylin and eosin (H and E) and mounting the sections on a slide (10). All tissue processing were done in Leica TP1020 Semi-enclosed Bench top Automatic Tissue Processor. The tissue-embedded block was used for sectioning using rotary microtome to obtain a tissue section of 5 μ thickness.

Gene expression studies

Reverse transcription-polymerase chain reaction

The effects on the expression level of some genes involved in production, secretion, and regulation of insulin were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). After 6 and 24 h treatment, total RNA from cells was extracted using GENEzol^{TM} reagent as described by the manufacturer (Geneaid, Taipei, Taiwan). Complimentary DNA (cDNA) of the *slc2a2, PCK1,* and β-*actin* was synthesized from total RNA using specific reverse primers by reverse transcriptase enzyme (Superscript reverse transcriptase IV, Thermo Fisher Scientific, Xinjiang, China) as shown in **Table 1**. Complimentary DNA (cDNA) of the control gene was synthesized from HeLa total RNA using specific reverse primers by reverse transcriptase enzyme which was supplied by Thermo Fisher Scientific, Xinjiang, China. RT-PCR was performed using SYBR Premix Ex Taq technology (TaKaRa Bio Inc., Otsu, Shiga, Japan) on the Applied Biosystems StepOne RT-PCR system. PCR products were analyzed using 1.5% agarose gel electrophoresis under UV transilluminator (BioRad, USA).

Results

The yield of crude extract was 0.8% (ethanol extract). The body weight of diabetic control group was significantly reduced as compared to the normal control group. The animals treated with glibenclamide (6 mg/kg) and *S. torvum* extract for 28 days significantly increased the body weight as compared to the diabetic control group. The body weight of normal control

TABLE 1 Gene name, amplicon size (bp), and forward and reverse primer sequences that were used in polymerase chain reaction (PCR).

Gene name	Amplicon size in bases	Annealing temp	Forward primer 5′-3′	Reverse primer 5′-3′
NM_031144.2 (Actb)	97	49°C	ATGGTGGGTATGGGTCAG	CAATGCCGTGTTCAATGG
NM_012879.2 (*slc2a2*)	162	50.3°C	TCTGTGCTGCTTGTGGAG	ACTGACGAAGAGGAAGATGG
NM_198780.3 (*PCK1*)	171	51.5°C	AACGTTGGCTGGCTCTC	GAACCTGGCGTTGAATGC
Control gene	353	52°C	GCTCGTCGTCGACAACGGCTC	CAAACATGATCTGGGTCATCTTCTC

TABLE 2 Effect of ethanoic extract of *Solanum torvum* fruits on body weight of streptozotocin (STZ)-induced diabetic rats.

Groups	Day 0	Day 14	Day 28
Group–I	181.67 ± 1.63^{a}	192.67 ± 3.26^{b}	202.50 ± 3.78^{b}
Group–II	205.00 ± 8.98^{c}	176.33 ± 8.73^{a}	154.67 ± 10.32^{a}
Group–III	189.33 ± 4.84^{ab}	204.50 ± 6.97^{cd}	221.33 ± 2.65^{c}
Group–IV	184.33 ± 2.87^{a}	197.00 ± 3.68^{bc}	206.50 ± 4.37^{b}
Group–V	192.83 ± 9.60^{b}	204.17 ± 9.17^{cd}	219.17 ± 3.54^{c}
Group–VI	187.33 ± 6.50^{ab}	206.33 ± 6.37^{d}	227.67 ± 1.86^{d}

The values are expressed as mean ± SD ($n = 6$). Values in the column having similar superscripts are not statistically different ($P < 0.05$) (one-way ANOVA followed by Duncan's multiple comparison test).

group was significantly increased on day 14 and 28 compared to day 0, while in the diabetic control group which was significantly decreased on day 14 and 28 compared to day 0. However, the body weight of diabetic rats treated with ethanol extract of *S. torvum* fruit in different doses (120, 160, and 200 mg/kg) was significantly increased (Table 2).

The results indicated that the fasting blood glucose levels during the experimental period (day 0–28) were significantly higher in diabetic control group as compared with the normal control group. Significantly decreased blood glucose levels were observed in glibenclamide group (6 mg/kg) from day 0 to 28 (101.83 ± 3.76 mg/dl) when compared to diabetic control group (280.83 ± 7.026 mg/dl). Similarly, blood glucose levels were significantly reduced in ethanolic extract of *S. torvum* fruit-treated rats (120, 160, and 200 mg/kg) compared to diabetic control group. Statistically lowest blood glucose level was observed in glibenclamide group (101.83 ± 3.76 mg/dl). Significantly, decreased blood glucose levels were observed in *S. torvum* (200 mg/kg)-treated group compared to other *S. torvum* (120 and 160 mg/kg)-treated groups. A dose-dependent effect on fasting blood glucose levels was recorded in groups IV, V, and VI (Table 3).

Total cholesterol, triglycerides, low-density lipoproteins, and very low-density lipoproteins increased significantly in diabetic control group as compared with normal control. On the contrary, the level of high-density lipoproteins decreased significantly in diabetic control group compared to normal control. Serum TC, TG, LDL, and VLDL were significantly reduced, whereas HDL was significantly increased in *S. torvum* fruit (120, 160, and 200 mg/kg)-treated groups in a dose-dependent manner. The anti-hyperlipidemic effect of glibenclamide (6 mg/kg) was comparable with the treatment of 200 mg/kg body wt of ethanolic fruit extract of *S. torvum* (Table 4).

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were increased significantly in diabetic control group compared to normal control group. There was a significant reduction of ALT and AST in glibenclamide group (6 mg/kg) compared to diabetic control group. Similarly, a dose-dependent reduction of ALT and AST was observed in *S. torvum* fruit extract-treated (120, 160, and 200 mg/kg) groups compared to diabetic control group (Table 5).

Photomicrograph (Figures 1A,B) of the normal control group showed normal acini and the β-cells of islets of Langerhans. Contrastingly, an extensive cellular damage of the β-cells was observed (Figures 2A,B) in STZ-induced diabetic rats. However, the diabetic rats treated with glibenclamide (6 mg/kg) showed an increased number of regenerated β-cells (Figures 3A,B). Histopathological evidence of pancreas of animals treated with ethanolic extract of *S. torvum* fruits showed comparable regeneration of β cells comparable to that of glibenclamide-treated group.

Interestingly, the diabetic rats treated with ethanolic extract of *S. torvum* fruits (120, 160, and 200 mg/kg) showed a dose-dependent effect on regeneration of β-cells (Figures 4–6A,B). It is evident that the ethanolic extract of *S. torvum* fruits at 200 mg/kg showed pronounced regeneration of β-cells as compared to 120 and 160 mg/kg.

In the present investigation, the molecular mechanism of *S. torvum* action as a hypoglycemic agent was studied by analyzing the expression levels of GLUT-2 and PCK1 genes in normal, diabetic, and diabetic-treated rats using RT-PCR assay. Significant decrease in GLUT-2 gene expression was observed in STZ-induced diabetic group compared to normal rats probably due to insulin deficiency (Figure 7). After administering *S. torvum* ethanolic fruit extract, the diabetic rats showed lower expression of GLUT-2 gene despite of decreased blood glucose level.

Discussion

Diabetes mellitus is a disorder in which the body tissues failed to utilize the glucose which leads to increased utilization of proteins responsible for reduction in body weight (16). It has been suggested that an increase in the body weight of *S. torvum* fruit extract-treated rats might be due to an enhancement in glycemic control and increased synthesis of structural protein (17). This may be achieved *via* the inhibition of hepatic gluconeogenesis and glucose output from the liver, which is accompanied by the suppression of lipolysis in adipose tissue (18). Abu-Odeh and Talib (19) suggested that the possible mechanism for body weight gain in plant extract-treated rats may be due to extra pancreatic action which might have contributed to the increased utilization of glucose by the tissues.

Diabetes is a metabolic disorder caused by impaired metabolism of carbohydrates, proteins, and lipids predisposing to hyperglycemia (20). Glycemic control is the main target of the treatment to prevent micro- and macrovascular and neurological complications of diabetes (21). The medicinal

TABLE 3 Effect of ethanol extract of *Solanum torvum* fruits on fasting blood glucose level in streptozotocin (STZ)-induced diabetic Sprague–Dawley (SD) rats.

	Fasting blood glucose mg/dl				
Groups	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Group–I	93.33 ± 2.582^{a}	92.67 ± 4.45^{a}	93.67 ± 4.32^{a}	93.00 ± 1.78^{a}	90.43 ± 2.36^{a}
Group–II	292.00 ± 5.13^{d}	278.83 ± 12.33^{e}	277.00 ± 9.77^{f}	280.33 ± 6.56^{f}	280.83 ± 7.026^{f}
Group–III	294.83 ± 5.87^{d}	261.83 ± 9.17^{d}	165.67 ± 9.52^{b}	128.83 ± 6.85^{b}	101.83 ± 3.76^{b}
Group–IV	281.67 ± 9.18^{bc}	257.67 ± 5.46^{d}	244.17 ± 3.71^{e}	232.83 ± 3.37^{e}	210.00 ± 7.61^{e}
Group–V	280.83 ± 9.66^{b}	241.00 ± 7.72^{c}	211.83 ± 19.65^{d}	165.83 ± 18.35^{d}	137.33 ± 6.28^{d}
Group–VI	289.83 ± 6.73^{cd}	205.50 ± 13.03^{b}	184.00 ± 15.47^{c}	141.50 ± 14.57^{c}	118.16 ± 3.81^{c}

The values are expressed as mean ± SD ($n = 6$). Values in the column having different superscripts are statistically different ($P < 0.05$) (One-way ANOVA followed by Duncan's multiple comparison test).

TABLE 4 Effect of ethanolic extract of *Solanum torvum* fruits on lipid profile in streptozotocin (STZ)-induced diabetic Sprague–Dawley (SD) rats.

	Lipid profile (mg/dl)				
Groups–Treatment	**Serum total cholesterol mg/dl**	**Triglycerides mg/dl**	**S.HDL mg/dl**	**S.LDL mg/dl**	**S.VLDL mg/dl**
Group–I	94.31 ± 2.64^{a}	84.31 ± 2.64^{a}	33.10 ± 3.50^{c}	44.35 ± 4.64^{a}	16.85 ± 0.52^{a}
Group–II	130.84 ± 5.03^{e}	123.58 ± 3.24^{e}	23.01 ± 1.63^{a}	83.11 ± 4.86^{e}	24.71 ± 0.64^{e}
Group–III	106.13 ± 4.79^{b}	92.30 ± 2.52^{b}	36.42 ± 2.58^{d}	51.25 ± 4.33^{b}	18.45 ± 0.50^{b}
Group–IV	124.17 ± 3.77^{d}	111.18 ± 3.58^{d}	29.43 ± 1.38^{b}	72.51 ± 3.87^{d}	22.23 ± 0.71^{d}
Group–V	117.54 ± 3.06^{c}	106.23 ± 3.52^{c}	31.27 ± 1.43^{bc}	65.26 ± 1.99^{c}	21.24 ± 0.70^{c}
Group–VI	107.82 ± 3.63^{b}	93.01 ± 4.12^{b}	33.58 ± 2.60^{c}	55.64 ± 4.33^{b}	18.59 ± 0.82^{b}

The values are expressed as mean ± SD ($n = 6$). Values in the column having different superscripts are statistically different ($P < 0.05$) (One-way ANOVA followed by Duncan's multiple comparison test).

TABLE 5 Effect of ethanolic extract *Solanum torvum* fruits on liver enzymes in streptozotocin-induced diabetic Sprague–Dawley (SD) rats.

	Liver enzymes	
Groups–Treatment	**ALT (U/L)**	**AST (U/L)**
Group–I	35.68 ± 2.62^{ab}	56.63 ± 3.18^{b}
Group–II	56.17 ± 3.57^{d}	67.56 ± 2.63^{e}
Group–III	33.83 ± 2.97^{a}	52.57 ± 2.97^{a}
Group–IV	42.4 ± 3.00^{c}	65.54 ± 1.17^{de}
Group–V	38.41 ± 1.09^{b}	63.99 ± 1.21^{cd}
Group–VI	35.11 ± 2.02^{ab}	60.95 ± 1.41^{c}

The values are expressed as mean ± SD ($n = 6$). Values in the column having different superscripts are statistically different ($P < 0.05$) (One-way ANOVA followed by Duncan's multiple comparison test).

plants are widely used as a prophylaxis and for curing of human diseases due to the presence of phytochemicals such as flavonoids and phenols in *S. torvum* fruit extract (22).

Many research reports showed that medicinal plants that possess hypoglycemic activity act through various mechanisms include improvement of insulin sensitivity of target cells, augmenting insulin secretion and stimulating the regeneration of β-cells of islets of Langerhans in pancreas (23). Several authors reported that the presence of flavonoids, steroids, terpenoids, and phenols is responsible for antidiabetic activity (24). Flavonoids have also been known to regenerate the damaged beta cells in alloxan-induced diabetic rats and act as insulin secretagogues (25).

The potential therapeutic use of polyhydroxylated alkaloids in the treatment of type 2 diabetes due to their ability to inhibit maltase-glucoamylase has been reported (26). In this study, the marked reduction in blood glucose levels may be due to regeneration of pancreatic beta cells leading to an increased secretion of insulin in *S. torvum*-treated groups. High-performance liquid chromatography revealed the presence of quercetin (flavonoid) in *S. torvum* fruit extract. It has been shown that quercetin possesses antidiabetic activity in streptozotocin-induced diabetic rats through regeneration of pancreatic islets which increases insulin secretion (27). This histological evidence may also account for the hypoglycemic activity of the *S. torvum* fruit extract. Biologically active, naturally occurring phytochemical compounds found in plants provide health benefit for humans (28).

Oxidative stress is one of the contributing factor in the pathogenesis of diabetes. Diabetes, by itself, increases the production of tissue damaging reactive oxygen species (ROS) by glucose auto-oxidation that inhibits enzymatic protein glycosylation (29). The antioxidant enzyme levels are affected by diabetes, which further increase oxidative stress (30).

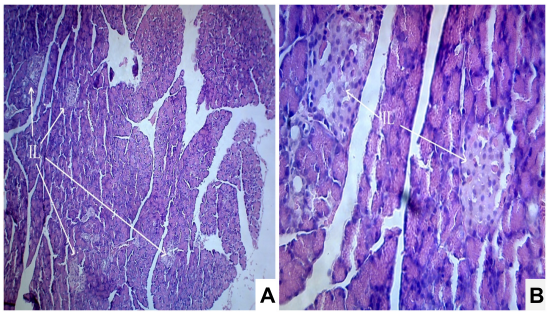

FIGURE 1
Effect of ethanolic extract *of Solanum torvum* fruit on histopathology of pancreas in streptozotocin (STZ) induced diabetic Sprague–Dawley rats (SD rats). **(A,B)** Photomicrograph of a section from pancreas (Normal control group) showed normal architecture. The acinar cells are seen to be normal. **(A)** H&E 10X; **(B)** H&E 40X. IL, islets of Langerhans cells.

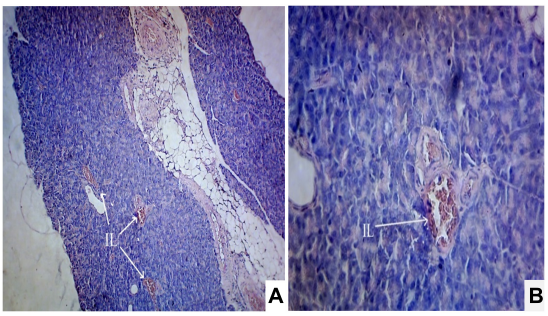

FIGURE 2
Effect of ethanolic extract *of Solanum torvum* fruit on histopathology of pancreas in streptozotocin (STZ)-induced diabetic Sprague–Dawley rats (SD rats). Photomicrograph of a STZ-induced diabetic rat section from pancreas (diabetic control group) showed severe decrease in number of islets of Langerhans cells and β-cells. The acinar cells are seen to be normal. **(A)** H&E 10X; **(B)** H&E 40X. IL, islets of Langerhans cells.

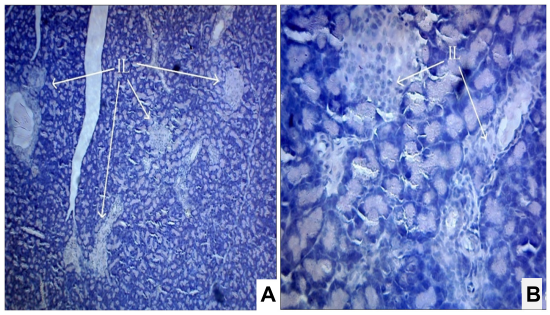

FIGURE 3
(A,B) Photomicrograph of pancreas from glibenclamide (6 mg/kg)-treated diabetic rat showing partial restoration of normal cellular population and size of islet cells. The acinar cells are seen to be normal. **(A)** H&E 10X; **(B)** H&E 40X. IL, islets of Langerhans cells.

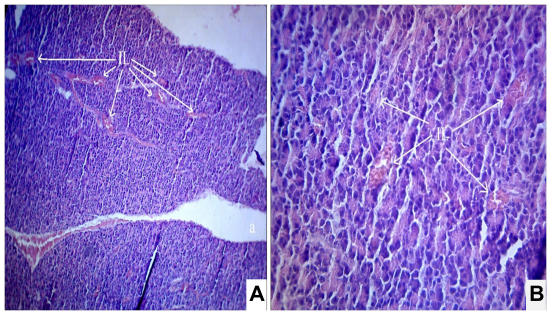

FIGURE 4
(A,B) Photomicrograph of a diabetic rats treated with *Solanum torvum* fruits extract (120 mg/kg/b.wt.) section from pancreas showed minimal restoration of normal cellular population and size of islet cells. The acinar cells are seen to be normal. **(A)** H&E 10X; **(B)** H&E 40X. IL, islets of Langerhans cells.

Hyperglycemia and hyperlipidemia are the main causes of oxidative stress in type 2 diabetes. Reactive oxygen species (ROS) formed in this process triggers tissue damage and has been shown to affect the two major mechanisms failing during diabetes: insulin resistance and insulin secretion (31). In diabetes, tissue damage is considered to be mediated by free radicals by attacking membranes through peroxidation of unsaturated fatty acids which lead to extensive membrane damage and dysfunction (32). Decreased lipid peroxidation and improved antioxidant condition could be one of the mechanisms to prevent complications of diabetes (33).

Poongothai et al. (34) reported that methanolic extract *Solanum xanthocarpum* leaves at a dose of 200 mg/kg significantly reduced the blood glucose level and increased the serum insulin level in alloxan-induced diabetic rats. Saponins at 1.3 mg/100 g from *Solanum anguivi* fruit strongly inhibited lipid peroxidation and increased the levels of antioxidant enzymes, thus preventing hyperglycemia-induced oxidative stress (35). Diabetic rats treated with *Solanum lycocarpum* fruit extract at 1000 mg/kg resulted in reduced levels of blood glucose and also reduced food and water intake (36).

Two varieties *Solanum melongena* such as white and graffiti showed antioxidant activity, reduced the hyperglycemia-related complications, and decreased glucose absorption in the intestine (37). Similarly, ethanolic root extract of *S. xanthocarpum* at 200 and 400 mg/kg showed antihyperglycemic activity in alloxan-induced diabetic rats (38).

A recent study by Ammulu et al. (39) indicated that *Solanum trilobatum* ethanolic leaf extract at 100 and 200 mg/kg lowered blood glucose and generation of free radicals in alloxan-induced diabetic rats. Aqueous fruit extract of *Solanum nigrum* reduced blood glucose levels and hyperglycemia-related vascular complications in STZ-induced diabetic rats (40). *Solanum pubescens* methanolic leaf extract at 300 mg/kg was reported

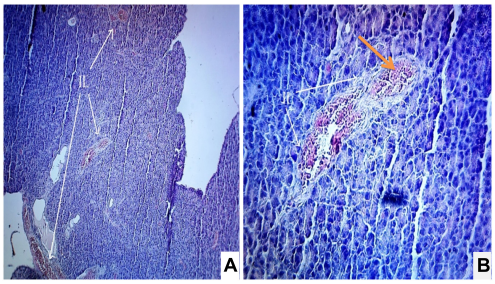

FIGURE 5
(A,B) Photomicrograph of a diabetic rats treated with *Solanum torvum* fruits extract (160 mg/kg/b.wt.) section from pancreas showed minimal restoration of normal cellular population and regeneration of β-cells of islets of Langerhans. The acinar cells are seen to be normal. **(A)** H&E 10X; **(B)** H&E 40X. IL, islets of Langerhans cells.

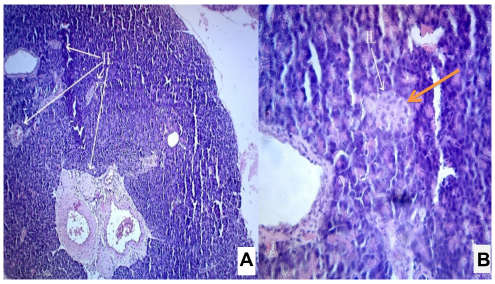

FIGURE 6
(A,B) Photomicrograph of a diabetic rats treated with *Solanum torvum* fruits extract (200 mg/kg/b.wt.) section from pancreas showed maximum restoration of normal cellular population and regeneration of islets of Langerhans. The acinar cells are seen to be normal. **(A)** H&E 10X; **(B)** H&E 40X. IL, islets of Langerhans cells.

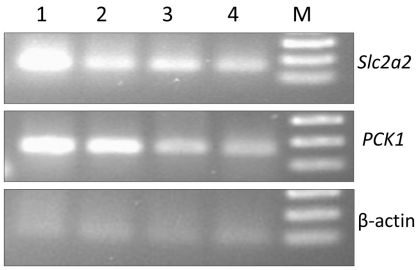

FIGURE 7
Effect of ethanolic extract of *Solanum torvum* fruit on glucose metabolism regulating gene (slc2a, PCK1, and β actin) expression study by reverse transcription-polymerase chain reaction (RT-PCR). NC, negative control; PC, positive control (genomic DNA); IC, internal control; 1, normal control group; 2, diabetic control group; 3, glibenclamide-treated group; 4, *S. torvum*-treated group; M, 100 bp DNA ladder (Thermo Fisher Scientific, Xinjiang, China).

to decrease the blood glucose levels in alloxan-induced diabetic rats (41).

Generally, the diabetes is accompanied by hyperglycemia and hyperlipidemia (42). Hypercholesterolemia and hypertriglyceridemia are major risk factors for atherosclerosis which could be prevented by hypocholesterolemic drugs (29). During diabetic condition, serum fatty acids are produced in excess and converted into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed at the same time in liver may be discharged into blood in the form of lipoproteins (43). The abnormal high concentration of serum lipids in the diabetic condition is mainly due to increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone-sensitive lipase.

In diabetes, the hyperlipidemia is the consequence of the uninhibited action lipolytic hormones on the fat depots (44). During normal metabolism, insulin activates lipoprotein lipase to hydrolyze triglycerides. However, in a state of insulin deficiency, lipoprotein lipase is not activated resulting in hypertriglyceridemia (45). In accordance with this study, the possible mechanism of antidiabetic and hypolipidemic activity of *S. torvum* fruit extract is mainly due to the presence of polyphenolic compounds. In addition to this, it also possesses antioxidant property which could be beneficial to diabetes (46).

Hepatoprotective, anti-inflammatory, and antioxidant effects of herbal medicines could be attributed to the presence of a variety of phytochemicals such as tannins, kaempferol, rutin, bergapten, psoralenes, flavonoids, coumarin, and phenolic glycosides (47). STZ-induced diabetic rats treated with *S. torvum* fruit extract showed decreased levels of ALT and AST indicating the hepatoprotective effect through maintenance of functional integrity of hepatic cell membrane and restoration of liver metabolism in diabetic rats (48).

The liver enzymes ALT and AST are considered to be good biomarkers of hepatotoxicity, wherein the elevated levels of these enzymes are indicative of liver cell damage (49). In diabetic rats, an increased level of these enzymes is due to the hepatic cell damage. Furthermore, the high levels of ROS play a critical role in the inflammatory damage of liver cells (50). Earlier studies have indicated the elevated levels of AST and ALT in diabetic condition including STZ-induced diabetics in experimental animals (51). It was also observed that STZ-induced diabetic rats showed a time-dependent rise in AST and ALT levels (52). Tripathi (47) reported hepatoprotective, anti-inflammatory, and antioxidant effects could be attributed to the presence of the various phytoconstituents including tannins, kaempferol, rutin, bergapten, psoralenes, flavonoids, coumarin, and phenolic glycosides. STZ-induced diabetic rats treated with *S. torvum* fruit extract showed decreased activity of ALT and AST enzymes that might support its hepatoprotective indicating maintenance of functional integrity of hepatic cell membrane,

and normalization capability of impaired liver metabolism in diabetic rats (17).

The endocrine capability of pancreas is determined by apoptosis, replication, and neogenesis of beta cells of islets of Langerhans (45). Oxidative stress plays an important role in beta cell dysfunction and apoptosis (53). Apoptosis is activated by stress factors including growth factor deprivation, cell cycle disturbance, and DNA damage, which lead to mitochondrial release of cytochrome c followed by stimulation of caspase-9, 8, 3, 6, and 7 in sequence, that promote DNA fragmentation and cell death (54).

In this context, the protective effect of some phytochemicals on pancreas has been found to be mediated through their antioxidant activity (55). Furthermore, some phytochemicals stimulate the proliferation and differentiation of progenitor cells involved in protection and regeneration of β-cells (56). Most of the plants possess natural antioxidants such as phenol and flavonoids. The regeneration of pancreas may be also attributable to the tannins in the plant extracts through their anti-inflammatory action (57). The phytochemicals and amino acids in the herbal plants are associated with regeneration of β-cells in diabetic rats (58).

Modulation in gene expression related to carbohydrate metabolism is an important component of the pathogenesis of diabetes (59). In liver tissue, carbohydrate metabolism is regulated by multiple transcription factors through insulin response (60). Glucose transport is the key step in carbohydrate metabolism which is facilitated by glucose transporters. GLUT-2 is a transmembrane carrier protein that enables passive glucose movement across cell membranes (61). GLUT-2 is principal glucose transporter among the 14 GLUT protein family (62). Antihyperglycemic effect of *S. torvum* may be through a different glucose transporter rather than GLUT-2. The histopathological evidence in the treated animals showed regeneration of beta cells which may lead to an increased insulin secretion that could induce a different glucose intake pathway than GLUT-2.

Gluconeogenesis is controlled by hormone-mediated gluconeogenic enzymes at the level of gene expression. In the liver, phosphoenolpyruvate carboxykinase (*PCK1*) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and is considered to be the major rate-controlling enzyme in the gluconeogenesis pathway from pyruvate, lactate, and alanine (63). This study revealed that the ethanolic extract of *S. torvum* fruit represses *PCK1* gene expression which limits the gluconeogenesis pathway. Deficiency of glucose in the cells triggers gluconeogenesis pathway (64). The lower expression of *PCK1* was observed in treated groups compared to normal and diabetic group suggesting decreased gluconeogenesis in treated groups. This could be due to the sufficient intake of glucose by the cells after the treatment with glibenclamide or *S. torvum* fruit extract. Most of the experimental studies suggested that increased gluconeogenesis is a main source of hyperglycemia in insulin deficiency (65). It is also suggested that insulin may inhibit *PCK1* gene transcription by activation of a possible insulin response factors (66). Thus, *S. torvum*-treated diabetic rats showed the decreased expression of *PCK1* gene possibly due to the availability of insulin.

Conclusion

The effect of *S. torvum* fruit extract on histopathology of pancreas has showed the regeneration of β cells of islets of Langerhans which may be responsible for increased secretion of insulin resulting in hypoglycemia. *S. torvum* fruit extract (200 mg/kg)-treated rats showed decreased blood glucose level may be due to lower expression of *PCK1* gene. The *PCK1* genes regulate phosphoenolpyruvate carboxykinase enzyme activity in the gluconeogenesis pathway. Thus, *S. torvum* fruits can be a good candidate for novel phytomedicine that can be used to treat several diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by AIMST University Animal Ethics Committee.

Author contributions

NS and SC have equally contributed to design and conceived the study. NS, SC, and PS carried out the experiments, conducted the data analysis, and interpreted the data. NS has procured the Institutional Ethical Approval for the study. All authors have drafted, edited, and reviewed the manuscript and given their consent for submission.

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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.

The reviewer SP declared a shared affiliation with the authors AU and BM to the handling editor at the time of review.

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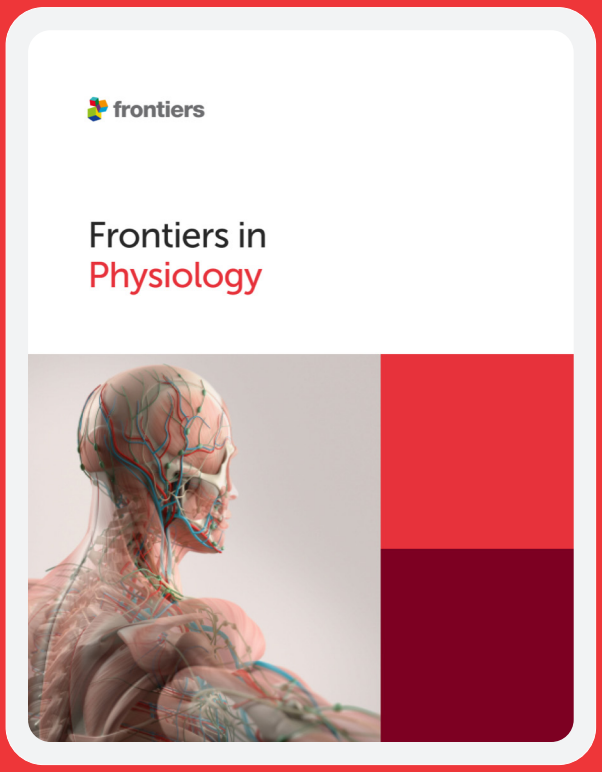

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