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Environmental threats to human reproduction

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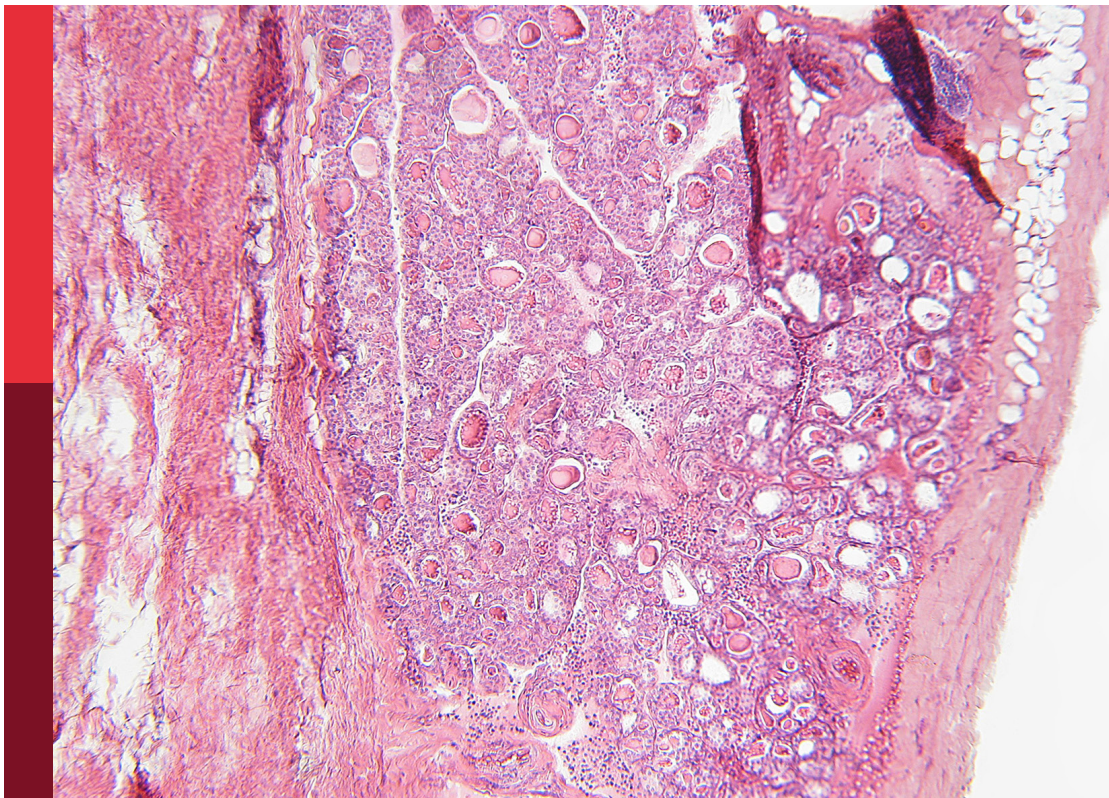

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Environmental threats to human reproduction

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Editorial: Environmental threats to human reproduction

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environmental toxicant, environmental stressors, oxidative stress, epigenetics, inflammation, apoptosis

Editorial on the Research Topic
Environmental threats to human reproduction

The human reproduction involves a cascade of complex events that is controlled by several social, biological, and environmental factors. Environmental factors include heavy metals such as arsenic and lead (1–3), pesticides (4), industrial chemicals like phthalates and bisphenol A (5–7), infection (8), and endocrine disruptors which include pharmaceuticals (9). Environmental toxicants disrupt menstrual cycles, and reduce ovarian reserve and oocyte quality (10, 11). These toxicants also reduce circulating testosterone and sperm quality (12).

More so, these stressors iinfluence pregnancy outcomes. They induce miscarriage and stillbirth, birth defects, preterm birth and low birth weight, and neurodevelopmental disorders (13, 14).

These environmental stressors act via multiple pathways. First, they disrupt the endocrine system by mimicking or blocking sex hormones (15). They may also induce oxidative stress by upregulating the generation of free radicals and suppressing antioxidants (16, 17), trigger inflammation and immune response (18, 19), and promote genotoxicity (20). This Research Topic provides emerging evidences linking environmental toxicants with human reproduction.

Wu et al. reviews the impact of taxanes on ovarian function in women and analyzed the possible reasons for different outcomes. They reported that taxanes-induced ovarian damage is associated with abnormal cell division, follicular cell apoptosis, and reactive oxygen species accumulation. Pan et al. observed an inverted U-shaped association of blood lead levels with oestrogen and a U-shaped association between blood lead levels and sex hormone-binding globulin in female adolescent, indicating that adjusting blood lead exposure to mitigate the effects of lead on growth and development is important for adolescents. He and Wan demonstrated a positive association between smoking and elevated infertility risk.

In a meta-analysis by Hamed et al., it was observed that organophosphate pesticides reduced sperm quality via a testosterone-independent mechanism. Odetayo et al. reported that omega 3 fatty acid attenuated bisphenol F-induced reductions in testosterone and

sperm quality by downregulating oxidative stress, inflammation, and apoptosis. Sustarsic et al. observed in a meta-analysis that lifestyle intervention may be beneficial in overweight and obese women diagnosed with infertility by improving ovulation, chances of pregnancy, and rate of live births.

Yao et al. demonstrated a positive association between phthalate exposure and antral follicular count, suggesting that this plasticizer may promote primordial follicle recruitment and depletion of ovarian reserve. Wang et al. demonstrated an inverse association between Life's simple 7 (LS7) metric scores and infertility. They showed that higher LS7 scores are associated with reduced fertility among women between 18 and 44 years. This finding provides a novel evidence linking cardiovascular status with reproductive health. Qi et al. observed a positive correlation between higher dietary inflammatory index (DII) score and female infertility.

Although SARS-CoV-2 remains quite novel, convincing evidences have been provided on its possible link with infertility (21, 22). Hu et al. showed that asymptomatic or mild SARS-CoV-2 infection during controlled ovarian stimulation had no adverse effect on assisted reproductive technique outcome. Although they observed mild inflammation in the serum, this was absent in the follicular fluid of the subjects. Liprino et al. showed that phase angle is positively associated with low sperm quality. This confirms the reliability of phase angle as a marker of membrane integrity (23). Yu et al. provided a review on the role of epigenetics in female reproduction. They revealed that environmental toxicants impair female reproductive functions via the induction of epigenetic modification.

Summing up, this Research Topic provides interesting data, from experimental to clinical and meta-analysis, demonstrating the influence of environmental stress on human reproduction.

Author contributions

RA: Conceptualization, Investigation, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing.

Conflict of interest

The author declares 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 effects and mechanism of taxanes on chemotherapy-associated ovarian damage: A review of current evidence

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Chemotherapy is often a cause of premature ovarian insufficiency and infertility since the ovarian follicles are extremely sensitive to the effects of chemotherapeutic agents. Different chemotherapeutic agents with varying mechanisms of action may damage ovarian function differently. Taxanes are widely used in clinical cancer treatment, but the specific reproductive toxicological information is still controversial. This review described the impact and duration of taxanes on ovarian function in women and analyzed the possible reasons for different conclusions. Furthermore, the toxicity of taxanes on ovarian function and its possible mechanisms were discussed. The potential protective strategies and agents against ovarian damage induced by taxanes are also reviewed.

KEYWORDS

taxanes, chemotherapy, ovarian function, amenorrhea, fertility preservation

Introduction

The global burden of cancer has been increasing in recent years, showing a trend towards onset at a younger age. There were approximately 19.3 million new cancer cases worldwide in 2020, and the number is expected to reach 28.4 million by 2040 (1). Fortunately, with the development of early diagnosis and multimodality treatments, the survival rates of cancer patients have improved significantly (2). Therefore, increasing attention has been paid to the quality of survival and the risk of long-term sequelae. Chemotherapy-associated ovarian damage (CAOD) is a well-recognized sequela in

females with cancer. It has been associated with a higher incidence of premature ovarian insufficiency (POI), leading to delayed puberty, infertility, and disease associated with secondary chronic estrogen deficiency, such as osteoporosis, cardiovascular dysfunction, and Alzheimer's Disease (3, 4). These consequences contribute to poor health and affect psychological and social well (5). By 2025, approximately 100 million women will be at risk of CAOD worldwide (6). Hence, understanding the biological mechanisms of ovarian damage caused by chemotherapeutic agents and developing new preservation strategies becomes paramount.

Chemotherapeutic agents include alkylating agents, alkylating-like platinum complexes, anthracyclanes, taxanes, topoisomerase inhibitors and vinca alkaloids. As a broad-spectrum anticancer drug, taxanes are used as systemic chemotherapy drugs, such as in breast, ovarian, lung, bladder, and other types of solid tumors cancer treatment. Nowadays, the taxanes family can be divided into three groups: paclitaxel, the synthetic derivatives of paclitaxel (docetaxel and cabazitaxel), nanoparticle albumin-bound paclitaxel (7, 8). Taxanes alter multiple cellular oncogenic processes, including mitosis, angiogenesis, apoptosis, inflammatory response, and oxygen species (ROS) generation, which result in cell death (9).

The detrimental consequences of taxanes chemotherapy on ovarian function have long been documented in clinical and animal experiments. Assessing the specific gonadotoxicity induced by taxanes is challenging, as they are frequently used sequentially or concurrently with other gonadotoxic drugs in clinical. Early clinical studies showed that adding taxanes to anthracycline-based chemotherapy had no additional adverse effect on the menstrual cycle in women younger than 40 years old. In contrast, a large scales prospective cohort found that adding taxanes to anthracycline-based chemotherapy increased the probability of amenorrhea (10). Then, late-phase studies using precise indicators of ovarian reserve function reported evidence of ovarian toxicity induced by taxane-based combined chemotherapy (11, 12). In addition, two recent clinical studies showed that taxanes monotherapy had a strong ovarian toxic effect by assessing amenorrhea and serum steroid hormone levels in premenopausal patients (13–15).

Animal studies exploring paclitaxel's effects on CAOD also showed divergent results. Both in rats and mice models, a high dose of paclitaxel (7.5 mg/kg) treatment caused the depletion of primordial follicular reserve (16, 17). In contrast, some studies in rodent models reported no effects on the number of follicles at pre-antral stage after repeated administration of paclitaxel; only the antral follicles decreased significantly, indicating that paclitaxel's ovarian toxicity is mild and transient (18, 19). In addition, *in vitro* culture of neonatal mouse ovaries shows that docetaxel adversely affects early growing follicles (20). *In vitro* culture of early secondary follicles, high concentration paclitaxel had detrimental impacts on the dynamics of follicle development (21). Furthermore, oocytes exposed to paclitaxel showed defective spindle organization and aneuploidy formation, especially in MII stage oocytes (19). Overall, the effect of taxanes on ovarian function remains controversial; more in-depth studies are needed to clarify its ovarian toxicity in humans and animals.

The mechanisms behind CAOD induced by taxanes chemotherapy have been extensively investigated, but they remain largely unclear. There is some evidence in animal models proved that taxanes may damage ovarian function through abnormal cell division (22), oxidative stress (OS) (23), and caspase-dependent apoptosis (20). Based on the mechanisms of ovarian damage, some possible compounds have been proposed to prevent taxanes' gonadotoxicity (17, 19, 22, 23). This review aims to elucidate the impact of taxanes on ovarian function in clinical and animal studies. In addition, we also discussed the underlying mechanisms of taxanes-induced ovarian damage and the promising strategies for fertility preservation.

The effects of taxanes on female ovarian function

The results of taxanes on ovarian function are mostly reported in women with breast cancer. Taxanes, combined with other chemotherapeutics, is widely used for breast cancer and significantly improves the disease-free and overall survival rates (24). However, the ovarian function of patients receiving taxanes chemotherapy remains uncertain.

The effects of taxanes on levels of ovarian steroid hormone

Steroid hormonal and ultrasound tests, including anti-mullerian hormone (AMH), estradiol (E2), follicle-stimulating hormone (FSH), inhibin B, and antral follicle count (AFC), are commonly used to evaluate ovarian function (25). We summarized the published clinical studies on the effects of taxanes alone or combined with other chemotherapy agents on levels of ovarian steroid hormone.

The ovarian toxicity of taxanes monotherapy was best illustrated by its effects on ovarian function. Furlanetto et al. found that 57% of premenopausal patients with early breast cancer suffered from chemotherapy-induced ovarian failure (CIOF) after taxanes monotherapy, defined as FSH>12.4 IU/L, E2<52.2 ng/L, amenorrhea, and AMH level <0.22 ng/mL after treatment. The incidence of CIOF with taxanes alone was 55.6% at six months, 33.3% at 12 months, and 25% at two years. Furthermore, the median number of AFC was extremely low at the end of treatment and did not appreciably improve during two years of recovery (13). Together, the above results showed that taxanes monotherapy decreased ovarian reserve, but more studies are needed to confirm this conclusion.

Taxanes were often used in combination with other chemotherapy drugs, especially the anthracycline-based regiment, which includes AC (doxorubicin, cyclophosphamide), EC (epirubicin, cyclophosphamide), FAC (fluorouracil, doxorubicin, cyclophosphamide) and FEC (fluorouracil, epirubicin, cyclophosphamide) (26). Seven case-control trials about the changes of hormonal levels when patients received anthracycline based regimens with or without taxanes are presented in Table 1 (11, 12, 27–30). Notably, Reh et al. showed that when women with breast cancer of stages I-IIIA received AC or AC followed by paclitaxel treatment, there were no significant differences in serum FSH or E2 levels at a mean of 28 months after chemotherapy (27). Perdrix et al. reported that the median AMH levels were lower in patients who received FEC sequentially with docetaxel than in patients who received FEC chemotherapy alone in women below 35 years with early breast cancer, indicating the combined use of paclitaxel increased the risk of ovarian damage. Besides, the AMH level did not restore to the age-matched level even after three to five years (30). In all, there is no consensus on whether adding taxanes to the AC regimen leads to a worse damage to ovarian endocrine function.

Multiple reasons may cause the uncertainty of ovarian damage induced by taxanes. First, some clinical data do not provide clear information about the ovarian function of patients while receiving taxanes chemotherapy, which may have essential impacts on evaluation results. In addition, the sensitivity of the AMH detection assay needs to be considered. AMH levels in most studies were undetectable or close to the detection limits due to their limited sensitivity, which may result in no difference between different chemotherapy groups (11). A considerable AMH decline related to adding taxanes to the AC regimen by employing an automated AMH immunoassay method (30). Indeed, since there is no international standard for AMH, all related data should be interpreted cautiously. Second, the median age of patients was around 40 years, when the ovarian reserve was gradually decreasing and sensitive to chemotherapy damage (31). Lastly, heterogeneity varies between studies, including small population size, high missed follow-up rates, and scarce studies with taxanes alone. Therefore, extensive prospective studies are needed in the future to ascertain the impact of taxanes on ovarian reserve.

The effects of taxanes on female menstrual cycle

The incidence of chemotherapy-induced amenorrhea (CIA) was easy to record and commonly used as an endpoint to evaluate taxanes' reproductive toxicity. Iwamoto et al. reported that 69.4% of patients (138/199) with breast cancer of stages I-III had no menstrual cycle for at least six months after taxanes monotherapy (eight cycles of docetaxel or paclitaxel alone) (14). However, taxanes monotherapy is not a standard treatment, and most studies explored its ovarian function when taxanes were used in combination with chemotherapeutic agents. Ruddy et al. showed that breast cancer patients treated with adjuvant paclitaxel and trastuzumab had a relatively low amenorrhea rate (28%) in HER2-positive breast cancer patients at a median age of 44 years (32). The available data indicate that trastuzumab is unlikely to be gonadotoxic (33, 34); the ovarian toxicity induced by the combined regimen may come from paclitaxel. The incidence of CIA varied across different taxanes combination regimens such as anthracycline, epirubicin, and cyclophosphamide is shown in Table 2 (14, 15, 27–29, 33, 35–43). Petrek et al. evaluated the ovarian function in women with a history of breast cancer of stages I-IIIA who received the standard therapy of AC alone or in combination with paclitaxel or docetaxel. The CIA rate drastically increased one month after the treatment, ranging from 10% to 30%. Patients with an AC regimen had the lowest CIA rate, and adding docetaxel to the AC regimen had the highest CIA rate (10). Sukumvanich et al. recruited 245 women with stage I to III breast cancer and prospectively examined the CIA rates after chemotherapy. They observed that AC sequential paclitaxel regimens caused a higher CIA rate than AC regimens at six months of follow-up (45% vs 37%) (38).

There is conflicting evidence regarding taxanes aggravated the gonadotoxicity of other cytotoxic drugs. In Turnbull's study, patients with early-stage breast cancer showed no difference in CIA rates at a median follow-up of 60 months after anthracyclines-based or anthracyclines-taxane-based chemotherapy (36). A meta-analysis published in 2014 regarded that taxane-based regimens significantly increased the rate of CIA regardless of the definition of CIA (44). Another meta-analysis described that when anthracycline-based chemotherapy combined with taxanes was not related to a higher risk of CIA, but the level of evidence was weak (45). In the latest meta-analysis, Wang et al. reported that the addition of taxanes to anthracycline-based regimens would significantly increase the CIA rates with no heterogeneity and publication bias (46).

Based on the above studies, the effects of taxanes on the female menstrual cycle are unclear. Possible explanations for this discrepancy may be due to the following points. First, the definition of CIA and the follow-up duration varied across studies (Table 2). The incidence of CIA decreases as ovarian function gradually recovers, which may cause different results regarding taxane-induced ovarian damage. Han et al. followed patients' menstrual cycles for up to three years, and a higher CIA rate was observed in taxane-based regimens compared with non-taxane-based regimens within the first year. No significant differences were observed after the second year (47). Second, the reproductive toxicity of taxanes is affected by their types, duration, and dosage. For example, docetaxel appears to have higher ovarian toxicity than paclitaxel (10). Third, a higher CIA rate is associated with the increasing female age. Tham et al.

TABLE 1 The changes of female hormone levels with taxane-based chemotherapy.

Study	Breast cancer diganosis	Treatment regimen and Number of patients	Age (y)	Median follow up time	The level of serum steroid hormone with and without taxane use		
					AMH (*P* value)	E2 (*P* value)	FSH (*P* value)
Al-Rawi et al. (11)	I-III	4c AC→4c T (n=28) 4c AC (n=30)	39 (25-45)	End of treament (EOT)	AC→T (0.06 ng/ml); AC (0.06 ng/ml) P> 0.05	AC→T (6.88 pg/ml); AC (14.49 pg/ml) P< 0.016	N/A
Reh et al. (27)	I-IIIA	AC→T (n=6) AC (n=5)	40 (37-44)	28 (15-86)	N/A	AC→T (95.3 mIU/ml); AC (45.2 mIU/ml) P> 0.05	N/A
Yoo et al. (28)	I-III	4c AC→4c T (n=77) 4c AC (n=103)	43 (30-52)	6	N/A	E2↓ P=0.02	FSH↑ P=0.004
Arslan et al. (29)	N/A	AC→T (n=67) AC (n=19)	AC→T (34.8) AC (34.5)	AC→T 32 AC 25.4	N/A	AC→T (73.5 pg/ml); AC (39.5 pg/ml) P >0.05	AC→T (21.2 mIU/ml); AC (11.8 mIU/ml) P >0.05
Perdrix et al. (30)	N/A	3c FEC→3c D (n=45)	31.5 (11-35)	12	FEC→D (0.09 ng/ml); FEC (0.39 ng/ml) P=0.007	N/A	N/A
		6c FEC (n=9)					
Lambertini et al. (12)	I-III	3c FEC→3c D (n=127)	35.5 (31.5-38)	12	FEC→D (0.04 μg/L); FEC (0.22 μg/L) P=0.0006	N/A	N/A
		6c FEC (n=21)		36	FEC→D (0.18 μg/L) FEC (0.06 μg/L) P> 0.05	N/A	N/A

AC, anthracycline/cyclophosphamide; FEC, fluorouracil/epirubicin/cyclophosphamide; D/DTX, docetaxel; T/PTX, paclitaxel; c, cycle; N/A, not available; DPC, (Diagnostic Products Corporation, Los Angeles, CA).

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TABLE 2 The incidence of CIA with taxane-based regiment.

Study	Breast cancer diganosis	Treatment regimen (Number of patients)	Age(y)	CIA definition (m)	Follow-up (m)	The incidence of CIA with andwithout taxane use (%)	*P* value
Reh et al. (27)	I-IIIA	AC→T (n=28) AC (n=17)	N/A	≥6	6	AC→T (96); AC (82)	P > 0.05
					28	AC→T (35.7); AC (9.1)	P < 0.05
Yoo et al. (28)	I-III	4c AC→4c T (n=120) 4c AC (n=192)	43(30-52)	Long-term CIA ≥12 and not recovery	17.5	AC→T (64.2); AC (53.6)	P < 0.05
				Temporary CIA ≥3 and recovery	17.5	AC→T (34.2); AC (37.5)	P > 0.05
Najafi et al. (15)	I-IV	4c AC→4c T (n=75) 4c AC/6cFAC (n=111)	40(25-56)	≥3	36	AC→T (78.7); AC or CAF (66.7)	P < 0.05
Iwamoto et al. (14)	I-III	4c AC→4c T (n=90) 4c AC→4c D (n=105) 8c PTX (n=94) 8c DTX (n=105)	44.2(24-62)	≥6	60	AC→T (76.9); AC→D (75.2); PTX (62.8); DTX (75.2)	P > 0.05
Okanami et al. (35)	I-III	AC/FAC→T (n=49) AC/FAC (n=17)	37(26-40)	Without menstruation during chemotherapy	27.6	AC→T (93.9); AC (70.6)	P < 0.05
				Persistent CIA≥12	27.6	AC→T (24.5); AC (11.8)	P > 0.05
Abusief et al. (33)	Early stage	AC→T (n=203) AC (n=228)	43(25-55.6)	≥6	33	AC→T (56.6); AC (54.8)	P >0.05
Turnbull et al. (36)	Early stage	3c FEC→3cD (n=66) 3c FEC (n=41)	43 (35-50)	Without menstruation during follow-up	60	FEC→D (77); FEC (76)	P > 0.05
Davis et al. (37)	N/A	AC→T (n=43) FAC→T (n=4) CMF→T (n=6) AC (n=59) FAC (n=14) CMF (n=33)	40.8(18-50)	≥12	N/A	AC→T/FAC→T/CMF→T (43.4); AC/FAC/CMF (51.9)	P > 0.05
Sukumvanich et al. (38)	I-III	AC→T (n=143)	38.5(20-45)	≥6	6	AC→T (45.4); AC (37.4)	P > 0.05
		AC (n=111)			12	AC→T (29.4); AC (19.4)	P > 0.05
					24	AC→T (23.7); AC (15.1)	P > 0.05
Berliere et al. (39)	I-III	3c FEC→3c D (n=70) 6c FEC (n=84)	43.5(28-58)	Without menstruation during chemotherapy	End of treatment	FEC→D (93); FEC (92.5)	P > 0.05
Tham et al. (40)	N/A	4c AC→3m T (n=117) 4c AC (n=74)	≤50	≥6	N/A	AC→T (61); AC (44)	P < 0.05
Abdel-Razaq et al. (41)	I-III	4c AC→4c/12c T (n=13) 4c AC→4c D (n=10) 4c AC (18)	35.7(22-44)	≥12	≥36	AC→T (69.2); AC→D (66.7); AC (38.9)	P < 0.05
Arslan et al. (29)	N/A	AC→T (n=67) AC (n=19)	AC→T (34.8) AC (34.5)	Without menstruation during follow-up	AC→T 32 AC 25.4	AC→T (67.2); AC (42.1)	P < 0.05
Narmadha et al. (42)	I-III	6c FEC (n=8) 6c DEC (n=6) 6c FAC (n=28) 6c DAC (n=8)	40 (26-50)	≥3	N/A	DAC/DEC (100); FAC/FAC (75)	P < 0.05
Zhou L et al. (43)	I-III	4c FEC→4c/6cT(n=18) 6c FEC/FAC (n=85)	45.4 (26-57)	Without menstruation during follow-up	4	FEC→4c/6c T (61.1); FEC/FAC (70.6)	P > 0.05

AC, anthracycline/cyclophosphamide; FEC, fluorouracil/epirubicin/cyclophosphamide; FAC, fluorouracil/doxorubicin/cyclophosphamide; CMF, cyclophosphamide/methotrexate/fluorouracil; EC,epirubicin/cyclophosphamide; D/DTX, docetaxel; T/PTX, paclitaxel; c,cycle; N/A not available.

found that women younger than 40 who received AC sequentially paclitaxel therapy had a higher CIA rate compared with those receiving AC alone. However, these differences were not statistically significant in women older than 40 (40). Fourth, many patients were treated with tamoxifen for a long time, and the use of tamoxifen appears to be associated with higher rates of CIA (46). Consequently, the reported CIA rates may be overestimated. Last, CIA is also affected by the menstrual cycle phase when chemotherapy begins. Women had a higher incidence of CIA when patients received chemotherapy during the follicular development phase than in other phases (48).

The long-term impact of taxanes on gonadotoxicity should also be given attention. Some clinical evidence showed that taxanes-induced amenorrhea had a better recovery rate during long-term follow-up, suggesting that ovarian damage induced by taxanes may be temporary. In a recent study, approximately 66.7% of patients with breast cancer receiving taxanes monotherapy recovered from CIA, and more than 70% restored E2 and FSH after two years after treatment (13), which suggested that ovarian damage induced by taxanes may be temporary. This is probably because human follicles are periodic cycled; it takes approximately six months for dormant follicles to develop into ovulatory follicles (49). Another reason may be related to the rapid elimination of paclitaxel, with a mean elimination half-time only of 2.44 h (50). We summarized the percentage of menstruation restoration and the duration of amenorrhea in patients with or without the addition of taxanes chemotherapy in Table 3 (15, 27–29, 35, 36, 39, 40, 42, 43). From the table, we found that contradicting results exist for the recovery and duration of CIA induced by taxane-based chemotherapy. The recovery of ovarian function after chemotherapy means that women have a chance to become pregnant, but it is with regret that fertility is rarely evaluated in clinical trials. Hamy et al. reported that women treated with anthracyclines and cyclophophamide-based regimens were more likely to get pregnant than taxane-based regimens (51). In short, CIA induced by taxanes may be transient and long-term effects are relatively small, while further studies are needed to confirm this conclusion.

Animal studies about the effects of taxanes on ovarian function

Understanding taxanes' ovarian toxicity is essential for developing ovarian preservation approaches. Here, we summarized animal studies associated with the effects of taxanes on ovarian fertility and endocrine function.

The effects of taxanes on follicular quantity

Ovaries contain follicles of various stages, including primordial, primary, secondary, antral, and preovulatory follicles. The following data obtained in animal models demonstrated that taxanes significantly affect the number of follicles. A single intraperitoneal injection of high dosage paclitaxel at 7.5 mg/kg significantly decreased the number of primordial follicles after one week of exposure (16, 17, 23, 52). However, in some studies, paclitaxel decreased antral follicles and increased atretic follicles, but did not affect the number of primordial follicles (18, 19). The main concern regarding

TABLE 3 The percentage of menstruation restoration after taxane-based chemotherapy.

Study	Breast cancer diganosis	Treatment regimen and Number of patients	Age (y)	Follow-up (m)	Menstrution restoration (%)	*P* value
Yoo et al. (28)	I-III	4c AC→4c T (120) 4c AC (192)	43 (30-52)	17.5 (7.5-29.2)	AC→T (34.7); AC (41.1)	P > 0.05
Najafi et al. (15)	I-IV	4c AC→4c T (75) 4c AC or 6c FAC (111)	40 (25-56)	36 (12-120)	AC→T (49.1); AC or FAC (27.1)	P < 0.05
Reh et al. (27)	I-IIIA	AC→T (28) AC (17)	N/A	28.2 (15-86)	AC→T (64); AC (57)	P > 0.05
Okanami et al. (35)	I-III	4c AC→4c T (49) 4c AC (17)	37 (26-40)	27.6 (10.6-64.8)	AC→T (75.5); AC (88.2)	P > 0.05
Turnbull et al. (36)	Early stage	3c FEC→3c D (66) 6c FEC (41)	43 (35-50)	60	FEC→D (44); FEC (32)	P >0.05
Berliere et al. (39)	I-III	3c FEC→3c D (70) 6c FEC (84)	43.5 (28-58)	12	FEC→D (35.5); FEC (23.7)	P < 0.05
Narmadha et al. (42)	I-III	6c FEC (n=8) 6c DEC (n=6) 6c FAC (n=28) 6c DAC (n=8)	40 (26-50)	N/A	DEC/DAC (35.8); FEC/FAC (51.9)	P < 0.05
Tham et al. (40)	NA	4c AC→3m T (n=117) 4c AC (n=74)	N/A	N/A	AC→T (37.8); AC (29.2)	P > 0.05
Arslan et al. (29)	NA	AC→T (n=67) AC (n=19)	AC→T (34.8) AC (34.5)	AC→T 32 AC 25.4	AC→T (82.2); AC (100)	P < 0.05
Zhou L et al. (43)	I-III	6c FEC/FAC (n=85) 4c FEC→4c/6c TEC (n=18)	45.44 (26-57)	N/A	AC→T(27.8); AC (20.0)	P > 0.05

AC, anthracycline/cyclophosphamide; FEC, fluorouracil/epirubicin/cyclophosphamide;FAC, fluorouracil/doxorubicin/cyclophosphamide; CMF, cyclophosphamide/methotrexate/fluorouracil; EC,epirubicin/cyclophosphamide; D/DTX, docetaxel; T/PTX, paclitaxel; c,cycle; N/A not available; m, month.

ovarian damage is whether it significantly affects the primordial follicle pool because it is non-renewable, and may lead to POI. The loss of primordial follicles may attribute to direct injury to the primordial pool or an indirect outcome of the accelerated primordial follicle activation due to a major loss of mature follicles, known as the burnout model (53). In contrast, Nicosia et al. reported that mature follicles were more prone to damage induced by chemotherapy than immature follicles (54). A recent study also showed single or repeated intraperitoneal injection of high dosage paclitaxel at 30 mg/kg decreased the number of antral follicles without reducing primordial follicles; and the reduction only maintained for 1-2 estrous cycles, suggesting that the reproductive toxicity of paclitaxel was mild and transient (19). Similarly, *in vitro* intervention of neonatal mouse ovaries, docetaxel reduced early growing follicles without affecting primordial follicles (20). The possible reason is that taxanes act on actively dividing proliferating cells, and the growing follicle is in a stage of rapid development. Furthermore, the reduction in follicle number induced by taxanes was concentration-dependent both *in vitro* and *in vivo*, and was usually observed at high concentrations but not at low concentrations (16, 20). Based on the above results in animals, Taxanes can reduce the number of follicles *in vitro* and *in vivo*, but there is no consensus on which type.

The effects of taxanes on follicles quality

The damage of taxanes to ovaries can be further studied in animals by detecting involved indicators of follicle quality. Severe follicular damage was observed even at low-dose docetaxel (0.1μM) treatment *in vitro* (20). Granulosa cells of growing follicles are the first cellular target of docetaxel-induced follicular damage, and oocyte damage followed as a downstream consequence of granulosa cells compromised (55). Approximately 30% of abnormal transitional follicles and more than 80% of abnormal primary follicles were manifested as eosinophilic, shrunken, heterogeneous cytoplasm or condensed nuclear chromatin. In another study, mouse pre-antral follicles were treated with 10^{-10} M paclitaxel for five days *in vitro*, the follicular survival and growth were significantly suppressed, and no ovulation was observed. The follicle survival rates decreased by approximately 50% compared with controls, showing morphological abnormalities such as follicular constriction and oocyte extrusion. Furthermore, the expression of follicle development-relevant genes, growth differentiation factor 9 (GDF9), and bone morphogenetic protein 15 (BMP15) were also repressed by paclitaxel (56). Another study investigated the effects of paclitaxel on early secondary follicles of mouse and treated these follicles with 2.5×10^{-10}, 2.5×10^{-9}, and 2.5×10^{-8} M paclitaxel for 12 days (21). The results showed that high concentrations of paclitaxel inhibited the growth of secondary follicles, which is consistent with the above study by Kim et al. (56). Furthermore, a recent animal study demonstrated that a high concentration of paclitaxel affects the quality of MI and MII stage oocytes *in vitro*, with disordered spindle organization, decreased maturation, increased aneuploid oocytes, and lower fertilization rate (19).. Based on the above results, taxanes treatment damages ovarian granulosa cells and oocytes, leading to follicular death or aneuploidy.

The effects of taxanes on the ovarian stroma

Ovarian stroma, typically the supporting tissue of follicles, includes stromal cells, immune cells, blood vessels, lymphatic vessels, nerves, and extracellular matrix components (57). The ovarian stroma has adverse effects on the health of the ovarian reserve, affecting normal follicle development (58). Early in 2007, the ovarian cortex of chemotherapy patients exhibited blood vessel damage and fibrosis (59). Cyclophosphamide, busulphan and doxorubicin have been reported to cause vascular and stromal damage in the ovary (58, 60, 61), which might impair ovarian function. However, the effects of taxanes on ovarian stroma have been less studied by previous scholars. A study on the time accumulation of the drugs showed that doxorubicin accumulated first in the ovarian stroma's core because of its close relationship to the blood supply (62). Some clinical evidence revealed that young breast cancer patients receiving taxane-based chemotherapy had ovarian vascular damage, with decreased ovarian blood flow and reduction in ovarian size at the end of treatment (63). Then, the same group reported the continuous prospective evaluation of ovarian function in these patients. They indicated that ovarian toxicity might derive from acute ovarian vascular damage and the ovarian blood flow was partially restored at long-term follow-up (64). In another animal study, docetaxel negatively affected ovarian stromal cells with the high expression of apoptotic indicators, including cleaved caspase 3, cleaved caspase 8, Bax and cleaved poly (ADP-ribose) polymerase (20). Future studies are warranted to further assess the role of taxane-induced vascular toxicity in the ovary.

The effects of taxanes on ovarian endocrine and fertility

Endocrine and fertility, as two main functions of the ovary, need to be taken seriously when considering the impact of taxanes. Chen et al. reported that paclitaxel produced an inhibitory effect on basal progesterone (P4) and E2 in a dose- and time- dependent manner in porcine ovarian granulosa cells (65). Nevertheless, Tarumi et al. showed that the serum E2 level was slightly lower than that of the control group after repeated paclitaxel injections in rats. At the same time, there was no

difference in the P4 level (18). Furthermore, the mating experiment was performed to evaluate the consequence of taxanes on female fertility. Tarumi et al. showed that rats significantly decreased fetuses and implantations rate when mated immediately after administration. Still, these adverse effects were not detected when mated 24 days after administration (18). In another research, paclitaxel contributed to a decrease in pregnancy rates and an increase in stillbirths. Besides, the karyotypes of the offspring were normal, indicating that the damage of paclitaxel to the offspring is embryonic lethal (19). In addition, *in vitro*–fertilized (IVF) embryos culture found that paclitaxel treatment resulted in lower cleavage and blastocyst development rates of bovine embryos (66). Overall, paclitaxel disturbed the secretion of endogenous ovarian hormone and fertility, but the damage may be transient and reversible.

The mechanism of taxane-induced ovarian damage

The primary pharmacological mechanism of taxanes was to promote microtubule assembly and resist depolymerization (67). Early studies indicated that cancer cells treated with taxanes cannot establish a normal mitotic apparatus and were arrested in the late G2/M phase of the cell cycle (67, 68). Further studies revealed that taxanes influence multiple processes in cancer cells, including mitosis, apoptosis (69), oxidative stress (70), and inflammatory response (71). However, the mechanism of ovarian damage caused by taxanes is unclear; current studies have shown that abnormal cell division, follicular cell apoptosis, and oxidative stress might be involved in the damage to ovarian function induced by taxanes, as shown in Figure 1.

Taxanes cause abnormal cell division of follicular cells

The follicle is composed of an oocyte surrounded by granulosa and thecal cells. Oocytes complete their first meiotic division before ovulation with the extrusion of the first polar body, during which they are more vulnerable to chemotherapy. Oocytes can be divided into the germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) phase according to the difference in the nucleus (71). In a mice model that received paclitaxel intraperitoneally immediately after injection of human chorionic gonadotropin (HCG) to promote ovulation, Mailhes JB et al. found that paclitaxel blocked the meiosis of oocytes with increased MI phase oocytes, diploid oocytes and polyploid zygotes (72). Similarly, oocyte with abnormal meiotic status was observed with high concentrations of paclitaxel (30mg/kg) treatment in mice (19). In addition, GV stage oocytes are impervious to the harmful effects of paclitaxel because microtubules have not yet assembled into a specific format at this stage (73), and MI and MII oocytes are vulnerable to paclitaxel since their maturation relies on the assembly of microtubules and the spindle formation (74, 75). These results suggested that paclitaxel affects the

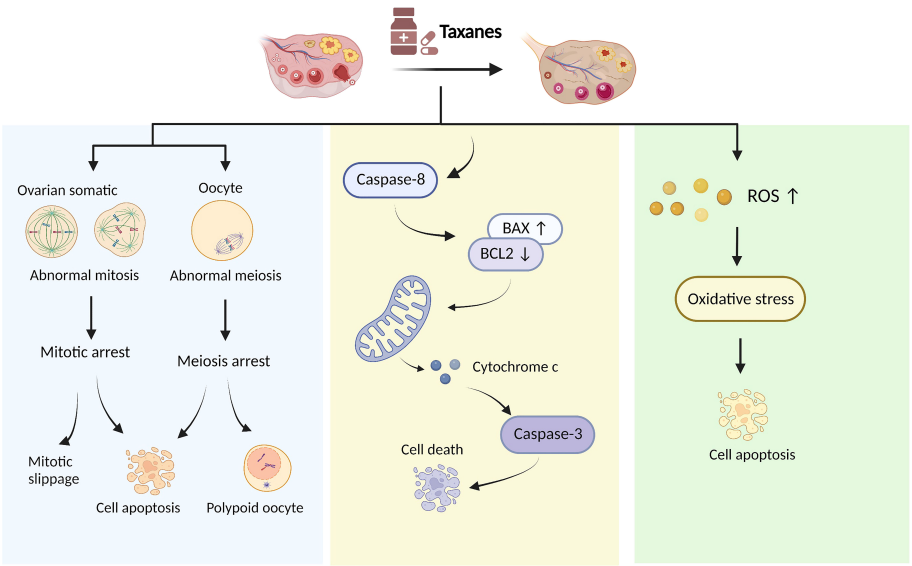

FIGURE 1
Proposed taxanes mechanisms of action in ovarian damage.

meiotic process of oocytes, especially in MI and MII oocytes. Ovarian granulosa cells are a kind of mitotically active cells located at the outermost layer of the follicle; thus, they are sensitive to chemotherapy. Cell cycle analysis has been performed on granulosa cells after 6, 12, 24, and 48 hours of paclitaxel treatment. These results showed that paclitaxel produced the characteristic G2 block at 12h (20%) and 24h (35%), which increased at 48h up to nearly 100%. In addition, the G2 cyclins A and B1, and their partner CDK1 were down-regulated at 48h paclitaxel exposure (22). Collectively, paclitaxel as a microtubule-targeting drug affects mitosis in granulosa cells and meiosis in oocytes.

Taxanes cause the apoptosis of follicular cells

Follicular apoptosis is a pivotal event in the depletion of follicles in chemotherapy-treated women (76, 77). Many studies have demonstrated that taxanes induce the apoptosis of follicular cells. Lopes et al. found that apoptosis-associated markers cleaved caspase 3 and cleaved caspase 8 expressed extensively in ovarian granulosa cells and stromal cells after low-dose docetaxel intervention *in vitro* (20). Moreover, the expression of anti-apoptosis genes Bcl2 and XIAP was significantly down-regulated in granulosa cells by the treatment with 10^{-10} M paclitaxel *in vitro* (56). XIAP is a known inhibitor of apoptosis protein 3 (IAP3), and overexpression of XIAP significantly improves the survival of pre-antral follicles (78). PI staining and TUNEL assay indicated a large proportion of cells underwent apoptosis after 48h of culture with paclitaxel in rat primary granulosa cells (22). Similarly, paclitaxel-induced follicular apoptosis was also observed in mice with high cleaved caspase 3 expression in granulosa cells (23). Lopes et al. indicated that docetaxel activated the mitochondria-dependent apoptotic pathway in ovarian granulosa cells, resulting in the upregulation of Bax and cytochrome C movement from mitochondria to the cytoplasm. The cytochrome C in the cytoplasm subsequently stimulated downstream effector caspases such as caspase 3, leading inactivation of cellular DNA repair followed by apoptosis (20). Indeed, paclitaxel-induced cell apoptosis was inextricably linked to cell cycle arrest. The checkpoint of mitotic spindle assembly and aberrant activation of cyclin-dependent kinases were shown to be involved in paclitaxel-induced apoptosis (79). Besides, G2 arrest of granulosa cells occurred after paclitaxel intervention for 12h, whereas apoptosis was evident only after 48h, indicating that apoptosis may be secondary to G2 arrest (22). Together, advances in apoptosis research have extended our understanding of the mechanisms of paclitaxel-induced follicular cell damage. However, the downstream biochemical events from paclitaxel's binding to microtubules that lead to follicular cell apoptosis are poorly understood.

Taxanes cause oxidative stress in ovaries

OS is a condition wherein pro-molecules, including ROS and nitrogen species (NOS), and antioxidant defence are out of balance. OS significantly negatively impacts ovarian cells and oocyte health (80). Excessive ROS accumulation leads to OS when ovaries are exposed to chemotherapeutic agents, γ-radiation, polycyclic aromatic hydrocarbons, or a poor lifestyle (80). For example, paclitaxel significantly induces mitochondrial ROS production by activating the STAT3 signalling pathway (81). ROS production is essential to paclitaxel cytotoxicity and is an early step before paclitaxel-induced cancer cell apoptosis (70). Qin et al. proposed that the loss of primordial follicles caused by paclitaxel may be related to OS. 4-hydroxynonenal (4-HNE), an established biomarker of OS, was significantly increased in oocytes and granulosa cells of paclitaxel-treated mice, especially in primordial follicles (23). These studies suggested that taxanes may generate oxidative metabolites, which increase OS and consequently triggers apoptosis in the ovary; however, it may be too early to draw a conclusion (82).

Protective approaches to ovarian damage during taxanes therapy

Some women still hope to have children after chemotherapies with the delay of first births and the trend of younger patients (83). Recently, there has been extensive discussion about preserving fertility and quality of life in tumor survivors. The American Society of Clinical Oncology (ASCO) recommends that individuals should seek fertility preservation before cancer treatment and update practice guidelines regularly (84–86). Here, we aim to review several ovarian protective strategies against paclitaxel-induced damage in the following text and Figure 2.

Oocyte, embryo, and ovarian tissue cryopreservation

Oocyte and embryo cryopreservation are currently considered standard practice according to the latest ASCO clinical practice guidelines, and ovarian cryopreservation is still an experimental method because of its immaturity (86). Compared to embryo cryopreservation, oocyte cryopreservation offers a better option for women without male partners. However, both techniques require ovarian stimulation to obtain oocytes, thereby delaying two weeks to six weeks in chemotherapy initiation. Fortunately, the problem is expected to be resolved. Von Wolff et al. developed an ovarian stimulation strategy in that patients received GnRH antagonists and recombinant FSH irrespective of the stage of the menstrual cycle, allowing oocyte collection within two weeks (87). Nowadays, random start ovarian stimulation is becoming more

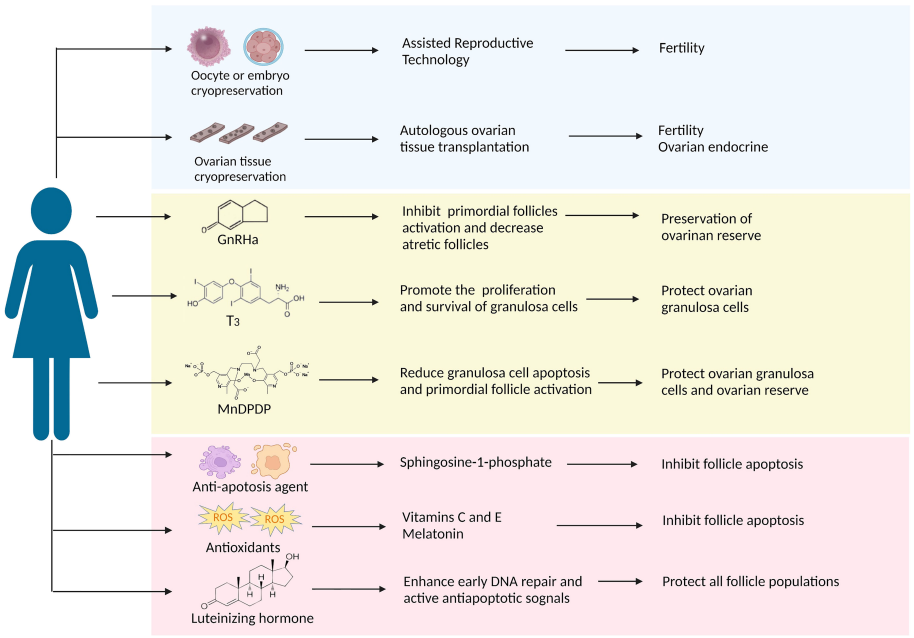

FIGURE 2
A summary of the protective strategies applied in clinical practice or have future potential against paclitaxel-induced ovarian damage. The approach discussed in this article is divided into three parts: the blue background box shows the methods that have been applied in clinical practice; the yellow background box shows potential strategies that have been proved to target taxane-induced ovarian damage in laboratory studies; the pink background box shows the potential strategies as presumed by the potential causative mechanism that can resist chemotherapeutic agents including taxanes.

popular, and it does not appear to delay the onset of chemotherapy (88). Besides, it may increase the potential risk of hormone-sensitive tumors with short-term exposure to high estrogen levels, although there is no clear evidence (89). The advantage of ovarian tissue cryopreservation is restoring ovarian endocrine function after transplantation (90) and not requiring ovarian stimulation. In a meta-analysis, Pacheco et al. autologous included nineteen studies thought that autologous ovarian tissue transplantation (OTT) showed higher reproductive performance, the rates of live birth and ongoing pregnancy were 37.7%, and the recovery rate of endocrine was 63.9% (91). Many experts believe that adequate evidence currently exists to support the use of OTT as a feasible and valid technique and will become standard therapy within the next few years (92). Following ovarian tissue transplantation, the most significant concern would be the potential for the re-introduction of cancer cells, especially hematological malignancies. Therefore, ovarian tissue screening should be performed to detect cancer cells, and patients at high risk of ovarian involvement should be cautiously selected for ovarian tissue cryopreservation. Altogether, these advances in oocyte, embryo, and ovarian tissue cryopreservation have contributed to fertility preservation for cancer patients before chemotherapy. Regrettably, there are no relevant reports about the above methods of fertility protection before and after paclitaxel therapy.

Gonadotrophin-releasing hormone analogs

GnRH analogs (GnRHa), comprising agonists and antagonists, produce a similar decline in GnRH secretion through different pathways (93). GnRHa inhibit the secretion of gonadotropins and prevent follicles development through the HPO axis, reducing chemotherapeutic drugs' damage to actively growing follicular cells (94). The underlying mechanism of ovarian protection may be the reduction of ovarian blood perfusion induced by GnRHa, thereby reducing chemotherapeutic drug accumulation (95). In addition, GnRHa also reduce cell apoptosis by directly activating ovarian GnRH receptors or indirectly on peripheral cumulus cells (96). Another revolutionary speculation is that GnRHa protect undifferentiated germline stem cells and eventually generate *de novo* primordial follicles (75). Many clinical studies showing the positive effect of GnRHa on ovarian function in women with malignancies who received chemotherapy (97–99), but do not prove in statistically in the meta-analysis that GnRHa combined with chemotherapy reduces gonadal toxicity due to conflicting results and substantial heterogeneity (100–102). GnRHa are not recommended as a preferred alternative to proven fertility preservation methods, such as oocyte and embryo cryopreservation, according to ASCO (86) and ASRM guidelines (103).

An RCT study showed a significant reduction in early menopause rates when patients received intramuscular triptorelin at a dose of 3.75 mg at least one week prior to chemotherapy, including taxane-based regimens, and then repeated every four weeks during chemotherapy (104). It is worth noting that GnRHa needs to be injected 1-2 weeks before chemotherapy; delaying chemotherapy may lead to deterioration of the disease process and possibly to rapid recurrence. Many animal studies have demonstrated that GnRH agonists protect against paclitaxel-induced ovarian damage *in vitro* and *in vivo*. GnRH agonists (2.5 μg/d) were injected in advance for 28 days in rat models until ovarian suppression, and a single dose of paclitaxel (7.5 mg/kg) was administered. Follicle counts indicated that primordial follicles could be preserved after paclitaxel chemotherapy (17). In another experiment, GnRH agonists effectively suppressed follicle maturation and decreased atretic follicles during paclitaxel chemotherapy. Furthermore, GnRH agonists shorten the time of paclitaxel-induced MII oocyte damage persisted (19). In addition, GnRH agonists protect the ovary from docetaxel-induced damage with the reduction of the total follicle and double-strand DNA breaks (105). Overall, given the current evidence, GnRHa is the most promising drug for taxane-associated chemotherapy damage.

Thyroid hormone

Thyroid hormones (THs), including L-triiodothyronine (T3) and L-tetraiodothyronine (T4), play an indispensable role in human growth and development (106). The cross-talk between the hypothalamic-pituitary-gonadal (HPO) axis and the hypothalamic-pituitary-thyroid (HPT) axis is vital in ovarian function (107). Abnormalities in the thyroid hormone can adversely affect female reproduction, causing menstrual disorders and infertility (108). T3 might have a direct role in ovarian physiology *via* its receptors that promote the proliferation and survival of ovarian granulosa cells (109, 110) and the development of pre-antral follicles (111, 112). About 40-60% of cells died after paclitaxel intervention by MTT and TUNEL assays, and T3 supplementation significantly reduced this ratio in rat primary granulosa cells. Besides, T3 could effectively overcome paclitaxel-induced G2 phase arrest, protecting granulosa cells from apoptosis and maintaining cell viability (22). Based on the above studies, thyroid hormone T3 is able to protect ovarian granulosa cells from paclitaxel-induced apoptosis, while more experiments need to be conducted for further verification.

Mangafodipir

Mangafodipir (MnDPDP) is a chelate of a paramagnetic manganese (II) ion and of the ligand fodipir (DPDP, a vitamin B6 derivate), a superoxide dismutase (SOD) mimetic with peroxidase and glutathione reductase activities, which plays a role in multiple stages of the ROS cascade, protecting cells from H_2O_2-induced apoptosis (113, 114). So far, several studies have demonstrated that MnDPDP is helpful for some diseases caused by oxidative damage or oxidative damage resulting from certain drugs or physical therapies, such as adjuvant cancer chemotherapy, acute myocardial infarction, and liver ischemia-reperfusion injury (115). Qin et al. proposed that MnDPDP might ameliorate ovarian injury caused by paclitaxel-induced oxidative stress. They confirmed that MnDPDP could partially reduce paclitaxel-induced granulosa cell apoptosis and primordial follicle activation *via* its SOD activity without affecting the antitumor activity of paclitaxel (23). But due to the limited studies, the protective effects of MnDPDP on ovarian function during chemotherapy are unclear.

Other candidates

Based on the mechanism of ovarian damage induced by taxanes, both antioxidants and anti-apoptotic agents are expected to play a protective role in ovarian function. Sphingosine-1-phosphate (S1P) is a metabolite of cell membrane sphingolipids that, as an anti-apoptotic agent, protects cells from ceramide-induced apoptosis (116). S1P has been shown to protect the ovary and preserve fertility from radiation and chemotherapy in mice and human ovary tissue *in vitro* (117, 118). Luteinizing hormone (LH), a steroid hormone that plays a cardinal role in follicular development and ovulation, has been proven to protect the ovarian reserve and ameliorate fertility during alkylating agents chemotherapy by generating anti-apoptotic signals and favoring DNA repair pathways in mice (119, 120). Further research found that some antioxidants, including vitamins C and E, melatonin, N-acetylcysteine, and coenzyme 10, also show ovarian protection during chemotherapy. Among them, melatonin, as a powerful antioxidant, prevents the loss of cisplatin-induced primordial follicles by inhibiting the overactivation of primordial follicles (121). Future explorations are needed to demonstrate their effectiveness in taxane-induced ovarian damage.

Conclusion and future prospects

Based on the available evidence, the exact influence of taxanes on ovarian function in clinical is still uncertain because taxanes are frequently combined with other cytotoxic agents. Relevant animal studies reveal that taxanes affect the quantity and quality of follicles, leading to endocrine disruption and adverse fertility consequences. The taxanes-induced ovarian damage is closely associated with abnormal cell division, follicular cell apoptosis, and ROS accumulation. Targeted strategies may protect ovarian function during taxanes chemotherapy, while future explorations are needed to demonstrate the effectiveness. Although there are still many unknown and unclear problems to be solved about the

taxanes' reproductive toxicity, it is expected to provide some ideas for developing fertility preservation strategies in the future.

Author contributions

MW and SXW conceived of the idea. CW performed the literature investigation and wrote the original draft of the manuscript. TW, DC, SMW, WT and LX designed the figures and table. JX, YH, YG and YC contributed to figure design and visualization. MW and SXW revised the manuscript. All authors contributed to the article and approved the submitted version.

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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. All authors contributed to the article and approved the submitted version.

Publisher's note

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Association of blood lead with estradiol and sex hormone-binding globulin in 8-19-year-old children and adolescents

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Background: Metals can interfere with hormonal functioning through indirect mechanisms and by binding at the receptor site; thus, they may be associated with hormonal changes. However, there have been few studies on the health impact of metal exposure among children and adolescents. Thus, we aimed to examine the associations of blood lead level (BLL) with estradiol (E2) and sex hormone-binding globulin (SHBG) among children and adolescents aged 8–19 years in the National Health and Nutrition Examination Survey (NHANES) database.

Methods: This was a cohort study of 2188 individuals from the NHANES. BLL was taken as independent variables, E2 and SHBG as dependent variable. We conducted weighted multivariate linear regression models and smooth curve fittings to evaluate the association between them.

Results: The BLL was significantly positively associated with serum SHBG level in females, especially when the LnBLL quartiles are between Q3 and Q4. There was an inverted U-shaped association between BLL and E2 with the point of inflection at 1.86 μg/L and a U-shaped association between BLL and SHBG with the point of inflection at 1.86 μg/L in female adolescents aged 16-19 years. Meanwhile, In males, there was a positive trend of correlation between BLL and E2 in the 8-11 years, and 16-19 years groups.

Conclusions: This study found an inverted U-shaped association of BLL with E2 and a U-shaped association between BLL and SHBG in female adolescents aged 16-19 years. This indicates that adjusting blood lead exposure to mitigate the effects of lead on growth and development is important for adolescents aged 16-19 years. Controlling the BLL below 1.86 μg/L may minimize the damage to E2.

KEYWORDS

blood lead, estradiol, SHBG, children, adolescents

1 Introduction

Estrogen (E) plays an important role in hormonal regulation in women (1). It maintains and promotes female secondary sexual characteristics and the function of gonads and serves as an important biomarker for the onset of puberty, menstrual status, and fertility (2). There are three types of estrogens: estrone (E1), estradiol (E2), and estriol (E3). Of these, E2 is the most physiologically relevant and has the highest affinity for estrogen receptors ERα and ERβ (3). In particular, E2 is directly related to menarche and helps breast maturation in puberty (4, 5). In males, E2 is also essential to reproductive function, body composition, and glucose metabolism (6). Furthermore, an experiment in female mice showed that E2 as a pubertal hormone is essential for the maturation of the frontal cortex, which regulates many cerebral functions, including executive functions, language, temporal integration, emotional behavior, and working memory (7). One study showed that E2 had a positive association with total testosterone (TT) in males, which is the most important male reproductive hormone (8).

Sex hormone-binding globulin (SHBG) is produced in the liver. As a transport carrier that binds E2 and regulates its biological activity, SHBG is thought to reflect metabolic levels and regulate the plasma levels and bioavailability of E2 (9, 10). E2 is tightly bound to SHBG in the circulation, and only a small free fraction is considered biologically active (11). E2 and SHBG have been shown to be influenced by various factors, such as insulin, thyroid hormone, and environmental endocrine disruptors, in which heavy metals like lead play an important role (12–14). Poisoning caused by lead, one of the ten most harmful metals listed by the World Health Organization (WHO), is a serious public health concern (15). Blood lead level (BLL) can be a homeostatic marker of lead exposure (16).

Lead exposure is unavoidable, and its sources include drinking water, contaminated soil particles, chemical industry, and mining (17–19). Among children and adolescents, lead exposure even at low levels can induce neuropsychological deficits in cognition, attention, behavior, intelligence, and memory (20). Blood lead has E receptor activity and has endocrine-disrupting properties even at low concentrations (21). Some animal experiment and child studies have shown that lead exposure may influence pubertal development through the hypothalamic–pituitary–gonadal axis (22, 23). In addition, children and adolescents are at the age when they are most sensitive to the adverse health effects of endocrine disruptors (24). Further, serum E2 and SHBG levels in puberty children are closely associated with growth and development. Thus, understanding the mechanism by which blood lead influences variations in E2 and SHBG levels may provide additional insight and opportunities for prevention lead exposure. However, there are few correlational studies on the effects of lead exposure on reproductive hormones in children and adolescents aged 8-19 years. Thus, the present study used BLL as the independent variable and E2 and SHBG as the dependent variables to determine the association of blood lead with E2 and SHBG in the general population of 8-19-year-old children and adolescents in the United States. Further, we explored the possible mechanisms of action of this association to provide evidence for avoiding and preventing diseases (e.g. delayed menarche, impaired gonadal development) and improving child-related health worldwide.

2 Materials and methods

2.1 Data source and study population

Data were collected from National Health and Nutrition Examination Surveys (NHANES), a large data registry of health and nutritional status of nationally representative samples in the United States. Information is available online (http://www.cdc.gov/nchs/nhanes.htm).

According to the guidelines published by the WHO in 2017, adolescents have been most recently defined as those aged 10-19 years. In this study, we selected children and adolescents aged 8-19 years in whom E2 and SHBG are the most associated with growth and development, as the study population (25). Moreover, we selected these two data cycles (2013-2014, 2015-2016) because the data on E2 for children and adolescents aged 8-19 years were only available for these two periods in data from 2011 to 2020. We screened 20146 participants from NHANES 2013-2016; of them, 4503 children and adolescents aged 8-19 years were included. After excluding those with missing BLL (n=1924), E2 (n=143), and SHBG (n=248) data, we finally enrolled 2188 eligible participants. The participant selection process is shown in Figure 1. The home interviews assessed the family and sample person demographics and other aspects at home. Adolescents aged over 16 years and emancipated minors were interviewed directly, while participants aged <16 years and those unable to answer questions themselves were provided with information by proxy.

All surveys have obtained National Center for Health Statistics (NCHS) Research Ethics Review Board (ERB) approval. The protocol numbers are available at https://www.cdc.gov/nchs/nhanes/irba98.htm, as accessed on January 4, 2023.

2.2 E2, SHBG, and BLL measurements

The level of serum E2 (all unconjugated, including free and protein-bound forms) was measured by quantitative analysis using isotope dilution high-performance liquid chromatography tandem mass spectrometry with stable isotope labeled internal standards and external calibrators (https://wwwn.cdc.gov/nchs/data/nhanes/2015-2016/labmethods/TST_I_MET_TST_EST.pdf). Meanwhile, the level of SHBG (bound and free types) in human serum and plasma matricesl was determined using the test based on the reaction of SHBG with immuno-antibodies and chemo-luminescence measurements of the corresponding reaction products (https://wwwn.cdc.gov/nchs/data/nhanes/2015-2016/labmethods/TST_I_MET_SHBG.pdf).

For BLL measurement, whole blood samples were stored frozen (-30° C) and then shipped to National Center for Environmental Health for testing. The BLL was quantitatively determined using inductively coupled plasma-dynamic reaction cell-mass spectrometry (https://wwwn.cdc.gov/nchs/data/nhanes/2015-2016/labmethods/PBCD_I_met.pdf).

2.3 Covariates

Covariates are variables that can affect the regression coefficient of the exposure variable by more than 10% when introduced into the

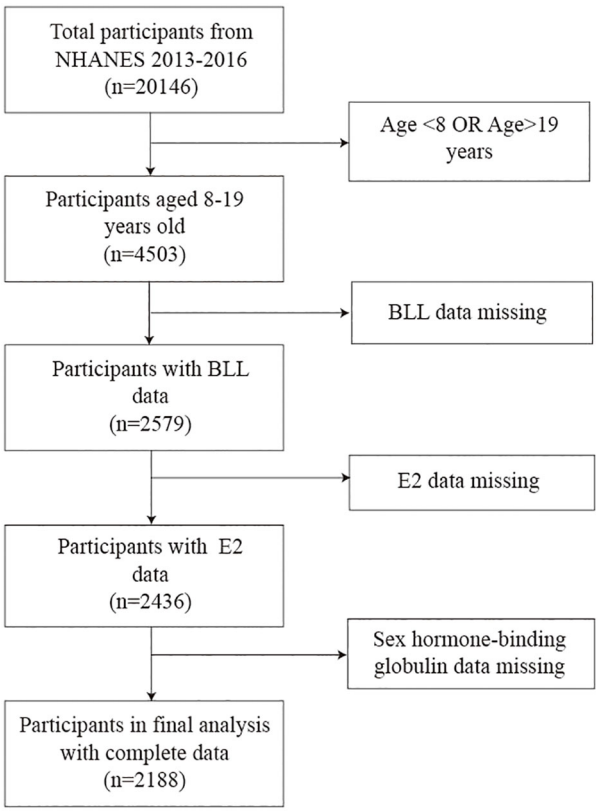

FIGURE 1
Flow diagram illustrating the process of participant selection. BLL, Blood lead levels; E2, Estradiol; SHBG, Sex hormone-binding globulin.

basic model or excluded from the full model. Based on previous studies (20, 21), we finally selected the following variables as possible confounders on the relationship between exposure and outcome variables: age, sex, race/ethnicity, ratio of family income to poverty; total energy, cholesterol, iron, and zinc intake on the first day, fish eaten during the past 30 days; moderate recreational activities; body mass index; haematocrit, serum cotinine; serum albumin, serum copper, and serum zinc. BLL is negatively correlated with HCT, and HCT-adjusted whole blood lead may be a better biomarker of lead hematotoxicity than the unadjusted BLL (26). As the primary metabolites of nicotine, serum cotinine can be used as markers for active smoking and as indices for secondhand smoke exposure, which is important in children and adolescents who are often passive recipients of tobacco. Dietary cholesterol was included because serum cholesterol may affect the binding of SHBG to E2. In addition, metal intake and data related to serum metal levels were also analyzed as several metals are known to have an association with reproductive hormones. Detailed interview and laboratory procedures of the above covariates are available on the website.

2.4 Statistical analysis

Considering the presence of missing values for covariates such as serum copper, serum zinc, and albumin, we used multiple interpolation to reduce the possible bias caused by missing values and the loss of test efficacy. Multiple linear regression analysis was performed on each of the five sets of random data formed by multiple interpolation, and the regression coefficients and standard errors of the five regression models were combined and tabulated. To investigate the effects of different levels of confounders, we constructed three multiple linear regression models as follows: Model 1 was the unadjusted model; model 2 was adjusted for the confounders of age, sex, and race/ethnicity as the minimally adjusted model; and model 3 was adjusted for all confounders associated with them as the fully adjusted model. Given the left-skewed distribution of blood lead, serum estradiol, and SHBG among the 8-19-year-old participants, they were ln-transformed to LnBLL, LnE2, and LnSHBG, respectively. Accordingly, serum copper, zinc, and albumin; total iron and zinc intake on the first day all underwent the same manipulation.

For the comparison of baseline data in different blood lead quartile groups, we used weighted linear regression models to calculate continuous variables and a weighted chi-square test to calculate categorical variables. Besides, multiple linear regression equations were used to explore the relationship between LnBLL and LnE2 or between LnBLL and LnSHBG, with three linear regression models for continuous variables (details of adjustment of confounders for each model in Supplementary Tables 1, 2) and chi-square tests for categorical variables achieving stratified analyses and *post hoc* subgroup comparisons. Furthermore, trend tests were performed for the LnBLL quartile grouping to enhance the strength of the evidence and the sensitivity of the test, and the results were considered positive when *P* for trend was also significant.

For the nonlinear relationship between the independent and dependent variables, we used threshold effect analysis to find the inflection points of the quantitative-effective relationship curves between LnBLL and LnE2, or LnBLL and LnSHBG in different age groups (27, 28). Therefore, we divided the participants into three age groups: 8 to 11 year-old age group, 12 to 15 year-old age group and 16 to 19 year-old age group. They consisted of 1152 people (576 males and 576 females), 550 people (269 males and 281 females), and 486 people (238 males and 248 females), respectively. The fitted model with the largest likelihood value was found by recursive experimental method, and the results were considered significant if the *P*-value was <0.05 in the log-likelihood ratio test, which indicated that there was an inflection point in the smooth fitting curve. Two-piecewise linear regression was then performed to further test the turning effect (27). All statistical analyses were performed using the Empower software (www.empowerstats.com; X&Y solutions, Boston MA), R version 3.4.3 (http://www.R-project.org, R Foundation) with weighted processing.

3 Results

3.1 Participant characteristics

There were significant differences in age, sex, race/ethnicity, ratio of family income to poverty, moderate recreational activities; total energy, iron, and zinc intake on the first day; fish eaten during the past 30 days; body mass index; hematocrit; serum cotinine; serum copper; serum zinc; estradiol; and SHBG between the different blood lead quartile subgroups. The results of the weighted characterization of the study population are presented in Table 1.

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3.2 Association of LnBLL with LnE2 and LnBLL with LnSHBG

Overall, the relationship between BLL and E2 is detailed in Supplementary Table 1, and results by age group are presented in Table 2. After stratifying the data by race/ethnicity, we found a significant negative association between BLL and E2 only among non-Hispanic Black and Mexican American participants in the unadjusted model (Model 1). However, no significant association was found after adjusting for covariates (Models 2 and 3). After sex stratification, there was a significant trend of varying LnE2 in males and females for each increase in LnBLL (*P* for trend <0.05) in model 3. This association was positive in males and negative in females (Figure 2) and was especially significant in females (*P* for trend <0.001 in all three models).

When stratified by age and adjusted for all covariates, we found an increase in LnE2 to varying degrees with LnBLL quartiles between Q2 and Q4 for male participants in all age groups compared with that in the lowest quartile group of LnBLL. There was a significant correlation between LnBLL and LnE2, especially a significant linear trend in the 8-11-year-old group (*P* for trend <0.001) and 16-19-year-old group (*P* for trend=0.006) (Table 2). In contrast, the association did not reach statistical significance for linear correlation among female children and adolescents in each of the three age groups.

TABLE 1 Characteristics and statistical description of children and adolescent participants aged 8-19 in NHANES 2013–2016 (n=2188).

	Blood lead level (μmol/L)					Pvalue
	Total	Q1 (0.002~0.016)	Q2 (0.017~0.024)	Q3 (0.025~0.035)	Q4 (>0.036)	
Age (years)	12.7 ± 3.4	13.4 ± 3.2	12.8 ± 3.4	12.3 ± 3.3	12.2 ± 3.6	<0.0001
8 to 11 years old	9.5 ± 1.1	9.7 ± 1.1	9.5 ± 1.1	9.6 ± 1.2	9.3 ± 1.1	<0.0001
12 to 15 years old	13.5 ± 1.1	13.7 ± 1.1	13.5 ± 1.1	13.5 ± 1.0	13.2 ± 1.1	<0.0001
16 to 19 years old	17.3 ± 1.1	17.1 ± 1.1	17.4 ± 1.1	17.4 ± 1.1	17.6 ± 1.2	<0.0001
Sex (%)						<0.0001
Male	50.2	33.7	43.7	58.2	67.3	
Female	49.8	66.3	56.3	41.8	32.7	
Race/ethnicity (%)						<0.0001
Non-Hispanic White	53.4	52.7	56.6	51.7	51.7	
Non-Hispanic Black	12.2	8.4	9.2	14.9	16.9	
Mexican American	16.3	22.0	15.1	16.4	12.2	
Other race/ethnicity	18.1	16.9	19.1	17.0	19.2	
Ratio of family income to poverty	2.3 ± 1.5	2.6 ± 1.6	2.5 ± 1.5	2.3 ± 1.5	1.9 ± 1.3	<0.0001
Moderate recreational activities (%)						<0.0001
Yes	28.9	27.0	34.7	29.0	23.2	
No	24.3	35.4	21.4	18.6	22.5	
Not recorded	46.8	37.7	43.9	52.4	54.3	
Total nutrient intake on the first day–Energy (kcal)	2017.7 ± 882.2	1985.5 ± 856.9	1965.2 ± 780.9	2019.7 ± 942.5	2117.5 ± 955.4	0.0248
Total nutrient intake on the first day–Cholesterol (mg)	241.3 ± 199.0	246.7 ± 214.5	237.9 ± 179.9	228.1 ± 209.4	254.0 ± 193.9	0.1776
Total nutrient intake on the first day–Iron (mg)	14.9 ± 9.5	15.0 ± 9.3	14.3 ± 8.8	14.6 ± 8.3	16.0 ± 11.6	0.0169
Total nutrient intake on the first day–Zinc (mg)	10.8 ± 7.7	10.7 ± 6.8	10.4 ± 6.9	10.4 ± 6.6	11.9 ± 10.1	0.0032
Fish eaten during the past 30 days (%)						<0.0001
Yes	40.4	38.4	38.0	39.9	46.1	
No	59.4	52.2	55.8	50.3	40.1	
Not recorded	9.2	9.3	6.2	9.7	13.8	
Body mass index (kg/m^2)	22.1 ± 5.9	23.5 ± 6.7	22.5 ± 6.0	21.5 ± 5.3	20.7 ± 5.2	<0.0001
Hematocrit (%)	40.5 ± 3.3	39.9 ± 3.1	40.7 ± 3.3	40.6 ± 3.4	40.7 ± 3.4	0.0001

(Continued)

TABLE 1 Continued

	Blood lead level (μmol/L)					*P*value
	Total	Q1 (0.002~0.016)	Q2 (0.017~0.024)	Q3 (0.025~0.035)	Q4 (>0.036)	
Serum cotinine (ng/mL)	5.8 ± 37.9	1.6 ± 15.5	3.7 ± 32.0	5.1 ± 30.0	13.5 ± 60.8	<0.0001
Serum albumin (g/L)	45.2 ± 3.0	45.0 ± 2.8	45.3 ± 3.1	44.9 ± 2.9	45.6 ± 2.9	0.0599
Serum copper (umol/L)	17.3 ± 4.0	18.1 ± 4.6	17.3 ± 3.6	16.8 ± 3.9	17.0 ± 3.7	0.0011
Serum zinc (umol/L)	12.5 ± 2.2	12.2 ± 2.1	12.8 ± 2.2	12.7 ± 2.4	12.4 ± 2.2	0.0238
Estradiol (pg/mL)	31.5 ± 54.4	47.7 ± 73.5	34.0 ± 51.1	23.8 ± 39.9	19.9 ± 43.0	<0.0001
SHBG (nmol/L)	69.1 ± 47.3	68.2 ± 55.4	63.7 ± 41.0	69.6 ± 44.9	76.6 ± 47.5	0.0001

Mean ± SD for continuous variables like age, ratio of family income to poverty; total energy, cholesterol, iron and zinc intake on the first day; body mass index, hematocrit, serum continine, serum albumin, serum copper, serum zinc, estradiol, SHBG. P value was calculated by weighted linear regression model.
% for Categorical variables like race/ethnicity, moderate recreational activities, fish eaten during the past 30 days. P value was calculated by weighted chi-square test.
SHBG, Sex hormone-binding globulin.

Smoothed curve fit plots (Figure 3) stratified by age supported the results.The multiple linear regression models for the association of BLL with SHBG are shown in Supplementary Table 2, as well as the results by age group in Table 3. BLL and SHBG were positively associated in model 2 (adjusted for sex, age, and race/ethnicity) (P <0.05), while no significant association was observed in neither model 1 (unadjusted model) nor 3 (fully adjusted model). After stratification by race/ethnicity, the association between BLL and SHBG was significantly positive among non-Hispanic Black and Mexican American populations. However, after adjusting for all covariates, this association was not observed across races. The results after sex stratification showed a significant relationship between LnBLL and LnSHBG for both sexes in each model (P for trend <0.001), indicating that LnSHBG tended to significantly increase with every quartile increase in LnBLL. Further, we found a more significantly positive correlation in female participants with LnBLL quartiles between Q3 and Q4, demonstrating a significant difference in all three models.

When stratified by age and sex (Table 3), we found that LnBLL and LnSHBG were correlated in male children and adolescents of all ages, with significant results as seen in trend tests; particularly, LnSHBG significantly increased with LnBLL in the Q4 range compared to LnSHBG in the lowest LnBLL quartile group (8-11-year-old group, 0.0510 [0.0055, 0.0965]; 12-15-year-old group, 0.0806 [0.0084, 0.1527]; and 16-19-year-old group, 0.0694 [0.0049, 0.1340]). Moreover, among male adolescents aged 16-19 years, we found a decrease in LnSHBG in the group with LnBLL of Q2 compared to that in group with LnBLL of Q1. Meanwhile, the relationship between LnBLL and LnSHBG was significant in female participants aged 8-11 years and 16-19 years. When LnBLL were between Q3 (0.2767 [0.1895, 0.3640]) and Q4 (0.2638 [0.1596, 0.3681]), LnSHBG

TABLE 2 Association between LnBLL (μmol/L) and LnE2 (pg/mL) Stratified by race/ethnicity and sex.

LnBLL (μmol/L) (Quartile)	LnE2 (pg/mL)		
	8 to 11 years old	12 to 15 years old	16 to 19 years old
Male			
Q1	Reference	Reference	Reference
Q2	0.0868 (0.0460, 0.1277) ***	1.7147 (0.4118, 3.0176) *	0.2173 (0.1510, 0.2837) ***
Q3	0.0430 (0.0037, 0.0823) *	3.0914 (1.7980, 4.3847) ***	0.1553 (0.0871, 0.2235) ***
Q4	0.1073 (0.0673, 0.1473) ***	1.6038 (0.2687, 2.9388) *	0.1560 (0.0906, 0.2215) ***
P for trend	<0.001	0.326	0.006
Female			
Q1	Reference	Reference	Reference
Q2	-0.0788 (-0.1760, 0.0183)	-3.3109 (-11.8286, 5.2069)	0.0795 (-0.0585, 0.2175)
Q3	-0.0756 (-0.1791, 0.0278)	-16.4467 (-27.4173, -5.4762) **	-0.0019 (-0.1705, 0.1668)
Q4	-0.0632 (-0.1739, 0.0476)	-3.1587 (-16.2987, 9.9813)	-0.1939 (-0.3980, 0.0102)
P for trend	0.308	0.377	0.171

In this chart, age, ratio of family income to poverty; total energy, cholesterol, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index, Ln(serum copper) were adjusted.
BLL, Blood lead levels; E2, Estradiol.
*P < 0.05, **P < 0.01, ***P < 0.001.

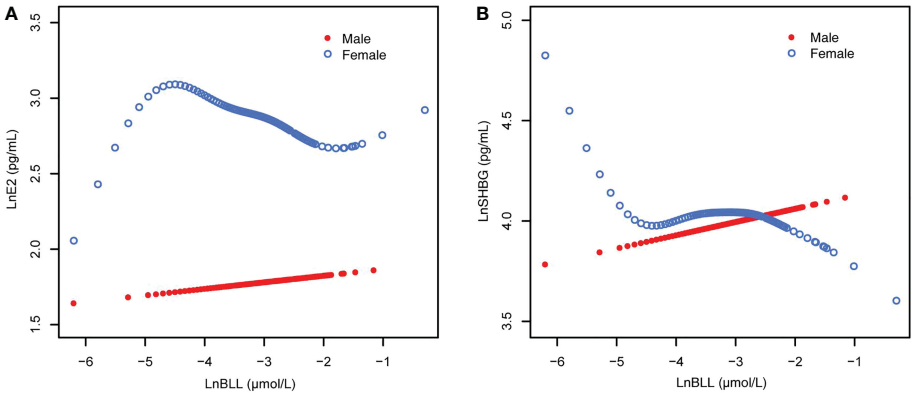

FIGURE 2
The associations of BLL with E2 and SHBG, stratified by age. **(A)** BLL and E2 dose–response relationship. Adjusted for age, ratio of family income to poverty; total energy, cholesterol, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index and Ln(serum copper). **(B)** BLL and SHBG dose–response relationship. Adjusted for age, ratio of family income to poverty; total energy, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index, hematocrit, serum continine, Ln(serum albumin), Ln(serum copper) and Ln(serum zinc). BLL, Blood lead levels; E2, Estradiol; SHBG, Sex hormone-binding globulin.

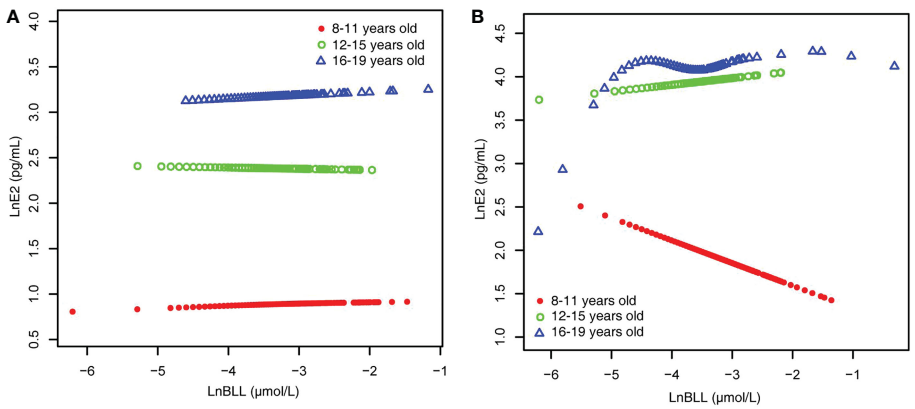

FIGURE 3
BLL and E2 dose–response relationship, stratified by age. **(A)** male. **(B)** female. Adjusted for ratio of family income to poverty; total energy, cholesterol, Ln (iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index and Ln(serum copper). BLL, Blood lead levels; E2, Estradiol.

showed a more prominent increase in female adolescents aged 16-19 years compared to LnSHBG with LnBLL quartiles in Q1. Smoothed curve fit plots (Figure 4) stratified by age supported these results.

Because the smoothed curve fit plots (Figure 4) after age stratification showed nonlinear relationships between LnBLL and LnE2 and between LnBLL and LnSHBG, we implemented threshold effects analysis and segmented linear regression models to fit the data and find meaningful inflection points in the dose-response curves of the independent and dependent variables for male and female children and adolescents of different ages. Among female participants aged 16-19 years, a significant difference between the two segmented regression coefficients was found for LnBLL and LnE2 before and after -4.7 μmol/L, and the log-likelihood ratio test was = 0.029<0.05, showing that LnBLL increased with LnE2 in adolescents aged 16-19 years before LnBLL was -4.7 μmol/L. In contrast, opposite effects were observed after -4.7 μmol/L (Table 4; Figure 3). An inverted U-shaped curve association was found. Similarly, there was a turning point in the LnBLL and LnSHBG dose-response relationship curve in female adolescents aged 16-19 years (log-likelihood ratio test <0.001), showing a U-shaped curve (Table 4; Figure 4). Beyond that, the LnBLL and LnE2 relationship curve in female participants aged 16-19 years in the remaining zones and the LnBLL and LnSHBG relationship curve in male participants aged 12-15 years, female participants aged 12-15 years, and female participants aged 16-19 years (in the remaining zones) all presented a linear relationship ($P > 0.05$).

4 Discussion

Lead exposure may be associated with hormonal changes, particularly in children, but there have been few studies on the health impact of metal exposure among children and adolescents (21). Our results showed an overall positive trend between BLL and

TABLE 3 Association between LnBLL (μmol/L) and LnSHBG (pg/mL) Stratified by race/ethnicity and sex.

LnBLL (μmol/L) (Quartile)	LnSHBG (pg/mL)		
	8 to 11 years old	12 to 15 years old	16 to 19 years old
Male			
Q1	Reference	Reference	Reference
Q2	-0.0035 (-0.0497, 0.0427)	-0.0311 (-0.1016, 0.0394)	-0.1099 (-0.1755, -0.0444) **
Q3	-0.0331 (-0.0774, 0.0113)	0.0559 (-0.0147, 0.1264)	-0.0458 (-0.1133, 0.0216)
Q4	0.0510 (0.0055, 0.0965) *	0.0806 (0.0084, 0.1527) *	0.0694 (0.0049, 0.1340) *
P for trend	0.030	0.001	<0.001
Female			
Q1	Reference	Reference	Reference
Q2	0.0002 (-0.0415, 0.0419)	0.0266 (-0.0344, 0.0877)	0.0019 (-0.0689, 0.0728)
Q3	0.0665 (0.0224, 0.1106) **	-0.0124 (-0.0913, 0.0665)	0.2767 (0.1895, 0.3640) ***
Q4	0.0467 (-0.0011, 0.0945)	0.1327 (0.0400, 0.2255) **	0.2638 (0.1596, 0.3681) ***
P for trend	0.003	0.051	<0.001

In this chart, age, ratio of family income to poverty; total energy, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index, hematocrit, serum continine, Ln(serum albumin), Ln(serum copper), Ln(serum zinc) were adjusted.
BLL, Blood lead levels; SHBG, Sex hormone-binding globulin.
*P < 0.05, **P < 0.01, ***P < 0.001.

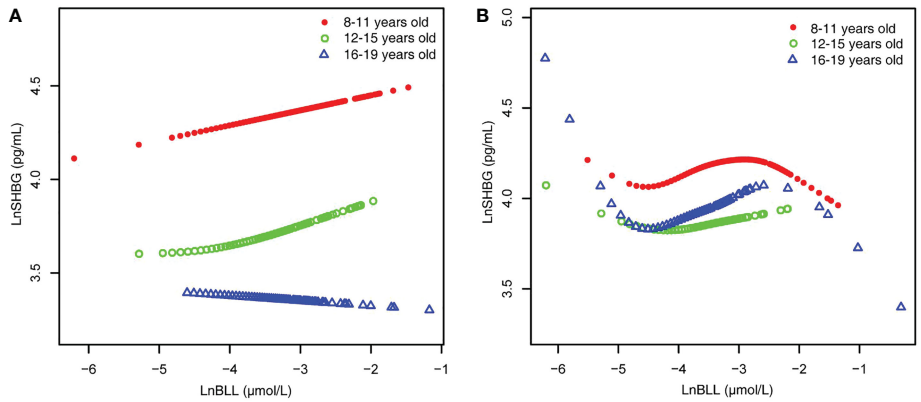

FIGURE 4
BLL and SHBG dose–response relationship, stratified by age. **(A)** male. **(B)** female. Adjusted for ratio of family income to poverty; total energy, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index, hematocrit, serum continine, Ln (serum albumin), Ln(serum copper) and Ln(serum zinc). BLL, Blood lead levels; SHBG, Sex hormone-binding globulin.

TABLE 4 Threshold effect analysis and two-piecewise linear regression of LnBLL (μmol/L) on LnE2 (pg/mL) or LnSHBG (pg/mL).

	LnE2	LnSHBG	Adjusted ß (95% CI), *P*-value
Female			
16-19 years old	LnBLL <-4.7 (μmol/L)		1.1845 (0.0525, 2.3164) 0.0414
	LnBLL >-4.7 (μmol/L)		-0.1306 (-0.3898, 0.1286) 0.3243
		LnBLL <-4.7 (μmol/L)	-0.8556 (-1.4203, -0.2909) 0.0033
		LnBLL >-4.7 (μmol/L)	0.1945 (0.0644, 0.3245) 0.0037

For two-piecewise linear regression model of LnBLL on LnE2, age, sex, race/ethnicity, ratio of family income to poverty; total energy, cholesterol, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index, Ln(serum copper) were adjusted.
For two-piecewise linear regression model of LnBLL on LnSHBG, age, ratio of family income to poverty; total energy, Ln(iron) and Ln(zinc) intake on the first day; fish eaten during the past 30 days, moderate recreational activities, body mass index, hematocrit, serum continine, Ln(serum albumin), Ln(serum copper), Ln(serum zinc) were adjusted.

E2 in males aged 8-11 years and 16-19 years. This association in the former age group was consistent with the findings of Mohamed A.M. Khalaf et al. (29). The mechanism may be that blood lead disrupts the balance of sex hormones in the hypothalamic-pituitary-gonadal system, especially at the hypothalamic level (29). In contrast, we found a significant trend of decreasing E2 in females for each elevated BLL after stratification for sex, consistent with other reports (30). Interestingly, we found an inverted U-shaped association between BLL and E2 with the point of inflection at 1.86 μg/L in female adolescents aged 16-19 years. That is, a low BLL promoted elevated levels of E2 while an excessive BLL caused a decrease in E2. There are two possible mechanisms by which a low BLL can promote an elevated E2 level. First, animal experiments found that a low BLL promoted a significant increase in gonadotropin-releasing hormone mRNA, which may stimulate the secretion of Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH), thereby causing an increase in the E2 levels (31). Second, blood lead increases homocysteine concentrations (32). Homocysteine is an N-methyl-D-aspartate agonist (33) that stimulates FSH and LH release (34). The reason for the decrease in E2 caused by high BLL may be the inhibition of steroid synthesis at the ovarian level. In an animal experiment of female rats, Prakash Pillai et al. found that lead could inhibit ovarian steroidogenesis by downregulating steroidogenic acute regulatory protein (StAR) expression, inhibiting ovarian steroidogenic enzymes, and increasing lipid peroxidation, leading to a decrease in serum E2 levels (35). Another possible mechanism is that lead could affect the expression of cyclin B1, a peptide related to ovarian granulosa cell proliferation and induce the expression of caspace-3, an apoptosis-related peptide, ultimately increasing the percentage of apoptosis in ovarian granulosa cells (36). Our findings differed from a study by Pollack who found that lead was positively with E2 (14). They enrolled 252 premenopausal women in Buffalo, New York and examined the associations of lead, cadmium, and mercury with reproductive hormones. The inconsistent results could be because their study population was premenopausal women, while we evaluated children. Further large-scale and longitudinal studies are needed to confirm this. E deficiency will result in bone demineralization, and the lead in the bone will be released into the blood in advanced bone demineralization (37). This can ultimately cause a vicious cycle, and thus, E2 suppression by BLL is important.

The results of our study showed that LnSHBG significantly increased with LnBLL in the Q4 range compared to that in the lowest LnBLL quartile group in male participants of all ages. Meanwhile, there was a significant positive correlation between BLL and SHBG in females, especially when the LnBLL quartiles are between Q3 and Q4. In addition, we found a U-shaped relationship between BLL and SHBG with an inflection point of 1.86 μg/L in female adolescents aged 16-19 years, indicating an inverse relationship between BLL and SHBG when BLL was <1.86 μg/L, and lead overload could lead to reduced SHBG. Previous studies have shown that excessive lead exposure can lead to hepatotoxicity by inducing oxidative injury and inflammation (38). Lead and other toxic metals have pro-oxidant and endocrine-disrupting properties, and studies have demonstrated that lead exposure may interfere with thyroid function by accumulating in the thyroid gland or affecting its regulation (39, 40). The production and maturation of thyroid hormones can increase blood SHBG levels (41). Thus, high BLL may indirectly affect the blood SHBG levels. These factors mentioned above may partially explain the negative correlation between them. Nevertheless, the mechanisms involved in the elevation of SHBG if the BLL is >1.86 μg/L have not been elucidated.

Our findings differed from a study reporting no relationship of blood lead with E2 and SHBG in men aged 50-75 years by Rotter et al. (42). They evaluated 313 men aged 50-75 years, and performed enzyme-linked immunosorbent assay to determine the concentrations of SHBG, E2, free testosterone (FT), and TT. Meanwhile, our study population comprised 8-19-year-old children and adolescents.

5 Limitations

To the best of our knowledge, this is the first study of relatively large sample size to assess the association of blood lead with E2 and SHBG in 8-19-year-old children and adolescents. Our findings provide prospective evidence for future clinical studies. We found U-shape and inverted U-shape patterns for blood lead and E2 with SHBG respectively for the first time. Moreover, we performed a subgroup analysis and found the inflection point for BLL to be 1.86 μg/L, which is lower than the safe reference value of 3.5 μg/dL set by the Centers for Disease Control (CDC) for blood lead in children (43). However, this study also has some limitations. First, there are restrictions on real-world research such as the data are often incomplete, inaccurate and biased. Second, our study was limited to children and adolescents, and the age stratification could be more detailed to identify sensitive age groups. Third, age at menarche is considered one of the indicators of estrogen exposure, and some studies have shown that the later the age at menarche, the lower the E2 level (44). The use of estrogen products may also have an impact on the result of serum E2 level. However, these factors could not be included as covariates in the analysis because of insufficient data from NHANES 2013-2016, making it difficult for us to assess their impact on the results obtained. Fourth, although SHBG is recognized to regulate estradiol transport and bioavailability, and the BLL values at the turning points of the two curves are almost equal, whether lead affects the affinity of SHBG for E2 in the circulation and affects the binding of the two has rarely been reported, and the mechanism still remains unclear and warrants further exploration (45).

6 Conclusions

This study found an inverted U-shaped association of BLL with E2 and a U-shaped association between BLL and SHBG in female adolescents aged 16-19 years. This indicates that adjusting blood lead exposure to mitigate the effects of lead on growth and development is important for adolescents aged 16-19 years. Controlling the BLL below 1.86 μg/L may minimize the damage to E2. Meanwhile, the optimal BLL to eliminate its effect on SHBG is still unclear. The almost coincidental overlap of BLL values at the turning points of the two curves is worthy of further investigation to determine the optimal level of blood lead.

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

KP and CZ contributed to conception and design of the study. YH extracted the data and organized the database. ZC and CZ performed the statistical analysis. KP wrote the first draft of the manuscript. CZ, RT, ZC, and YH wrote sections of the manuscript. All authors contributed to to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1096659/full#supplementary-material

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Associations between smoking status and infertility: a cross-sectional analysis among USA women aged 18-45 years

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Background: Although many studies have proven the harmful effects of smoking on human health, the associations between smoking status and infertility are limited in large epidemiologic studies. We aimed to investigate the associations between smoking status and infertility among child-bearing women in the United States of America (USA).

Methods: A total of 3,665 female participants (aged 18-45) from the National Health and Nutrition Examination Survey (NHANES) (2013-2018) were included in this analysis. All data were survey-weighted, and corresponding logistic regression models were performed to investigate the associations between smoking status and infertility.

Results: In a fully adjusted model, the risk of infertility was found to be increased by 41.8% among current smokers compared to never smokers (95% CI: 1.044-1.926, P=0.025). In the subgroup analysis, the odds ratios (95% CI) of the risk of infertility for current smokers were 2.352 (1.018-5.435) in the unadjusted model for Mexican American, 3.675 (1.531-8.820) in the unadjusted model but 2.162 (0.946-4.942) in fully adjusted model for people aged 25-31, 2.201 (1.097-4.418) in the unadjusted model but 0.837 (0.435-1.612) in fully adjusted model for people aged 32-38.

Conclusion: Current smokers was associated with a higher risk of infertility. The underlying mechanism of these correlations still needs more research. Our findings indicated that quitting smoking may serve as a simple index to reduce the risk of infertility.

KEYWORDS

smoking status, infertility, NHANES, cross-sectional analysis, population-based study

1 Introduction

Infertility is a top public health concern which is defined as the failure to conceive within a year of unprotected sexual activity (1, 2). The United States' Centers for Disease Control and Prevention (CDC) underlined that infertility was a serious public health concern with significant quality-of-life effects, such as psychological suffering, social stigma, financial strain, and marital discord (3). 15% of couples who are of childbearing age were struggling with infertility in the world (4). Although infertility is a widespread health problem, seldom are modifiable risk factors identified. Infertility has a complicated etiology that involves both male and female components, as well as a mixture of both. As of this writing, the CDC has designated infertility diagnosis and treatment as a national public health priority (5).

Cigarette smoking is a leading and preventable cause of morbidity and mortality worldwide (6–9). In the United States of America (USA), 34.1 million of individuals were reported to be smokers in 2019 (10). Smoking has so far been repeatedly shown to contribute to a wide range of human ailments, including reproductive abnormalities (11–13). About 4000 different chemicals, including alkaloids, heavy metals, and polycyclic aromatic hydrocarbons, all of which have reproductive toxicity are present in cigarette smoke (14, 15). Most research indicates that women who are current smokers and those who were exposed to parental smoking before conception had lower natural fertility (16).

By estimating an overall 60% increase in the probability of infertility, a meta-analysis highlighted a significant correlation between smoking and infertility (17). On the other hand, after controlling for relevant confounders, a prospective study was unable to find any discernible difference in fertility between smokers and non-smokers (18). In conclusion, there is conflicting evidence in the literature about the relationship between smoking and infertility. Other than that, the majority of earlier studies, however, used clinic-based samples, and only a few of them concentrated on sizable population-level samples. Considering the inconsistent and limited evidence on the associations between smoking status and infertility, based on a large national population-based representative survey, the objectives of this study were to evaluate the associations between smoking status and female infertility and determine which type of smoking status was linked with the highest infertility risk based on a population-based study. Age and race/ethnicity difference were further studied in the subgroup analysis because previous studies had demonstrated that they had an effect on the prevalence of infertility (19–22). The decision-making process by health authorities regarding programs for health promotion and intervention to avoid infertility in women of reproductive age may be aided by knowledge of the associations.

2 Methods

2.1 Data source and study population

NHANES, administered by the CDC and Prevention, is a nationally representative, cross-sectional survey conducted incessantly in 2-year cycles through questionnaire surveys, physical examinations, household interviews, and laboratory tests, designed to evaluate and assess the health and nutrition status of Americans. The included samples in this study have good representativeness because of the stratified multistage probability sampling approach used (23). The public can access all NHANES data at www.cdc.gov/nchs/nhanes/.

In the present study, NHANES data from 2013-2014, 2015-2016 and 2017-2018 were used. A total of 29,400 participants were incorporated at first; after the exclusion of males (n = 14,452), individuals aged <18 or >45 (n = 10,625) (24, 25), missing the smoking status or infertility data (n = 658), 3,665 participants were included in our final analysis (Figure 1). The National Center for Health Statistics Ethics Review Board approved human subjects for the conduction of NHANES, and all participants provided their written informed consent.

2.2 Study variables

In our analysis, the main exposure of interest is smoking status. Information on this exposure was obtained from relevant NHANES questionnaire items, which defined never smokers as having smoked fewer than 100 cigarettes in their lives, and former smokers as having smoked at least 100 cigarettes in their lives but not currently. These inquiries were made by trained interviewers using the Computer-Assisted Personal Interview (CAPI) technology at the mobile examination center (MEC). For these inquiries, both interpreters and proxy interviewers were accepted. Those who responded "Every day or certain days" to the question "Do you now smoke cigarettes?" were considered current smokers. Infertility is the key outcome of interest. These inquiries were made in the questionnaire on reproductive health using computer-assisted personal interviews conducted by qualified interviewers.

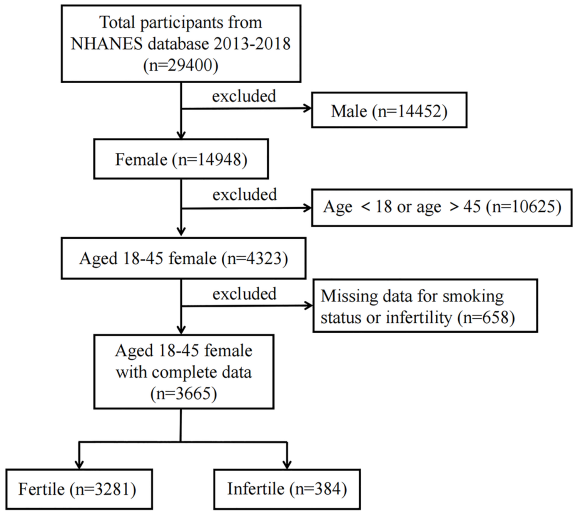

FIGURE 1
Flow chart of the study population. Describes how the sample of participants was composed. NHANES, National Health and Nutrition Examination Survey.

Women who responded "yes" to the question "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?" were labeled as experiencing infertility, with the answer "no" as the opposite category.

Our multivariable-adjusted models outlined relevant factors that might obscure the relationship between smoking status and infertility based on prior research (26). In our study, covariates including age (years), race/ethnicity (Mexican American/other Hispanic/non-Hispanic White/non-Hispanic Black/other races), education level (less than high school/high school/more than high school), marital status (married/living with partner, widowed/divorced/separated, never married) and ratio of family income to poverty (PIR), body mass index (BMI), diabetes, and physical activity were examined. According to Physical Activity Guidelines for Americans, 2nd edition of 75 min/week of vigorous or 150 min/week of moderate physical activity (27), participants were divided into three groups, including active, less active, and inactive. All detailed measurement procedures of the above variables are available on the NHANES website (www.cdc.gov/nchs/nhanes/).

2.3 Statistical analysis

For all statistical analyses, Stata (version 16.0), and EmpowerStats (version 2.0) were utilized with a determination of $P < 0.05$ for statistically significant. All estimates were computed using sample weights in accordance with National Center for Health Statistics' analytical standards since NHANES seeks to create data that is representative of the civilian noninstitutionalized population in the USA. Continuous variables were characterized by mean ± SD if they were normally distributed and by median values and interquartile ranges otherwise. Percentages were used to characterize categorical variables which were compared by χ^2 testing. To evaluate the relationship between smoking status and infertility, multivariable logistic regression was performed and the odds ratio (OR) values and 95% confidence intervals (95%CI) were calculated (28–32). Three models were built for the multivariate test. Model 1 had no variables adjusted. In Model 2, age and race/ethnicity were adjusted. Model 3 was adjusted for all covariates. Subgroup analysis stratified by age and race/ethnicity was carried out using stratified multivariate regression analysis.

3 Results

3.1 Baseline characteristics of participants

The weighted baseline characteristics of the included participants are displayed in Table 1. A total of 3,665 female participants were incorporated, including 384 with infertility and 3665 without infertility, with an average age of 31.438 ± 8.122 years. Infertility was significantly more prevalent among women who were older at the time of the survey (35.367 years vs. 30.924 years, $P < 0.001$), had higher BMI (≥30 kg/m^2: 55.061% vs 36.871%, $P < 0.001$) and higher family income (PIR >1.85: 65.737% vs 57.792%, $P = 0.008$), and they tended to be never married (Married/Living with partner: 77.152% vs. 57.500%, $P <0.001$). In addition, they were more inclined to suffer from diabetes (7.955% vs 7.955%, $P <0.001$) and more intend to be current smokers (23.859% vs 18.139%, $P = 0.001$).

3.2 Associations between smoking status and infertility

Using binary logistic regression with single and multiple variables, we constructed three models to investigate relationships between smoking status and infertility. The pertinent effect size OR, 95%CI, and *P*-values are displayed in Table 2. There was a substantial correlation between smoking status and infertility in Models 1, 2, and 3, which was positive regardless of the kind of adjusted covariates. In the initial model (Model 1), the risk of infertility among current smokers increased by 54.9% than that among never smokers (OR=1.549; 95% CI: 1.189-2.017, *P*=0.001), 33.6% in Model 2 (OR: 1.336, 95% CI: 1.013-1.763, *P*=0.040) and 41.8% in Model 3 (OR: 1.418, 95% CI: 1.044-1.926, *P*=0.025).

3.3 Subgroup analysis

Subgroup analysis revealed that the connection between smoking status and infertility was mostly present in Mexican Americans and participants aged 25 to 38 after controlling for variables. Tables 3, 4 provide comprehensive information on the subgroup analysis. For Mexican American, the association is similar in Model 2 (OR: 2.304, 95% CI: 0.971-5.470, *P*=0.058) and Model 3 (OR: 1.883, 95% CI: 0.829-4.278, *P*=0.13), but not in Model 1 (OR: 2.352, 95% CI: 1.018-5.435, *P*=0.045) (Table 3). For people aged 25-31, there is a significant positive association between smoking status and infertility in Model 1 (OR: 3.675, 95% CI: 1.531-8.820, *P*=0.004), Model 2 (OR: 2.501, 95% CI: 1.225-5.105, *P*=0.012) but not in Model 3 (OR: 2.162, 95% CI: 0.946-4.942, *P*=0.067). For people aged 32-38, there is a significant positive association in Model 1 (OR: 2.201, 95% CI: 1.097-4.418, *P*=0.026), but not in Model 2 (OR: 0.659, 95% CI: 0.363-1.195, *P*=0.169) and Model3 (OR: 0.837, 95% CI: 0.435-1.612, *P*=0.595) (Table 4).

4 Discussion

In this cross-sectional study, which included 3,665 people, we found that current smokers had a higher risk of infertility. An examination of subgroups revealed that populations with Mexican American heritage and those between the ages of 25 and 38 shared this connection. Our findings imply that smoking status should be taken into account while treating infertile individuals in therapeutic settings.

Clinical investigations on the connection between smoking status and infertility in females are still limited and controversial. Three studies indicated a substantial link between smoking and infertility, with the risk being 1.85 (95% CI: 1.08-3.14) times greater for smokers than for non-smokers (33–35). The relevant literature

TABLE 1 Characteristics of the study population, National Health and Nutrition Examination Survey (NHANES) 2013–2018.

Characteristic	Total (n=3665)	No infertility (n=3281)	Infertility (n=384)	*P* value
Age (years)	31.438 ± 8.122	30.924 ± 8.107	35.367 ± 7.099	<0.001
Race/Ethnicity (%)				0.116
Mexican American	11.995	12.218	10.284	
Other Hispanic	7.908	8.075	6.631	
Non-Hispanic White	55.979	55.184	62.058	
Non-Hispanic Black	13.479	13.645	12.216	
Other race	10.639	10.878	8.810	
Education (%)				0.795
Less than high school	11.544	11.682	10.556	
High school	19.185	19.146	19.464	
More than high school	69.271	69.172	69.980	
Marital status (%)				<0.001
Married/Living with partner	29.766	32.279	11.832	
Widowed/Divorced/Separated	10.319	10.222	11.016	
Never Married	59.915	57.500	77.152	
PIR (%)				0.008
≤ 1.30	29.636	30.210	25.369	
1.30- ≤ 1.85	11.630	11.998	8.894	
> 1.85	58.734	57.792	65.737	
BMI(%)				<0.001
<25	36.806	38.036	27.382	
25≤-30	24.223	25.093	17.557	
≥30	38.971	36.871	55.061	
Diabetes (%)				<0.001
No	96.502	97.085	92.045	
Yes	3.498	2.915	7.955	
Physical activity (%)				0.698
Inactive	54.379	54.159	56.055	
Less active	7.342	7.440	6.598	
Active	38.279	38.401	37.347	
Smoking status(%)				0.001
Never smokers	69.427	70.439	61.686	
Former smokers	11.773	11.422	14.456	
Current smokers	18.800	18.139	23.859	

Mean ± SD for continuous variables: P value was calculated by one-way ANOVA; % for categorical variables: P value was calculated by χ^2 test.
PIR, family income to poverty ratio; BMI, body mass index.

from 1966 through late 1997 was found by a meta-analysis, which revealed an OR of 1.60 for infertility among female smokers compared to non-smokers across all research designs (23). Since the publication of this meta-analysis, more extensive population-based studies have shown that smoking has a detrimental effect on fecundity, regardless of other factors. The largest of these studies found that active smoking was linked to an increased failure to conceive within both the 6- and 12-month trial periods (36).

TABLE 2 Association between smoking status and infertility.

	Model 1: OR (95%CI) *P*	Model 2: OR (95%CI) *P*	Model 3: OR (95%CI) *P*
Smoking status			
Never smokers	reference	reference	reference
Former smokers	1.629 (1.179, 2.252) 0.003	1.312 (0.939, 1.832) 0.112	1.152 (0.806, 1.646) 0.437
Current smokers	1.549 (1.189, 2.017) 0.001	1.336 (1.013, 1.763) 0.040	1.418 (1.044, 1.926) 0.025
P for trend	<0.001	<0.001	<0.001

Model 1 adjusted for none.
Model 2 adjusted for age and race/ethnicity.
Model 3 adjusted for age, race/ethnicity, education level, marital status, PIR, BMI, diabetes, and physical activity.

However, this study divided smoking into active, passive, or both and the number of cigarettes smoked instead of different status and we are unclear about the difference between the relationship of past and current smoking and infertility. An Ontario, Canada, retrospective cohort study of farm couples found no difference in the risk of infertility between current smokers and non-smokers (37). Additionally, based on data from a North American internet-based preconception cohort study that enrolled participants from 2013 to 2018, a prospective analysis of cigarette smoking and fecundability found both female current smoking and previous smoking were related to slight declines in fecundity (38). Both of the above studies are consistent with the findings of Model 3 in our study, indicating that the relationship between smoking and infertility varies from current smoking status. In our study, the relationship between former smokers and infertility in Table 2 was significant in Model 1 but not in Models 2 and 3 after adjusting for the covariate. The effect of former smokers on the outcome event infertility reflected not only the pure effect of exposure factor but also the effect of confounding factors. By constructing a multiple regression model in Model 2 and Model 3, i.e., "adjusting" for the

TABLE 3 Association between smoking status and infertility stratified by race/ethnicity.

Race/Ethnicity (%)	Model 1 OR (95%CI) *P*	Model 2 OR (95%CI) *P*	Model 3 OR (95%CI) *P*
Mexican American			
Never smokers	reference	reference	reference
Former smokers	2.146 (0.860,5.356) 0.102	2.081 (0.824,5.254) 0.121	1.673 (0.579,4.831) 0.342
Current smokers	2.352 (1.018,5.435) 0.045	2.304 (0.971,5.470) 0.058	1.883 (0.829,4.278) 0.130
Other Hispanic			
Never smokers	reference	reference	reference
Former smokers	2.269 (0.813,6.338) 0.118	2.008 (0.699,5.768) 0.196	2.065 (0.673,6.342) 0.205
Current smokers	1.030 (0.316,3.362) 0.960	1.133 (0.343,3.748) 0.838	1.130 (0.284,4.496) 0.862
Non-Hispanic White			
Never smokers	reference	reference	reference
Former smokers	1.204 (0.690,2.100) 0.514	0.908 (0.503,1.639) 0.749	0.821 (0.445,1.513) 0.527
Current smokers	1.310 (0.837,2.049) 0.238	1.180 (0.743,1.874) 0.482	1.231 (0.723,2.098) 0.444
Non-Hispanic Black			
Never smokers	reference	reference	reference
Former smokers	1.405 (0.557,3.546) 0.471	1.239 (0.482,3.184) 0.656	1.112 (0.446,2.772) 0.820
Current smokers	1.726 (0.987,3.020) 0.056	1.608 (0.910,2.84) 0.1020	1.495 (0.786,2.843) 0.221
Other Race			
Never smokers	reference	reference	reference
Former smokers	1.652 (0.672,4.063) 0.274	1.523 (0.612,3.788) 0.365	1.305 (0.445,3.829) 0.627
Current smokers	1.696 (0.593,4.851) 0.324	1.787 (0.577,5.539) 0.314	1.921 (0.472,7.823) 0.362

Model 1 adjusted for none.
Model 2 adjusted for age and race/ethnicity.
Model 3 adjusted for age, race/ethnicity, education level, marital status, PIR, BMI, diabetes, and physical activity.

TABLE 4 Association between smoking status and infertility stratified by age.

Age	Model 1 OR (95%CI) *P*	Model 2 OR (95%CI) *P*	Model 3 OR (95%CI) *P*
Age (25-31)			
Never smokers	reference	reference	reference
Former smokers	2.587 (0.864,7.751) 0.089	1.620 (0.647,4.058) 0.303	1.225 (0.448,3.351) 0.693
Current smokers	3.675 (1.531,8.820) 0.004	2.501 (1.225,5.105) 0.012	2.162 (0.946,4.942) 0.067
Age (32-38)			
Never smokers	reference	reference	reference
Former smokers	1.523 (0.609,3.808) 0.368	1.404 (0.745,2.646) 0.293	1.511 (0.775,2.946) 0.226
Current smokers	2.201 (1.097,4.418) 0.026	0.659 (0.363,1.195) 0.169	0.837 (0.435,1.612) 0.595
Age (39-45)			
Never smokers	reference	reference	reference
Former smokers	1.488 (0.801,2.764) 0.208	0.636 (0.302,1.338) 0.233	0.633 (0.301,1.331) 0.228
Current smokers	0.711 (0.398,1.269) 0.248	1.266 (0.706,2.268) 0.429	1.619 (0.843,3.109) 0.148

Model 1 adjusted for none.
Model 2 adjusted for age and race/ethnicity.
Model 3 adjusted for age, race/ethnicity, education level, marital status, PIR, BMI, diabetes, and physical activity.

effects of other confounding factors, the effect of the confounding factor was actually separated from the effect of the exposure factor. After eliminating the effect of the confounding factors, the spurious association between former smokers and the dependent variable disappeared, and there was no significant correlation between former smokers and infertility in Model 2 and Model 3. However, the population included in those studies did not distinguish between race and age. Thus, both age and race were the limitations of their study. In our study, the relationship between smoking status and infertility was found to be different across race and age groups by performing subgroup analysis.

Age and race/ethnicity have been proven in prior research to have an impact on the prevalence of infertility (19–22). Fecundity reportedly decreased for females in their late thirties and early forties. The likelihood of infertility rose from 10%-20% after age 35 to 45% in the early forties among women with previously confirmed fertility. Women who had never given birth were more likely to experience infertility at any age (19). Additionally, a study found that American Indians and Alaska Natives had a 1.30 times higher prevalence of decreased fecundity than white people (95% CI: 1.04 -1.62) (22). Thus, we conducted stratified analyses by age and race/ethnicity in the subgroup analysis.

Although the mechanisms underlying smoking and the risk of infertility have not been entirely understood, some evidence can support the negative association between them. Strong evidence suggested that smoking might impact natural female fertility by affecting several female reproductive function elements such as the ovary, oviduct, and uterus (39–41). In addition to clinical observational research in people, experiments on human tissues and cells as well as animal models have been used to study how smoking affects female reproductive function and fertility (42). But there is still debate over the outcomes. According to several research, smoking lowered the number of oocytes that may be obtained for assisted reproductive technology (ART) (43, 44). Those that are collected have a lower chance of becoming fertile, which lowers the quality of the resulting embryos (44, 45). However, when smokers are compared to age-matched controls, other studies have not discovered any differences in oocyte number, fertilization, embryo quality, clinical pregnancy, or birth rates (46, 47). The precise mechanism of the association between smoking status and infertility in our study remains unclear.

This study has a number of advantages. First, this study was based on data from NHANES, which are population-based sampling data collected across the country following a set procedure. The study samples were more representative since all analyses took into account the proper NHANES sampling weights. To make the results from the current study more trustworthy, the authors additionally made adjustments for confounding factors. However, it is impossible to disregard the study's limitations. First, a clear causal association cannot be established by the authors because of the cross-sectional study methodology. Second, we were constrained in our secondary analysis due to our inability to gather fresh data. Therefore, there is a chance that unmeasured factors will cause residual confounding. For instance, because these data were not obtained, we were unable to control for the family history of infertility, a potentially significant confounder. To learn more about the harmful effects of smoking, it is crucial to investigate the relationship between smoking status and female infertility. More studies are still required to produce definitive pieces of data.

5 Conclusion

This study demonstrated that current smoking was associated with elevated infertility risk. In subgroup analyses, the associations of smoking status with infertility were only found in women aged

25-38 and in Mexican Americans. Further studies are still needed to validate our findings.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/index.htm.

Author contributions

SH designed the research, analyzed the data, and wrote the paper. LW assisted in manuscript preparation. 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.

Publisher's note

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Omega-3 fatty acid ameliorates bisphenol F-induced testicular toxicity by modulating Nrf2/NFkB pathway and apoptotic signaling

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Introduction: Bisphenol F (BPF) has been shown to disrupt testicular functions via perturbation of testicular redox balance, while omega-3 fatty acid (O3FA) has been established to exert antioxidant and anti-inflammatory activities. Therefore, this study focused on the role and associated molecular mechanism of O3FA in BPF-induced testicular dysfunction in male Wistar rats.

Methods: Twenty-four (24) rats were randomly grouped after two weeks of acclimatization into four (4) groups (n=6/group); the vehicle-treated control group, BPF treated group received 30 mg/kg of BPF, and the intervention groups received 30 mg/kg BPF + 100 mg/kg O3FA (BPF+O3FA-L) and 30 mg/kg BPF + 300 mg/kg of O3FA (BPF+O3FA-H). All treatment lasted for 28 days.

Results: Low and high doses of O3FA ameliorated BPF-impaired sperm quality, and induced hormonal imbalance, accompanied by a distortion in testicular histology and elevated testicular injury markers. Furthermore, co-administration of BPF with both doses of O3FA blunted BPF-induced redox imbalance, inflammatory response, and apoptosis.

Discussions: In conclusion, our present findings show that O3FA improves testicular functions in BPF-treated rats by improving sperm quality and reproductive hormones via the maintenance of testicular redox balance.

KEYWORDS

omega-3 fatty acid, bisphenol F, bisphenol analogs, endocrine disruptors, testicular functions, apoptosis

1 Introduction

Plastics and cans are used in almost every facet of daily life. They are utilized in transportation, telecommunications, clothes, footwear, and, most importantly, as packaging materials for various foods, beverages, and other commodities. Numerous researches have been conducted on various elements of plastics and cans, particularly

their environmental effects and risks to natural environments, wildlife, and, most significantly, human health. One of the major raw materials in the production of plastics and cans is bisphenol A (BPA) (1).

BPA is ubiquitous in the environment, resulting in high rate of human exposure to this chemical. The concern of widespread exposure and established adverse effects on human health has led to strict restrictions on the production and usage of BPA in Canada, France, and the European Union in 2008, 2010, and 2011 respectively (2). This consequently led to the introduction of alternative substitutes for BPA. As the focus has switched to producing "BPA-free" products, bisphenol F (BPF) has become the major replacement for BPA. It is now widely used to produce everyday consumer products such as plastics, cans, thermal papers, and inner linings of food containers, infant bottles, and toys. Unfortunately, BPF, which is expected to be a safer alternative to BPA, displays a similar gonadotoxic effect to BPA. BPF is gradually becoming a ubiquitous chemical, and investigations have revealed that BPF may harm the reproductive system (3–5). BPF exposure has been implicated in the increased production of free radicals (oxidative stress) and pro-inflammatory cytokines (4, 6, 7), which is a major cause of testicular toxicity.

Nuclear Factor Erythroid Related Factor 2 (Nrf2) and Nuclear Factor-Kappa B (NFκB) are key regulators of the body's response to oxidative stress and inflammatory response (8). During excessive and continuous exposure to external stresses, the body produces excess free radicals and reactive oxygen species (ROS), leading to the downregulation of endogenous antioxidants, enzymes, and proteins, thereby damaging the body's cellular components such as proteins, DNA, and lipids (9). Nrf2 is a major endogenous antioxidant controlling various aspects of cellular homeostasis in response to oxidative stress (10). The decline in Nrf2 due to external stressors can upregulate NFκB expression, leading to an inflammatory response. Also, the increase in NFκB expression can also lead to a further decrease in Nrf2. Hence, Nrf2 and NFκB are important players in the crosstalk between oxidative stress and inflammation (11). The excessive decrease in the endogenous antioxidant system and increased inflammatory response can possibly trigger an apoptotic response (12). On the other hand, supplementation of exogenous antioxidants can target oxidative stress by inhibiting the production of free radicals and ROS and bolstering the endogenous antioxidant capacity.

Omega-3 fatty acid (O3FA) is a polyunsaturated fatty acid (PUFA) and an antioxidant with favorable effects against various diseases such as cardiovascular disorder (13) and reproductive dysfunction (14). O3FA can protect organs such as the testis via its antioxidant (15), anti-inflammatory, and antiapoptotic (14) properties. These data suggest that O3FA could be a promising cytoprotective agent against extrinsic toxic stimuli. Despite these established protective functions of O3FA, no study has investigated the effectiveness of O3FA on testicular dysfunction in BPF-induced reproductive toxicity. Hence, this study was designed to investigate the ameliorative effect of O3FA on BPF-induced gonadotoxicity.

2 Methods

2.1 Chemical

O3FA was purchased from Gujarat Liqui Pharmacaps Pvt. Ltd. Vadodara, Gujarat, India, and each O3FA capsule contains eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the ratio of 3:2. BPF was purchased from Sigma-Aldrich, St. Louis, MO, USA, CAS: 620-92-8. All other chemicals except otherwise stated were purchased from Sigma Aldrich.

2.2 Animals

Twenty-four (24) male Wistar rats of age 10 ± 2 weeks with comparable weights (160-180 g) were obtained from the University of Ilorin. The animals were randomly separated into clean wooden cages under natural conditions and were allowed unlimited free access to feed and water ad'libitum. The designed experimental protocol was approved by the University of Ilorin Review and Ethical Committee, and in accordance with the "National Institute of Health guidelines using the guide for the care and handling of laboratory animals (NIH Publication No. 80–23; amended 1978)". The experimental protocol was under the National Research Council's guidelines for the Care and Use of Laboratory Animals, and ARRIVE guidelines for reporting experimental findings were followed. Animals were randomly grouped after two weeks of acclimatization into four (4) groups (n=6/group); the vehicle-treated control group, BPF treated group received 30 mg/kg of BPF, and the intervention groups received 30 mg/kg BPF + 100 mg/kg O3FA (BPF+O3FA-L) and 30 mg/kg BPF + 300 mg/kg of O3FA (BPF+O3FA-H).

2.3 Sample collection

The dose of BPF was calculated and dissolved in corn oil, and 0.5 ml of the solution containing the appropriate calculated dose was administered for each animal. The 28 days administrations were carried out using an oro-pharyngeal cannula via the oral route to mimic the main route of human exposure. Overnight fasted animals were sacrificed 24 hours after the last dose of BPF and O3FA with ketamine (40 mg/kg) and xylazine (4 mg/kg) i.p (16). Blood samples were collected via cardiac puncture while the left and right testes and left epididymides were harvested. The blood samples were centrifuged at 3000 rpm to obtain the serum for hormonal analysis, while the left testes were homogenized in cold Phosphate Buffer for biochemical assays. The right testes were used for histological examination, while the left epididymides were harvested for sperm analysis.

2.4 Epididymal sperm parameters

Each caudal epididymis was carefully cut into small pieces in a clean petri dish and sperm count, motility, and abnormal sperm morphology were determined as previously described (14, 17).

2.5 Reproductive hormones

Serum luteinizing hormone (LH) (Catalogue no.: B-1-121032301), follicle-stimulating hormone (FSH) (Catalogue no.: B-1-121040801), testosterone (B-1- 121071602), and estradiol (B-1-122042001) were quantified using an ELISA method following the manufacturer's guidelines (Bio-Inteco, UK).

2.6 Histology

Testicular histopathological analysis was performed based on documented methods (14, 18). The testis was fixed in bouin solution, dehydrated with ethanol series, and cleared with toluene. It was then embedded at room temperature of 37°C and blocked in paraffin wax incubated overnight in a 60°C incubator. Afterward, hematoxylin and eosin (H&E) stain was applied to the testes' 5 µm thick paraffin sections.

Testicular histoarchitecture was determined as established by Cosentino et al. (19) scoring system as follows:

"4: Irregular and distorted seminiferous tubules engorged by coagulative necrosis in the germ cells.

3: Disordered and sloughed germ cells with shrunken and pyknotic nuclei and impaired borders of the seminiferous tubules.

2: Loss of cohesion in germ cells, closely packed seminiferous tubules.

1: Normal testicular tissue with an orderly arrangement of germ cells".

The Mean testicular biopsy score (MTBS), which is an index of spermatogenesis, was determined at 400 X microscopic field as earlier established by Johnsen (20) scoring system as follows:

"10: Complete spermatogenesis with many spermatozoa.

9: Many spermatozoa present but disorganized germinal epithelium.

8: Only a few spermatozoa (<5–10) are present.

7: No spermatozoa but many spermatids present.

6: No spermatozoa and only a few spermatids (<5–10) present.

5: No spermatozoa or spermatids but several or many spermatocytes present.

4: Only a few spermatocytes (<5) and no spermatids or spermatozoa present.

3: Spermatogonia are the only germ cells present.

2: No germ cells, but Sertoli cells are present.

1: No cells (either germ cell or Sertoli cell) in the tubular section"

Mean seminiferous tubular and luminal diameter and epithelial height were estimated as reported earlier (14, 21, 22). "Mean Seminiferous Tubular Diameter (MSTD) of each testis was determined by measuring 20 separate roundest seminiferous tubules with a light microscope-adaptable micrometer. The mean of the values obtained was regarded as the MSTD of the testis.

2.7 Testicular injury markers

Gamma-glutamyl transferase (GGT) activities were estimated according to the manufacturer's instructions (Agape Diagnostics Ltd., CAT: 31070095), while Lactate dehydrogenase activities were also determined following the manufacturer's instructions (Agape Diagnostics Ltd., CAT: 31060230) using a spectrophotometer. The testicular lactate concentration was also estimated according to the manufacturer's guidelines (EnzyChrom, ELAC-100).

2.8 Steroidogenic enzymes

Testicular 3 beta-hydroxysteroid (3β-HSD) and 17 beta-hydroxysteroid (17 β-HSD) enzymatic activities were determined as previously documented (23) and (24) respectively.

2.9 Inflammatory markers

Standard ELISA kits were used to assay the concentrations of interleukin-6 (IL-6) (Solarbio, China, CAT: SEKH-0013) and tumour necrosis factor-α (TNF- α) (Solarbio, China, CAT: SEKH-0047), NFkB (Elabscience Biotechnology Inc., USA, CAT: E-EL-R0673) were determined using ELISA kits. Testicular Myeloperoxidase (MPO) and nitric oxide were determined based on established methods (25) and (26), respectively.

2.10 Markers of oxidative stress

Testicular malondialdehyde (MDA) (27) levels were assayed as previously reported. In addition, testicular glutathione (GSH), glutathione peroxidase (GPx), Glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) (10, 14, 28) activities were assayed by colorimetric methods as previously reported. In addition, testicular Nrf2 was determined using an ELISA method according to the manufacturer's guidelines (Elabscience Biotechnology Inc., USA). Testicular xanthine oxidase (XO) activities were based on a previously established method (22, 29).

2.11 Apoptotic markers

A spectrophotometric assay using diphenylamine (DPA) methods (14, 22) was employed in estimating the DNA fragmentation index, while testicular caspase 3 activities were estimated according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., USA).

2.12 Statistical analysis

Graph Pad Prism, version 7.00, was used for statistical analysis. To analyze data from various groups, one-way analysis of variance (ANOVA) was employed, followed by Tukey's *post hoc* test for multiple comparisons. Data are presented as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Epididymal sperm parameters

O3FA ameliorated the BPF-induced decrease in sperm count, motility, and normal morphology compared with the control (Table 1). While there was no significant difference in abnormal sperm morphology and motility of rats treated with low and high doses of O3FA, animals treated with high doses of O3FA showed an improved sperm count compared with their counterparts treated with a low dose.

3.2 Hormonal imbalance

As shown in Table 2, O3FA blunted the observed BPF-induced hormonal imbalance by significantly increasing serum LH ($p<0.0001$), FSH ($p<0.0001$), and testosterone ($p<0.0001$) and decreasing serum estradiol ($p<0.0001$) in BPF-exposed rats. Although low and high doses of O3FA significantly blunted the was observed BPF-induced hormonal imbalance, a more ameliorative effect was observed in animals treated with a high dose than their counterparts treated with a low dose.

3.3 Histopathological findings

As shown in Figure 1, BPF distorted the normal testicular histology, evidenced by a distorted histoarchitecture, scanty sperm cells in the lumen of the seminiferous tubule, and reduced Sertoli cells and Leydig cell mass compared with the control. This was accompanied by an increase in testicular histoarchitecture and seminiferous luminal diameter and a decrease in biopsy score, epithelial height, and seminiferous tubular diameter (Table 3). These observed alterations were ameliorated by co-administration of BPF with both doses of O3FA.

3.4 Testicular injury markers

BPF exposure led to a significant increase in testicular LDH ($p<0.0001$), GGT ($p<0.001$), and lactate ($p<0.0001$) and a decrease in testicular SDH (Figure 2) compared with the control. In contrast, co-administration of BPF with low and high doses of O3FA prevented the observed alterations in testicular injury markers activities.

3.5 Steroidogenic enzymes

BPF administration led to a significant decrease in 3β-HSD ($p<0.001$) and 17β-HSD ($p<0.001$) compared with the animals in the control group (Figure 3). This observed decrease was ameliorated by the co-administration of BPF with both doses of O3FA. Although both doses of O3FA blunted the observed decrease, the animals treated with high doses exhibited better ameliorative effects than their counterpart treated with low doses.

3.6 Inflammatory markers

Testicular IL-6 ($p<0.0001$), TnF-α ($p<0.001$), MPO ($p<0.001$), NO ($p<0.001$), NFκB ($p<0.0001$), and XO ($p<0.001$) were significantly increased in the animals treated with BPF alone compared with their counterparts in the control group (Figure 4). The observed increase was abolished by low and high-dose treatment of O3FA. The ameliorative effect of O3FA was more pronounced in animals treated with a high dose except in testicular

TABLE 1 Effect of BPF on sperm parameters.

Parameters	Control	BPF	O3FA-L	O3FA-H
Sperm Count (x106/ml)	9.760±0.143	6.300±0.148[a]	9.080±0.097[a,b]	9.640±0.093[b,c]
Motility (%)	86.00±0.548	63.40±0.510[a]	83.40±1.939[b]	86.00±1.095[b]
Abnormal Sperm Morphology (%)	79.578±1.047	49.895±1.894[a]	64.895±1.904[a,b]	68.904±1.894[a,b]

[a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose.

TABLE 2 Effect of BPF on reproductive hormones.

Parameters	Control	BPF	O3FA-L	O3FA-H
Serum LH (mIU/mL)	6.125±0.129	2.500±0.321 [a]	4.313±0.449 a[,b]	5.806±0.374[a,b,c]
Serum FSH (mIU/mL)	4.123±0.0648	2.677±0.149 [a]	3.457±0.236 a[,b]	4.046±0.179 [b,c]
Serum Testosterone (ng/mL)	2.29±0.083	1.229±0.068 [a]	2.078±0.069 a[,b]	2.150±0.100 a[,b,c]
Serum Estradiol (pg/mL)	4.541±0.130	6.998±0.155 [a]	4.889±0.138 a[,b]	4.956±0.149 a[,b]

[a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose; LH, Luteinizing hormone; FSH, Follicle stimulating hormone.

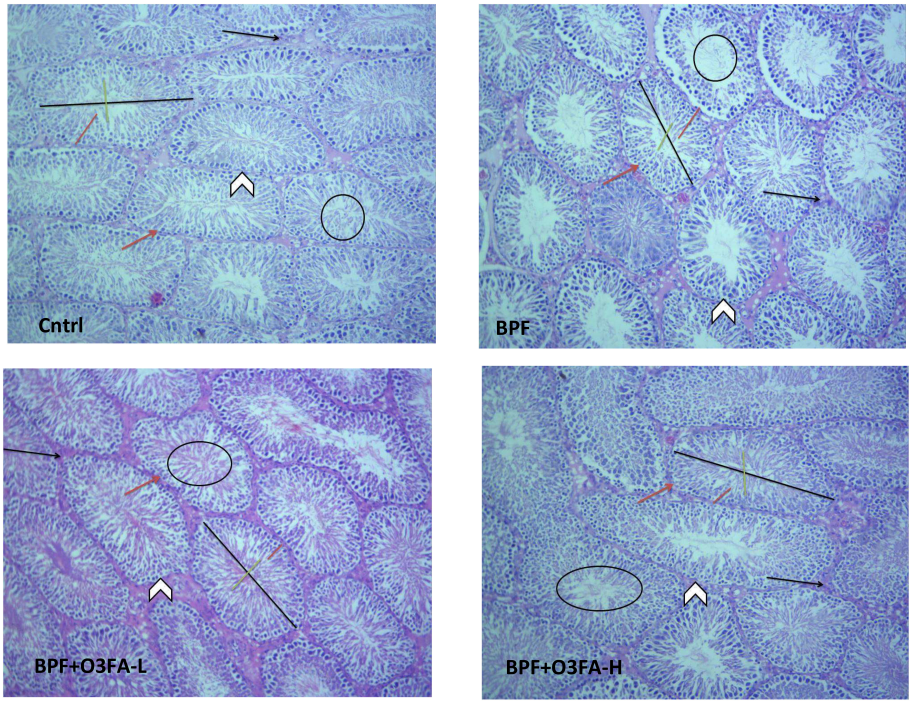

FIGURE 1
Cntrl: The testicular histoarchitecture appears preserved. The seminiferous tubules are normal with germ cells at varying degree of maturation (arrow head). The lumen of the seminiferous tubules shows normal sperm cells (black circle). The Sertoli cells appear normal (red arrow). The interstitial space appears normal with normal Leydig cell mass (black arrow). BPF: The testicular histoarchitecture appears distorted. The seminiferous tubules show germ cells at varying degree of maturation (arrow head). The lumen of the seminiferous tubules shows scanty sperm cells (black circle). The Sertoli cells appear reduced (red arrow). The interstitial space appears normal with reduced leydig cell mass (black arrow). BPF +O3FA-L and BPF+O3FA-H: The testicular histoarchitecture appears preserved. The seminiferous tubules are normal with germ cells at varying degree of maturation (arrow head). The lumen of the seminiferous tubules shows normal sperm cells (black circle). The Sertoli cells appear normal (red arrow). The interstitial space appears normal with normal leydig cell mass (black arrow). Black span: diameter of the seminiferous tubules; red span: epithelial height; green span: diameter of the seminiferous lumen. Stain H and E; x100. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose.

MPO, where there was no significant difference between animals in the BPF+O3FA-L and BPF+O3FA-H group.

3.7 Oxidative stress markers

As shown in Figure 5, BPF exposure led to a significant increase in testicular MDA and a decrease in CAT ($p<0.0001$), SOD ($p<0.0001$), GSH ($p<0.001$), GST ($p<0.001$), GPx ($p<0.001$), and Nrf2 ($p<0.0001$) compared with the control. This observed increase in testicular pro-oxidant and decrease in testicular antioxidants was blunted by co-administration of BPF with low and high doses of O3FA.

3.8 Apoptotic markers

Bisphenol F administration significantly increased DFI ($p<0.001$) and caspase 3 ($p<0.0001$) activities compared with the control group (Figure 6). These observed increases in apoptotic markers were ameliorated by the co-administration of BPF and low and high doses of O3FA.

TABLE 3 Effect of BPF on testicular cytoarchitecture.

Parameters	Control	BPF	O3FA-L	O3FA-H
Testicular histoachitecture	1.333±0.211	3.500±0.224[a]	1.833±0.307[a,b]	1.500±0.224[b,c]
Testicular biopsy score	9.667±0.211	6.833±0.307 [a]	8.667±0.211[a,b]	9.333±0.211[b,c]
Epithelial Height (μm)	68.910±3.863	40.020±2.065 [a]	71.070±6.016[a,b]	73.910±2.050[a,b,c]
Seminiferous Tubular Diameter (μm)	324.7±10.39	189.3±7.35 [a]	300.3±6.30[a,b]	336.0±19.54[a,b,c]
Seminiferous Luminal Diameter (μm)	38.26±5.102	141.5±5.440 [a]	41.62±0.558[a,b]	43.36±3.518[a,b,c]

[a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose.

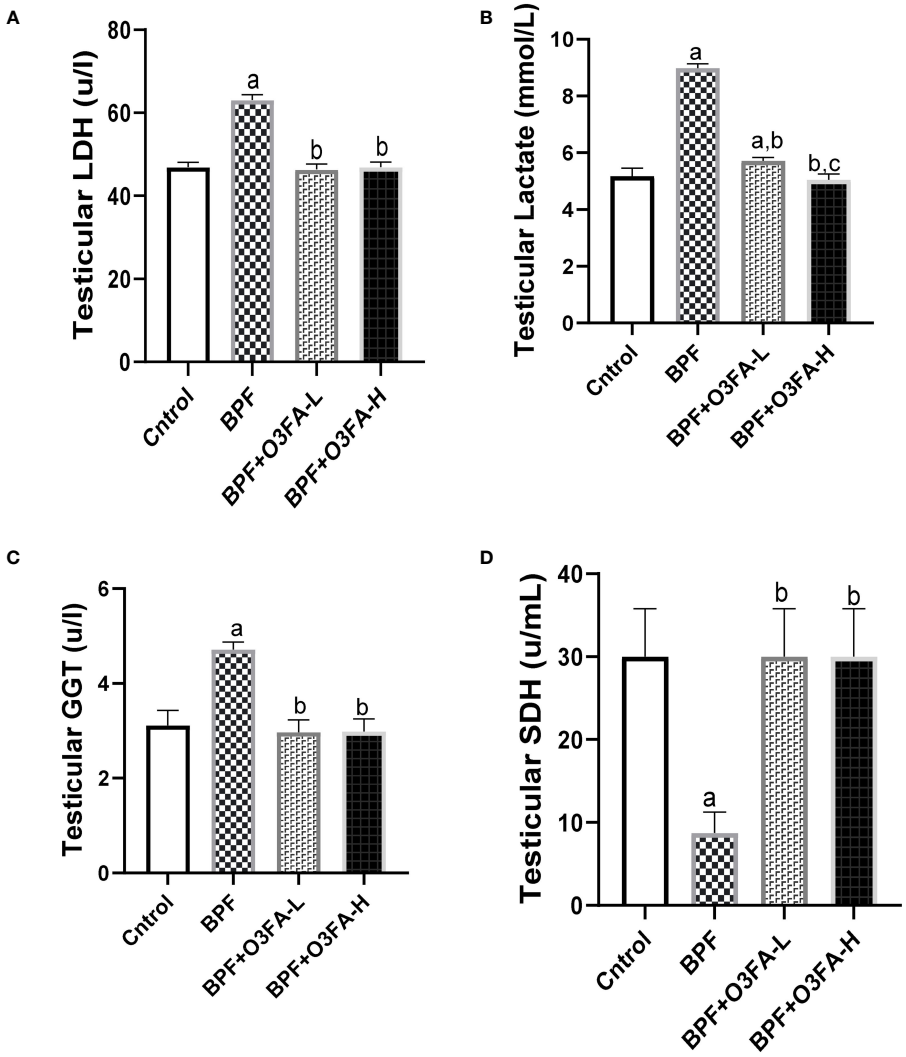

FIGURE 2
Effect of O3FA on testicular **(A)** LDH **(B)** Lactate **(C)** GGT **(D)** SDH in BPF exposed rats. [a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparison. BPF: Bisphenol F, O3FA-L: omega-3 fatty acid low dose, O3FA-H, omega-3 fatty acid high dose; GGT, Gamma glutamyl transpeptidase; SDH, Sorbitol Dehydrogenase.

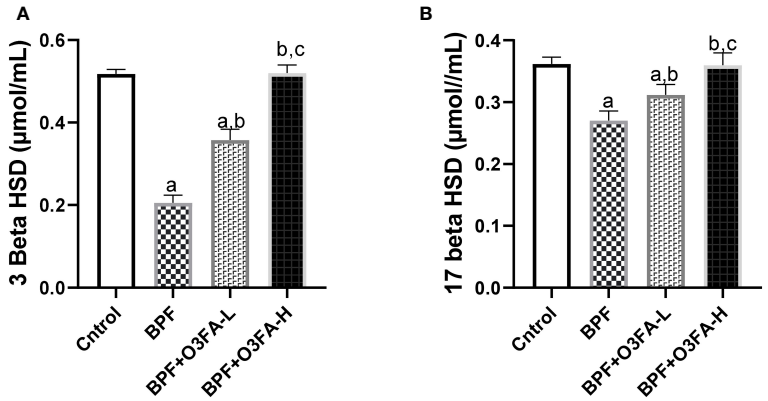

FIGURE 3
Effect of O3FA on testicular **(A)** 3 β-HSD **(B)** 17 β-HSD in BPF exposed rats. [a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose; 3 Beta HSD, 3-Beta–hydroxysteroid dehydrogenase; 17 Beta HSD, 17-Beta hydroxysteroid dehydrogenase.

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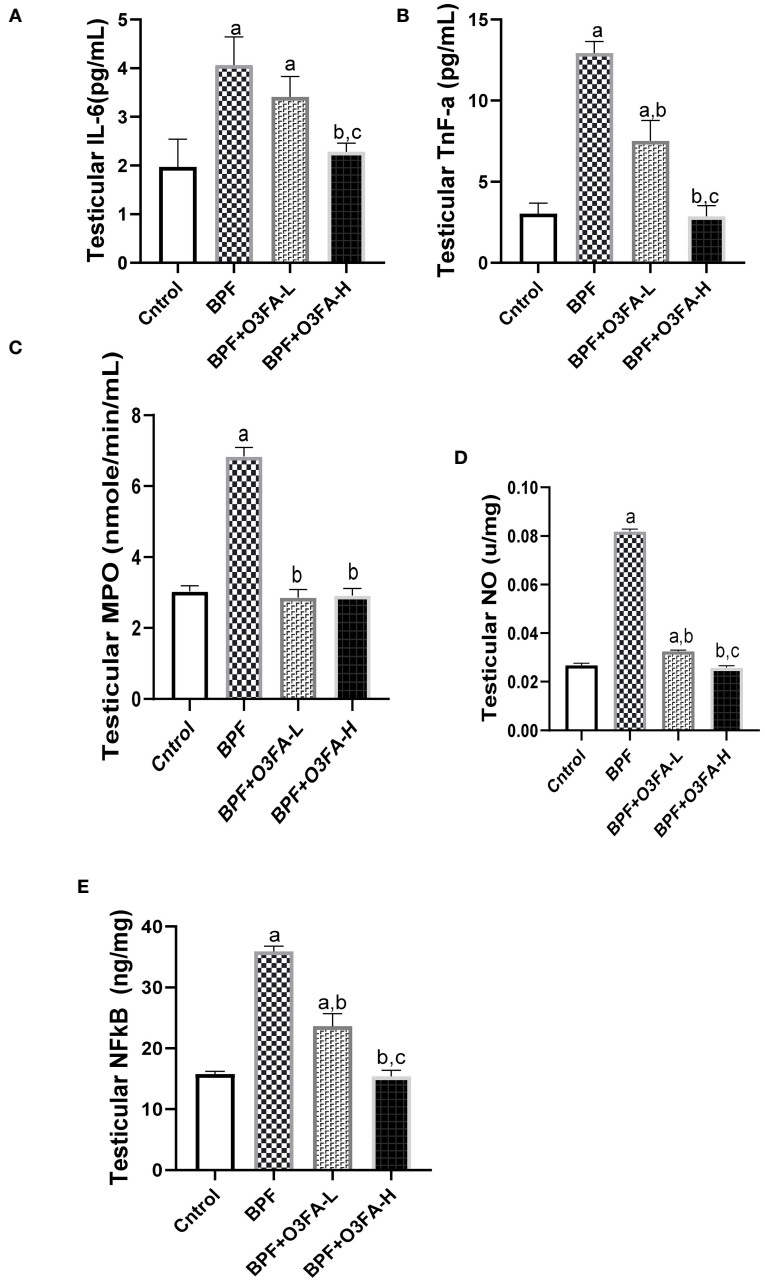

FIGURE 4
Effect of O3FA on testicular **(A)** IL-6 **(B)** TnF-α **(C)** MPO **(D)** NO **(E)** NFκB in BPF exposed rats. [a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose; IL-6, Interleukin-6; TnF-α, Tumor necrosis factor alpha; MPO, Myeloperoxidase; NO, Nitric Oxide; NFκB, Nuclear factor kappa-light-chain-enhancer of activated B cells.

4 Discussion

This study showed that BPF exposure disrupted testicular functions and induced testicular injury in male Wistar rats. BPF-induced hormonal imbalance and impaired sperm quality were associated with impairment in steroidogenic enzyme activities, inflammation, and oxidative stress. The alterations were associated with impaired testicular cytoarchitecture and increased testicular injury markers activities. It was also accompanied by the Nrf2/NFκB pathway distortion and upregulation of caspase 3-mediated apoptosis. Also, this study established the protective role of O3FA in BPF-impaired sperm quality, hormonal imbalance, and oxido-inflammatory injury via the modulation of the Nrf2/NFκB pathway and repression of the caspase 3 pathway.

In the present study, a significant decline in sperm count, motility, and normal morphology of rats exposed to BPF was observed. Furthermore, there was a significant decrease in serum testosterone which was accompanied by a decline in steroidogenic enzyme activities, which are consistent with our previous findings (5, 7). Different mechanisms may explain the reduced sperm quality

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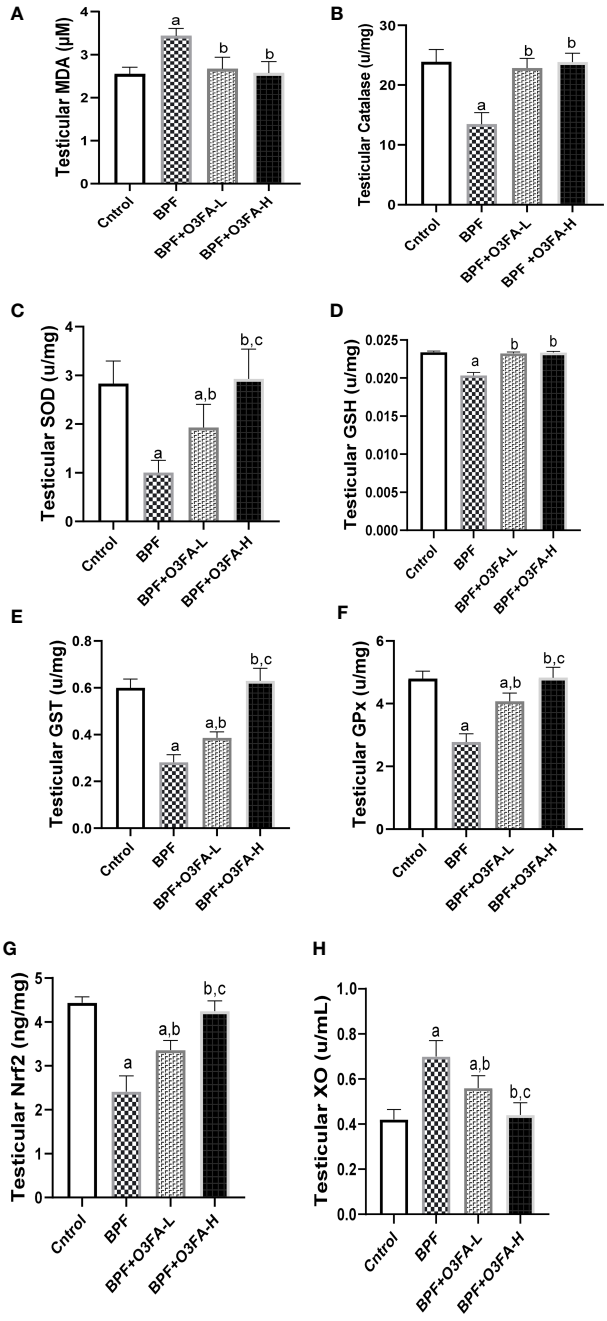

FIGURE 5
Effect of O3FA on testicular **(A)** MDA **(B)** Catalase **(C)** SOD **(D)** GSH **(E)** GST **(F)** GPx **(G)** Nrf2 **(H)** XO in BPF exposed rats. [a]p <0.05 versus control, [b]p < 0.05 versus BPF, [c]p < 0.05 versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose; MDA, Malondialdehyde; SOD, Superoxide dismutase; GSH, Glutathione; GST, Glutathione S-transferases; GPx, Glutathione peroxidase; Nrf2, nuclear factor erythroid 2–related factor 2, XO: Xanthine oxidase.

and circulatory testosterone. The impaired sperm parameters and decline in serum testosterone could be associated with the direct effect of BPF on the testicular tissue leading to male reproductive dysfunction (30). The findings from this study showed that BPF distorted the normal testicular histology by disrupting testicular histoarchitecture and reducing sperm cells in the lumen of the seminiferous tubule, which was accompanied by a distortion in testicular histoarchitecture, mean testicular biopsy score, seminiferous tubular and luminal diameter, and epithelial height. These suggest that BPF-impaired sperm quality via direct testicular damage. Also, BPF-impaired sperm quality and declined testosterone could be due to its endocrine-disrupting activities. The findings from this study that BPF disrupted the hypothalamic-pituitary-gonadal (HPG) axis are consistent with the findings of 31. The HPG axis forms a closed loop, and it is the major signaling pathway controlling reproductive hormone

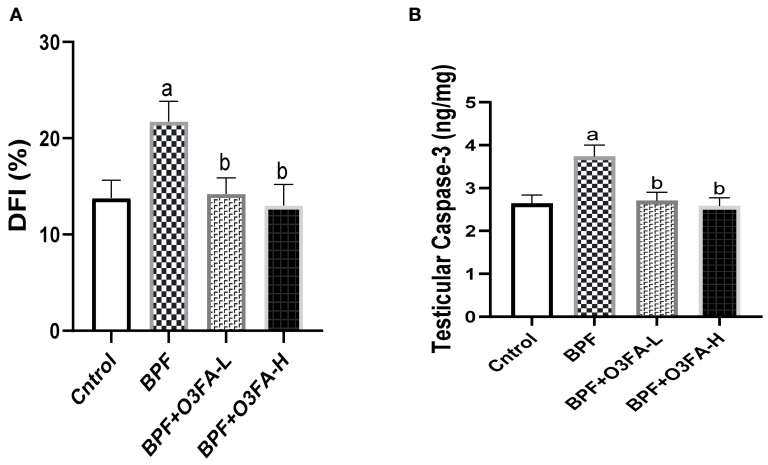

FIGURE 6
Effect of O3FA on testicular **(A)** DFI **(B)** Caspase 3 in BPF exposed rats. [a]p <0.05 versus control, [b]p < 0.05 versus BPF versus BPF + O3FA-L using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparison. BPF, Bisphenol F; O3FA-L, omega-3 fatty acid low dose; O3FA-H, omega-3 fatty acid high dose; DFI, DNA Fragmentation Index.

secretion (32). The hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the production of LH and FSH from the pituitary gland. LH is responsible for stimulating the synthesis of testosterone (steroidogenesis), and FSH stimulates sperm production (spermatogenesis) from the testis suggesting that the observed decline in sperm quality, serum testosterone, and steroidogenic enzymes activities could be via the endocrine disrupting activities of BPF.

Also, the findings that BPF impaired testicular functions via direct testicular cell damage are consistent with the observed increase in testicular injury markers. Testicular activities of LDH, GGT, and SDH are markers of energy balance, spermatogenesis, and Sertoli functions (16). The observed significant increase in testicular lactate following BPF exposure indicates energy imbalance (33) and could result from a BPF-induced increase in the activities of LDH, which is an index of testicular degeneration.

Redox balance plays an integral role in testicular functions (34), and a disturbance in the redox balance leads to oxidative stress. Oxidative stress can activate various transcription factors leading to the activation of inflammatory pathways (35–37). Antioxidant defense systems have been identified to protect against oxidative stress, and Nrf2 is the major transcription factor responsible for regulating redox balance (38). Nrf2 maintained redox balance by regulating antioxidant enzymatic activities responsible for detoxifying and eliminating ROS. In addition to its antioxidant activities, Nrf2 is an anti-inflammatory agent by inhibiting NF-κB activities. NF-κB is responsible for proinflammatory gene induction, which increases inflammatory response (39). The observed decrease in Nrf2 following BPF exposure in this study agrees with the findings of Zhou et al. (40), which associated BPF exposure with decreased Nrf2 expression. This may account for the observed increase in oxidative stress (evidenced by an increase in testicular MDA and a decrease in CAT, SOD, GSH, GST, GPx) and Nf-κB-mediated inflammatory response (evidenced by an increase in testicular IL-6, TnF-α, MPO, NO, and XO).

Furthermore, excessive ROS and inflammation collaborate to stimulate caspase 3-mediated apoptosis (41), which is a contributing factor to male infertility (42). Caspase-3 is a major player in apoptosis initiation because of its involvement in receptor-mediated and the mitochondrial pathway, which are the major apoptotic signal transduction pathways (43). The increase in testicular caspase 3 could explain the increase in testicular DFI in this study since both have been positively related (43). The observed increase in caspase-3 and DFI in this study is similar to the findings of Ferreira et al. (44), which reported an increase in apoptotic markers activities following BPF exposure. Hence, BPF impaired hormonal balance and sperm quality by inducing oxidative stress, inflammation, and apoptosis via the modulation of Nrf2/NF-κB signaling and caspase-3 mediated apoptosis.

Another important finding from this study is the beneficial role of O3FA in BPF-induced testicular dysfunction. The present study revealed that O3FA alleviated BPF-induced testicular damage by suppressing testicular injury markers, oxidative stress, inflammatory response, and apoptotic markers, thus improving sperm qualities, reproductive hormones synthesis, and testicular cytoarchitecture. Although this study demonstrates for the first time that O3FA ameliorates BPF-induced testicular dysfunction, these findings concurred with previous findings that reported the antioxidant (45), anti-inflammatory (46), and antiapoptotic (47) activities of O3FA. The observed redox balance and decreased levels of NF-κB, IL-6, Tnf-α, MPO, and XO in the testicular tissues of O3FA-treated animals possibly explained O3FA-driven repression of apoptotic markers via the upregulation of Nrf2 activities. The gonado-protective effect of O3FA was accompanied by the restoration of testicular histoarchitecture and function by preventing distortion of histoarchitecture, scanty sperm cells in the lumen of the seminiferous tubule, and reduced Leydig cell mass, and normalization of sperm qualities and reproductive hormones.

5 Conclusion

The results from this study showed that O3FA co-treatment suppressed hormonal imbalance, poor sperm quality, oxidative stress, inflammation, and apoptosis via the modulation of Nrf2/NF-κB signaling and caspase-3 mediated apoptosis in BPF-treated rats. These findings suggest a possible insight into the protective molecular mechanisms of O3FA against BPF-induced testicular dysfunction.

6 Limitations and future perspectives

The BPF-treated rats testicular histology showed reduced Sertoli and Leydig cell count, which could result from BPF-induced apoptosis, and the TUNEL assay would establish which of the cells were more affected. However, this special staining was not done. This limitation opens a grey area for future exploration. Nevertheless, the in-depth testicular planimetry analysis and quantitative Sertoli and Leydig cells count in this study strengthened our findings on the distortive activities of BPF on testicular histology and cells.

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 designed experimental protocol was approved by the University of Ilorin Review and Ethical Committee, and in accordance with the National Institute of Health guidelines using the guide for the care and handling of laboratory animals.

Author contributions

AO: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LO: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. WA: Formal Analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – review & editing.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1256154/full#supplementary-material

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Impact of organophosphate pesticides exposure on human semen parameters and testosterone: a systematic review and meta-analysis

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Background: Organophosphate (OP) pesticides have been associated with a decline in semen quality, although there are still considerable arguments about the magnitude of the association.

Objective: This study provides a systematic review and meta-analysis of the impacts of OP pesticides on semen quality and male reproductive hormones.

Methods: This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocols. Strategic search was conducted using combined text words as search terms. The eligibility criteria were developed based on Population, Exposure, Comparator, Outcome, and Study designs (PECOS) framework. Relevant data were extracted, risk of bias was evaluated by The Office of Health Assessment and Translation (OHAT) tool, and certainty of evidence was assessed by the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Working Group guidelines. Quantitative meta-analysis was performed by using Review Manager.

Results: A total of 766 male subjects (349 exposed to OP pesticides and 417 unexposed controls) were included in the meta-analysis. There was no significant difference in the ejaculate volume, seminal fluid volume, sperm multiple anomaly index, sperm, and leukocytes levels of the OP-exposed subjects compared to the control. In addition, OP pesticides exposure did not significantly affect serum concentrations of FSH, LH, and testosterone in subjects who were exposed to OP pesticides compared to their unexposed counterparts. However, we found a significant reduction in the sperm count, sperm concentration, progressive sperm motility, total sperm motility, and normal sperm morphology of OP pesticides-exposed subjects compared to the unexposed subjects. However, after subtype and sensitivity analyses, exposure

to OP pesticides did not reduce sperm count. Also, after sensitivity analysis, OP pesticides exposure did not alter progressive sperm motility.

Conclusion: This study demonstrates that OP pesticides exposure reduced sperm count, concentration, total and progressive motility, and normal sperm morphology, possibly via a testosterone-independent mechanism.

KEYWORDS

endocrine disruptors, environmental toxicants, hormone imbalance, male infertility, organophosphate, pesticides, sperm, testosterone

Introduction

An estimate of about one in six (approximately, 15%) couples are affected by infertility globally, and about 50% of this is due to male factor only and in combination with female factor (1–3). This has been associated with the global decline in sperm quality (4, 5), which occurs in concert with hormonal disruption (4). Testicular pathologies (such as cryptorchidism, testicular torsion and testicular cancer) (6–8), lifestyle factors, such as diets, smoking, energy dyshomeostasis and metabolic disorders (9–11), viral infections (12, 13), pharmaceuticals (14), and environmental toxicants, such as plasticizers and pesticides (15, 16) have been implicated in the pathogenesis of hormonal disruption and decline in sperm quality.

Although several human and experimental studies have shown that pesticides negatively alter normal physiological processes (17–20), they also act as endocrine-disrupting chemicals, leading to alterations in the normal hormonal milieu and reduced sperm quality (21, 22). Organophosphates are widely used pesticides for domestic and agricultural purposes (19, 20); however, they have been linked with endocrine disruption and poor sperm quality. A substantial body of evidence has demonstrated that organophosphate (OP) pesticides exert adverse effects on male reproductive hormones and sperm quality. However, most of these studies are on animal models and data on humans are limited with insufficient evidence to support this claim.

A cross-sectional study among Venezuelan farmer workers and unexposed control revealed that exposure to OP pesticides was negatively correlated with sperm concentration, morphology, and viability, while circulating testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were not altered (23). In another cross-sectional study among Peruvian pesticide sprayers, observed a significantly lower ejaculate volume, sperm motility, and normal morphology as well as serum LH and testosterone levels among OP-exposed workers when compared with the control. Padungtod et al. (24) documented that exposure to OP pesticides among Chinese pesticide factory workers led to reduced sperm concentration and motility. Recio-vega et al. (25) however observed that OP pesticides exposure significantly reduced ejaculate volume and sperm count, but not motility and viability, while Hossain et al. (26) showed that OP pesticides exposure significantly reduced sperm concentration, motility, viability, and normal morphology. Unexpectedly, GhafouriKhosrowshahi et al. (27) reported that OP pesticides markedly reduced sperm count and motility but increased serum testosterone while ejaculate volume, semen pH and normal sperm morphology were not significantly affected. This is similar to the findings of Kamijima et al. (28) that observed a marked increase in serum testosterone levels among OP pesticides users. Fascinatingly, Multigner et al. (29) did not observe any significant difference in sperm parameters and serum male reproductive hormones in banana plantation workers that were exposed to OP pesticides and the unexposed counterparts, although they found significantly reduced testosterone levels in rats captured in the banana plantations compared with the control rats.

The World Health Organization (WHO) and the International Labour Organization (ILO) recommend a systematic review and meta-analysis of studies with estimates of the effects of occupational exposure with disease risk to estimate the burden of a particular exposure. In a meta-analysis, Giulioni et al. (30) demonstrated a significant reduction in ejaculate volume [Weighted mean difference (WMD) −0.47ml, 95%CI −0.69 to −0.25; $p < 0.0001$), sperm count (WMD-40.03, 95%CI −66.81 to −13,25; $p = 0.003$), concentration (WMD-13.69 x10^6/mL, 95%CI −23, 27 to-4.12; $p = 0.005$) and motility (WMD −5.70%, 95%CI −12.89 to 1.50; $p = 0.12$) in OP pesticides-exposed workers. Although the negative association of organophosphates with spermatogenesis is noteworthy, the findings of Giulioni et al. (30) are with some shortcomings. First, some major studies were missing; only six studies were included in their study. This might have influenced their findings. In addition, Giulioni et al. (30) did not conduct a subtype and sensitivity studies to determine the source of heterogeneity. Moreso, the report of Giulioni and his colleagues did not appraise individual study included, thus the quality of evidence, publication bias, risk of bias, and certainty of evidence are unknown.

In a nutshell, human data on semen quality and male reproductive hormones in association with OP pesticides exposure are limited and inconsistent. Hence, the aim of this study was to analyze the association between OP pesticides exposure, sperm quality and testosterone levels through a systematic review and meta-analysis. Also, a comprehensive

review of the associated mechanisms of OP pesticides-induced male reproductive dysfunction was presented. This study provides an in-depth understanding of the effect and associated mechanisms of OP pesticides on male reproductive function. The research question was structured according to PECOS statement (Population, Exposure, Comparators, Outcomes, and Study design); "what is the effect of OP pesticides exposure on human semen parameters and testosterone?".

Methods

Literature search

This systematic review and meta-analysis was conducted on previously published articles that reported the impact of OP pesticides on semen quality and serum testosterone levels according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocols (31). We conducted a systematic electronic search on CNKI, Cochrane Library, EMBASE, Pubmed/Pubmed Central, Scopus, Science Direct/Elsevier, and Web of Science database to identify published studies from inception to October 2022. The language and study type were not restricted. The search terms combined text words. The search terms for OP pesticides were: 'OP pesticides', 'organophosphate chemical', 'organophosphate', 'OP chemical', an 'OP'. The search terms for semen parameters were: 'sperm', 'sperm cell', 'spermatozoa', 'semen analysis', 'seminal fluid analysis', 'sperm parameters', 'sperm variables', 'sperm count', 'sperm concentration', 'sperm motility', 'sperm viability', 'sperm vitality', 'sperm morphology', 'semen volume', 'ejaculate volume', 'seminal pH', 'seminal leukocyte'. The search terms for male reproductive hormones were: 'testosterone', 'luteinizing hormone', 'LH', 'follicle stimulating hormone', 'FSH', and 'male reproductive hormone'. All relevant articles and abstracts were retrieved. In addition, references cited in relevant articles were manually retrieved. The search strategy was pilot-tested and tested against benchmark papers.

Selection of studies and validity assessment

The eligibility criteria for studies included in the meta-analysis were developed based on PECOS framework denoting the Population, Exposure, Comparator, Outcome, and Study designs of interest as stated below.

Inclusion criteria:

i. Population: The population studied exclusively included male adults in their reproductive age group.
ii. Exposure: Studies that investigated the effect of one or more OP pesticides exposure, originating from domestic use or occupational exposure, for at least six months.
iii. Comparator: The studies must compare the OP-exposed individuals with normal age-matched unexposed male subjects.
iv. Outcomes: The association between OP pesticides exposure and semen parameters as well as serum testosterone levels is quantitatively reported. The mean and standard deviation could also be calculated from the provided data.
v. Study design: The study design is either case-control, cohort, cross-sectional or ecological. These studies must be designed to adequately answer the research question "what is the effect of OP pesticides exposure on human semen parameters and testosterone?".

Exclusion criteria:

i. Population: Studies on male animal models and *in vitro* studies were not considered eligible
ii. Exposure: Studies on prenatal OP pesticide exposure were excluded. Also, studies on adult male exposure to pesticides other than OP pesticides were not included in this study.
iii. Comparator: Studies without unexposed healthy control adult males were excluded.
iv. Outcome: Studies that did not report numerical exposure variable and has higher risk of exposure misclassification and residual confounding were not included in this study. In addition, studies reporting health outcome by self-diagnosis were excluded.
v. Study design: Studies that were not original studies (such as case reports, review articles, commentaries, letters, and editorials) were not considered eligible for inclusion.
vi. Conference abstract, thesis, preprint, or not peer reviewed/grey literature, literature review and systematic review articles were excluded.
vii. Retracted papers
viii. Studies that were not published in a peer-reviewed scholarly journal.
ix. Studies not written and published in English.

Two reviewers (ATM and AAE) independently screened the titles and abstracts of all the citations from the literature search. Relevant studies that met with the eligibility criteria were retrieved. The full text was analyzed if an equivocal decision was made on the basis of the title and abstract, and the final decision of eligible studies was made by reviewing the article. Disagreements were resolved by consensus or a third reviewer (HMA or ARE).

Data extraction

The following details were extracted from each eligible study:

i. Authors' names
ii. The year the study was published
iii. Study design
iv. Country
v. Type of OP pesticides
vi. Number of examined exposed and unexposed (control) subjects
vii. Age of subjects

viii. Duration of exposure to OP pesticides
ix. Outcomes/variables measured

Quality of evidence assessment

The quality of each study included in the meta-analysis was assessed using the ErasmusAGE quality score for systematic reviews. The five domains assess included study design, study size, method of measuring exposure, method of measuring outcome, and analysis with adjustment. These domains were scores as: study design (0 = cross-sectional study, 1 = longitudinal study, 2 = intervention study), study size (0 = <50, 1 = 50 to 150, 2 = >150 participants), method of measuring exposure (0 = not reported, 1 = moderate quality exposure, 2 = good quality exposure), method of measuring outcome (0 = no appropriate outcome reported, 1 = moderate

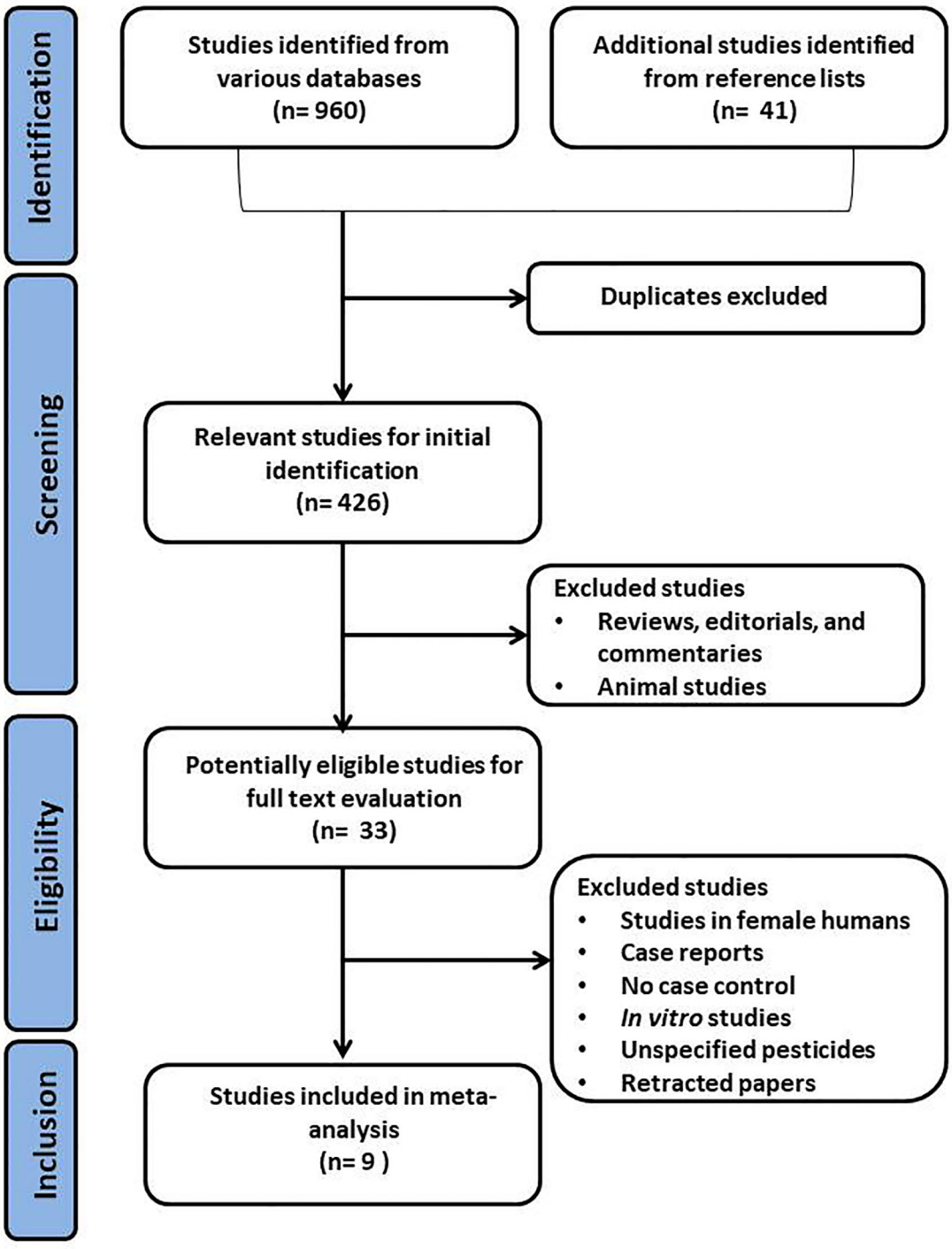

FIGURE 1
Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of the selection process for eligible studies. .

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outcome quality, 2 = adequate outcome quality), and analysis with adjustments (0 = no adjustments, 1 = controlled for key confounders, 2 = additional adjustments for confounders) (32).

Risk of bias assessment

The risk of bias (RoB) assessment was done by three reviewers (ATM, AAE, and HMA) for each study. Conflicts were resolved by the fourth reviewer (ARE). The Office of Health Assessment and Translation (OHAT) tool was used to assess the RoB for each included study. The six domains assess included selection bias, confounding bias, attrition/exclusion bias, deletion bias, selective reporting bias, and other bias. Each domain will be adjudged definitely low risk of bias, probably low risk of bias, definitely high risk of bias, or probably high risk of bias per study (33). Also, we visually assessed the total publication bias using the funnel plot generated by Review Manager (RevMan) software.

TABLE 1 Eligible studies included in the meta-analysis that reported the effects of organophosphate pesticides on semen quality and male sex hormones.

References	Study design	Country	Type of OP	Examined population	Age (years)	Duration of exposure (years)	Outcomes/variables measured
37	Cross-sectional	China	Ethyl parathion, methamidophis	13 pesticide industry workers and 16 unexposed control	19-50 vs 22-47	3 to 24	Sperm concentration, total motility, and morphology
24	Cross-sectional	China	Ethyl parathion, methamidophis, methyl parathion	32 pesticide industry workers and 43 unexposed control	31±9 vs 30±8	12±9	Ejaculate volume, sperm count, concentration, total motility, progressive motility, and morphology
28	Cross-sectional	Japan	Fentothion, dichlorvos, chlorpyrifos, chlorpyrifos-methyl, diazinon, propetanphis,	15 pesticide industry workers and 16 unexposed control in summer; 14 pesticide industry workers and 15 unexposed control in winter	33.8±7 vs 34.5 ±7.5	0.5 to 25	Ejaculate volume, sperm concentration, count, viability, total motility, progressive motility, and morphology; FSH, LH, and testosterone
	Cross-sectional	Peru	Methamidophis	31 pesticide industry workers and 80 unexposed control	29.7±7.1 vs 32.8 ±7.6	–	Ejaculate volume, seminal fluid pH, concentration, count, viability, total motility, progressive motility, morphology, leukocyte, LH, FSH, testosterone
29	Cross-sectional	France	Cadusaphos, ethoprophos, isazophos, pyrimiphos-ethyl, terbulos	42 banana plantation workers and 45 unexposed control	34.8±6.3 vs 38.4 ±7.6	–	Ejaculate volume, seminal fluid pH, sperm concentration, count, total motility, progressive motility, morphology, multiple anomaly index, viability, FSH, LH, and testosterone
38	Cross-sectional	Mexico	Methylparathion, metamidiphis, endosulfan, dimethoate, diazinon	46 agriculture workers and 47 unexposed control	19-46 vs 18-47	–	Ejaculate volume, concentration, count, total motility, progressive motility, and viability
26	Cross-sectional	Malaysia	Malathion, paraquat	62 rural farmers and 90 unexposed control	–	–	Ejaculate volume, seminal fluid pH, sperm concentration, motility, morphology, leukocyte
23	Cross-sectional	Venezuela	Unspecified	64 agricultural workers and 35 unexposed control	18-52 vs 18-42	<2 to >5	Ejaculate volume, sperm, seminal fluid pH, concentration, count, motility, morphology, viability, multiple anomaly index
27	Cross-sectional	Iran	Unspecified	30 rural farmers and 30 unexposed control	20-40	–	Ejaculate volume, seminal fluid pH, sperm count, total motility, progressive motility, morphology, FSH, LH, and testosterone

OP, Organophosphate pesticides; * study that reported outcomes/variables in two seasons, summer and winter.

Certainty of evidence assessment

The confidence in the body of evidence was rated using OHAT approach for systematic review and evidence integration for literature-based health assessment (34). This is based on the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Working Group guidelines (35). Four descriptors were used to indicate the level of confidence; high, moderate, low, and very low (36).

Meta-analysis

Quantitative meta-analysis was performed by using Review Manager (RevMan) software (version 5.4.1; the Nordic Cochrane Centre, the Cochrane Collaboration, 2012, Copenhagen, Denmark). Available data were analyzed in a meta-analysis, comparisons were made between the populations that were exposed to OP pesticides and the control groups and referred to as "exposed" and "unexposed".

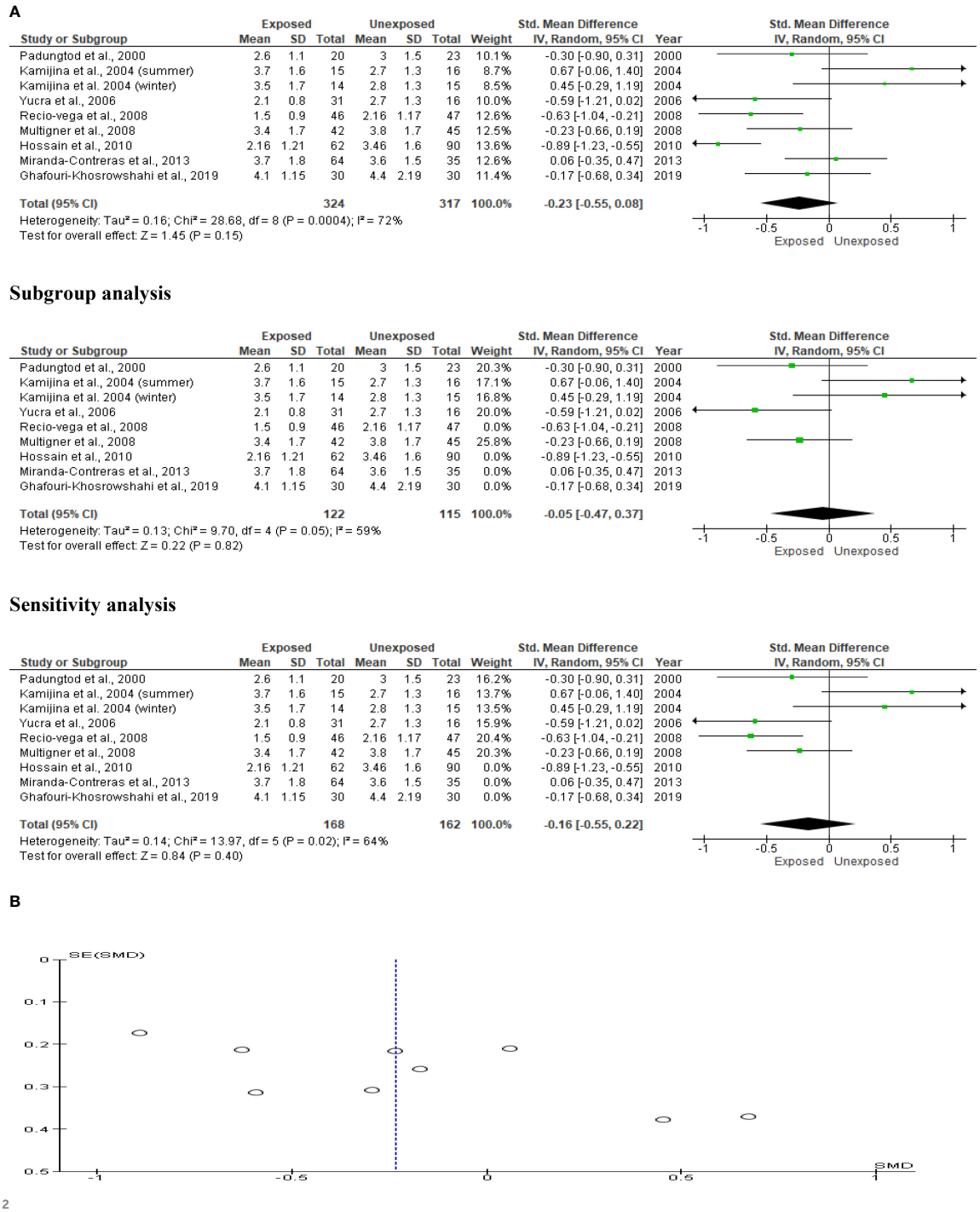

FIGURE 2
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on ejaculate volume (mL).

The standardized mean difference (SMD) of each reported variable was pooled from the included studies, which was identified with 95% confidence intervals (95% CIs). The P-value and I-square statistic (I^2) in the pooled analyses were used to determine the heterogeneity of the studies, representing the percentage of total variation across studies. The summary estimate was analyzed in a random-effects model if the P-value was less than 0.1 or the I^2-value greater than 50%; otherwise, a fixed-effects model was used. Visual symmetry of funnel plots was used to determine publication bias. The asymmetry of the funnel plot suggests possible publication bias.

Subgroup and sensitivity analysis

To investigate possible sources of heterogeneity, we conducted subgroup analyses, excluding studies with exposure to unspecified organophosphates (which included 23 and 27). Also, studies with exposure to non-OP pesticides in addition to OP pesticides were excluded (which included 25 and 26). In addition, the study with participants older than 50 years (23) was excluded.

Sensitivity analyses were performed excluding the study with the largest weight, studies with at least one domain with "definitely high risk of bias" or "probably high risk of bias", studies with low or very low confidence of evidence, studies with quality of evidence ≤ 5.

Systematic review on mechanisms from animal and human *in vitro* studies

A comprehensive review of animal and human *in vitro* studies related to the effects and the associated mechanisms of OP

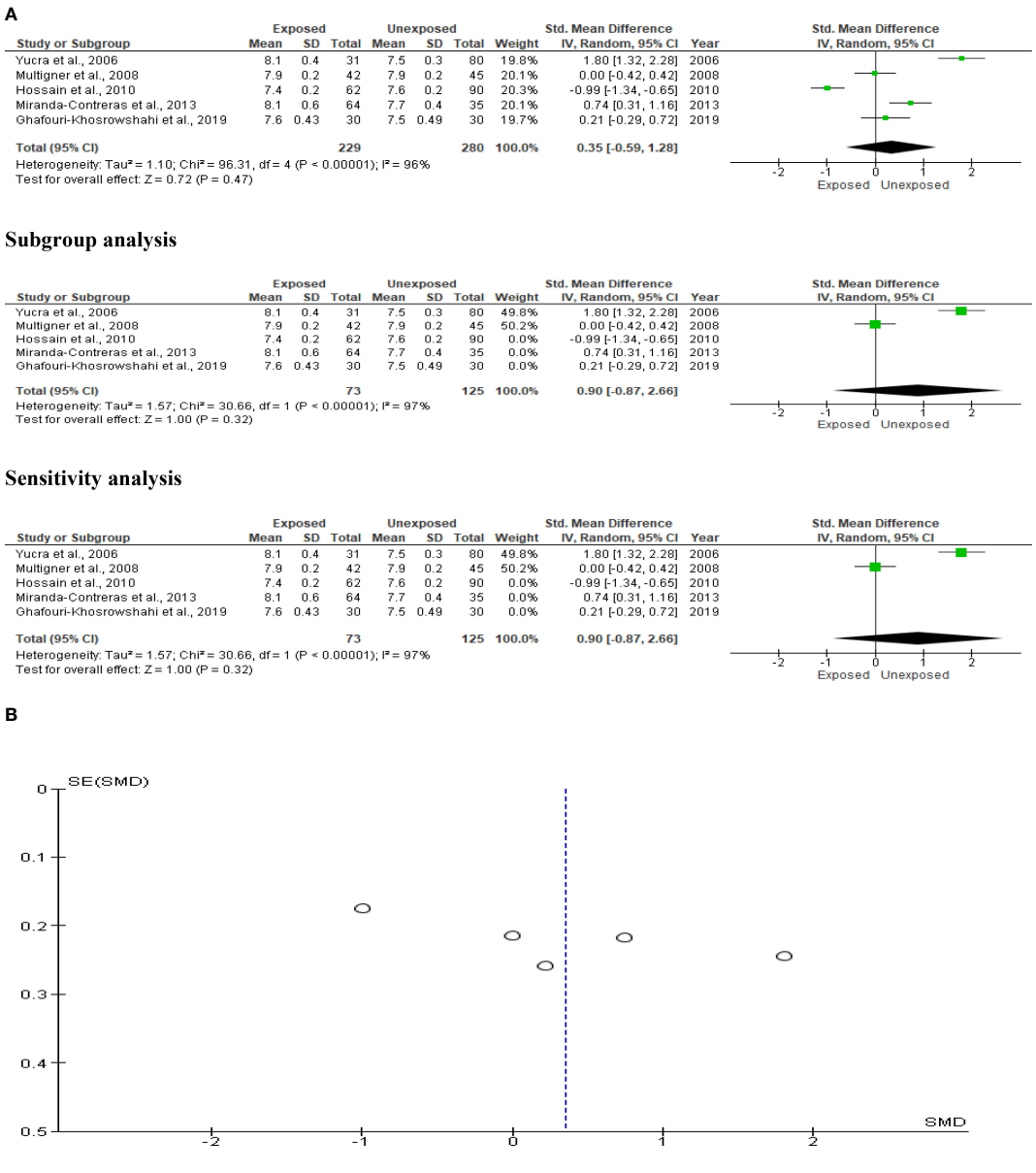

FIGURE 3
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on seminal fluid pH.

pesticides and sperm quality and testosterone levels was also conducted.

Results

Study characteristics

Using the above-mentioned search strategy, 9 articles were identified as eligible for this study (Figure 1). Two of the studies were from China, and one each from Japan, Peru, France, Mexico, Malaysia, Venezuela, and Iran. Two of the studies did not specify the types of OP pesticides used, while the remaining 7 did. The characteristics of the selected studies are presented in Table 1. The study consisted of a total of 766 male subjects (349 exposed to OP pesticides and 417 unexposed controls).

Ejaculate volume

Eight studies assessed the impact of OP pesticides exposure on ejaculate volume (324 in the exposed group and 317 in the unexposed

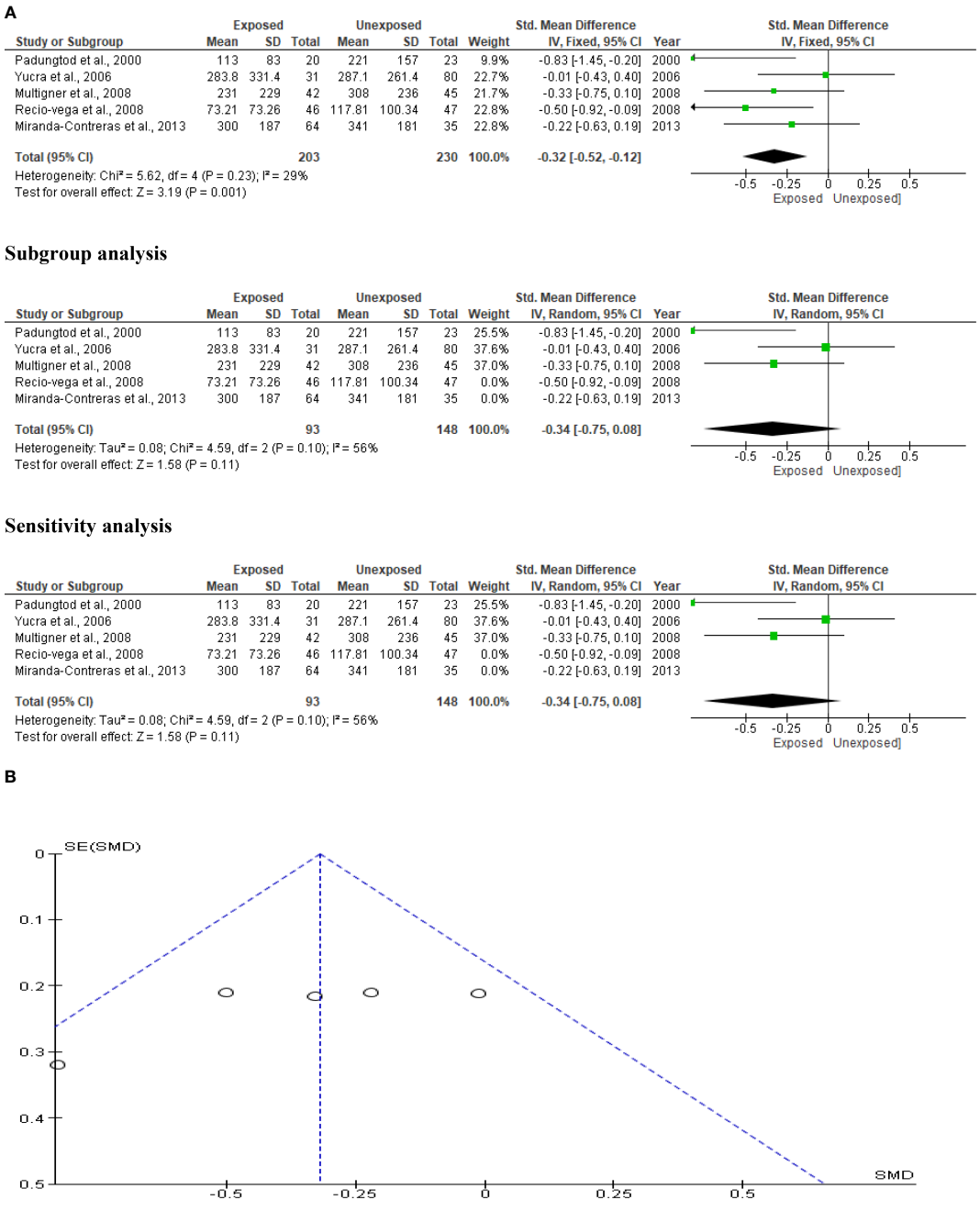

FIGURE 4
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on sperm count (x 10^6).

control group). Kamijina et al. (28) examined this in two seasons; summer and winter. There was no significant difference in the ejaculate volume of the OP-exposed subjects compared to the control (SMD -0.23 [95% CI: -0.55, 0.08]; p=0.1), with the presence of significant inter-study heterogeneity (I^2 = 72%; χ2 p=0.0004) (Figure 2). There was no significant publication bias. We also found out that OP exposure did not significantly alter ejaculate volume after subtype and sensitivity analyses were conducted (Figure 2).

Seminal fluid pH

Only five studies were included in the seminal fluid pH analysis, with a total of 229 exposed subjects and 280 unaffected controls. The analysis revealed that OP pesticide exposure had no effect on seminal fluid volume (SMD 0.35 [95% CI: -0.59, 1.28]; p=0.47), with significant inter-study heterogeneity (I^2 = 96%; χ2 p0.00001). There was evidence of publication bias. After performing subtype and sensitivity analyses, we found that OP exposure had no impact on seminal fluid pH (Figure 3).

Sperm count

The analysis included five studies that reported data on OP pesticide exposure and sperm count. In a total population of 433 subjects, we found a significant reduction in sperm count of OP pesticide-exposed subjects compared to unexposed subjects (SMD-0.32 [95% CI: -0.52, -0.12] p=0.001), with no significant inter-study heterogeneity (I^2 = 29%; χ2 p=0.23). There was no evidence of

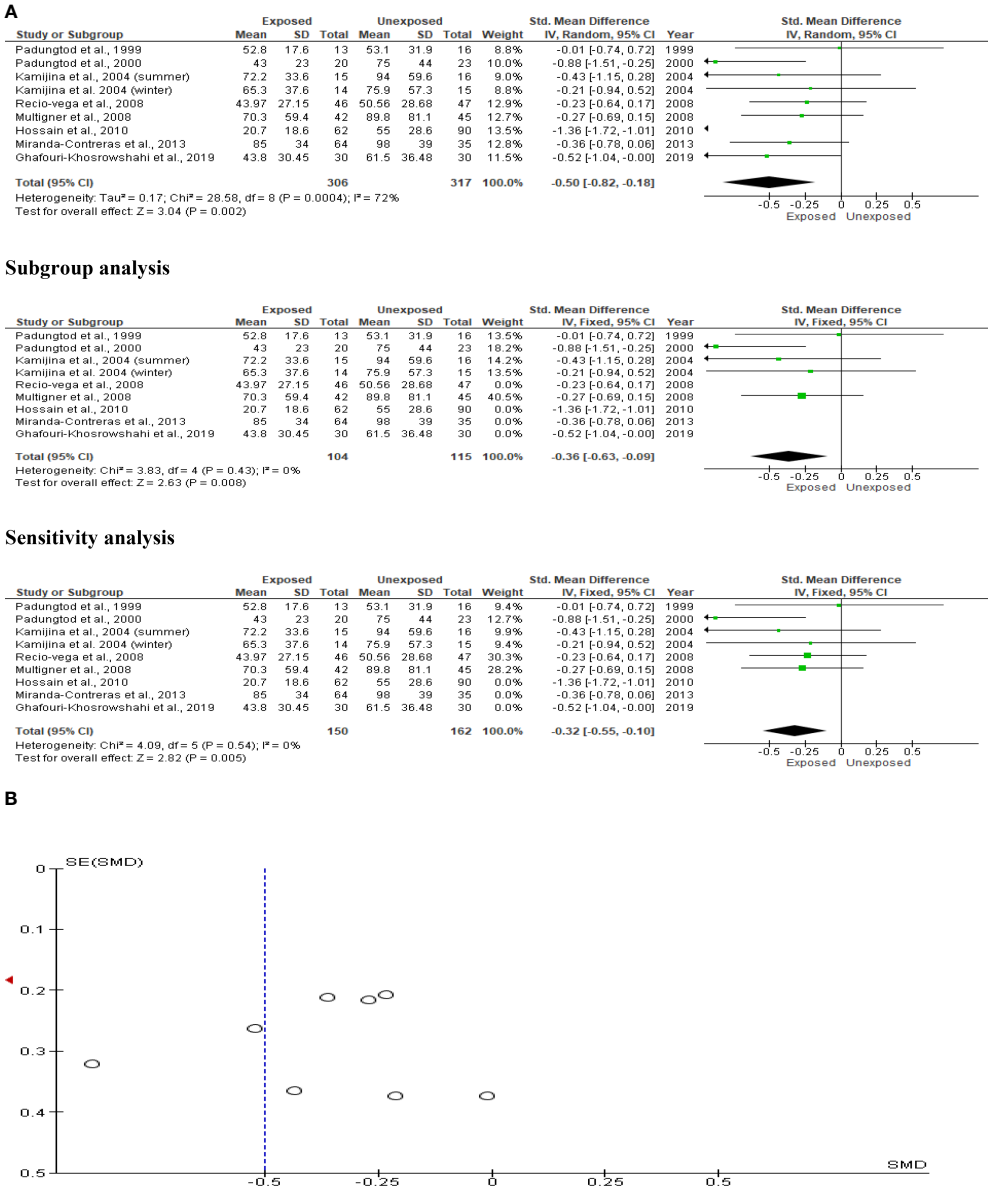

FIGURE 5
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on sperm concentration (x 10^6/mL).

publication bias. However, exposure to OP pesticides did not substantially decrease sperm count after subtype and sensitivity analyses (Figure 4).

Sperm concentration

Kamijina et al. (28) investigated this in two seasons, summer and winter, allowing them to analyze the results in 623 subjects (306 exposed subjects and 317 unaffected controls). The sperm concentrations of OP pesticide-exposed subjects were significantly lower than controls (SMD -0.50 [95% CI: -0.82, -0.18] p=0.002), with significant inter-study heterogeneity (I^2 = 72%; $\chi 2$ p=0.0004). The publication bias was significant. Even after subtype and sensitivity analyses, the observed significant reduction in sperm concentration persisted (Figure 5).

Progressive sperm motility

The effect of OP pesticides on progressive sperm motility was studied in six studies, with Kamijina et al. (28) reporting findings in

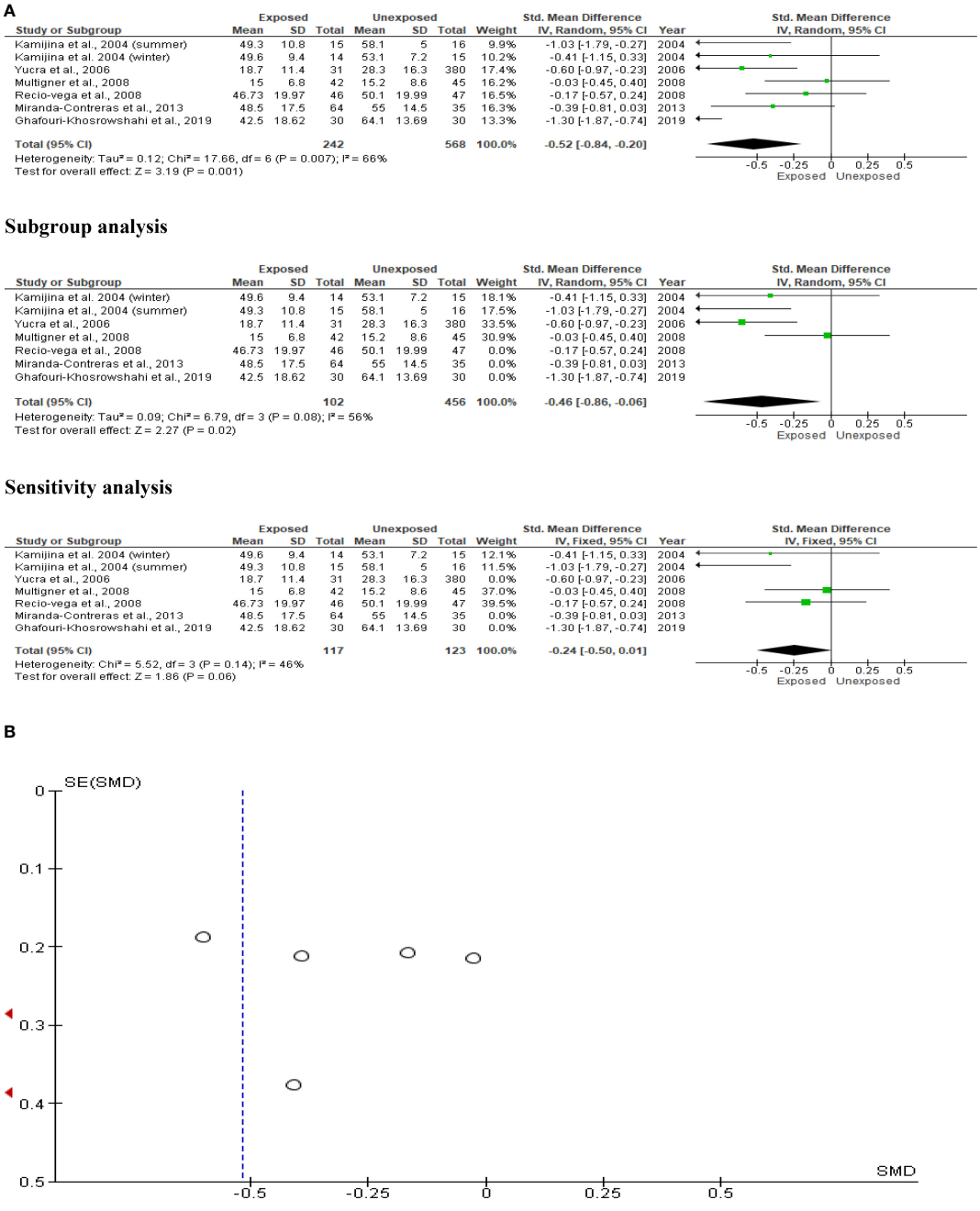

FIGURE 6
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on progressive sperm motility (%).

both the summer and winter seasons. There were a total of 810 subjects (242 exposed subjects and 568 unexposed controls). Subjects exposed to OP pesticides had significantly lower progressive sperm motility than those not exposed (SMD -0.52 [95% CI: -0.84, -0.20] p=0.001). Inter-study heterogeneity was significant (I^2 = 66%; $\chi 2$ p=0.007). The Funnel plot was significantly asymmetrical, indicating that publication bias was present. The observed significant reduction in progressive sperm motility remained after sensitivity analysis, but it became comparable between the OP-exposed and unexposed groups (Figure 6).

Total sperm motility

The total sperm motility analysis comprised nine studies with a total of 734 participants (337 exposed and 397 controls). Kamijina et al. (28) investigated this in the summer in addition to the winter. Total

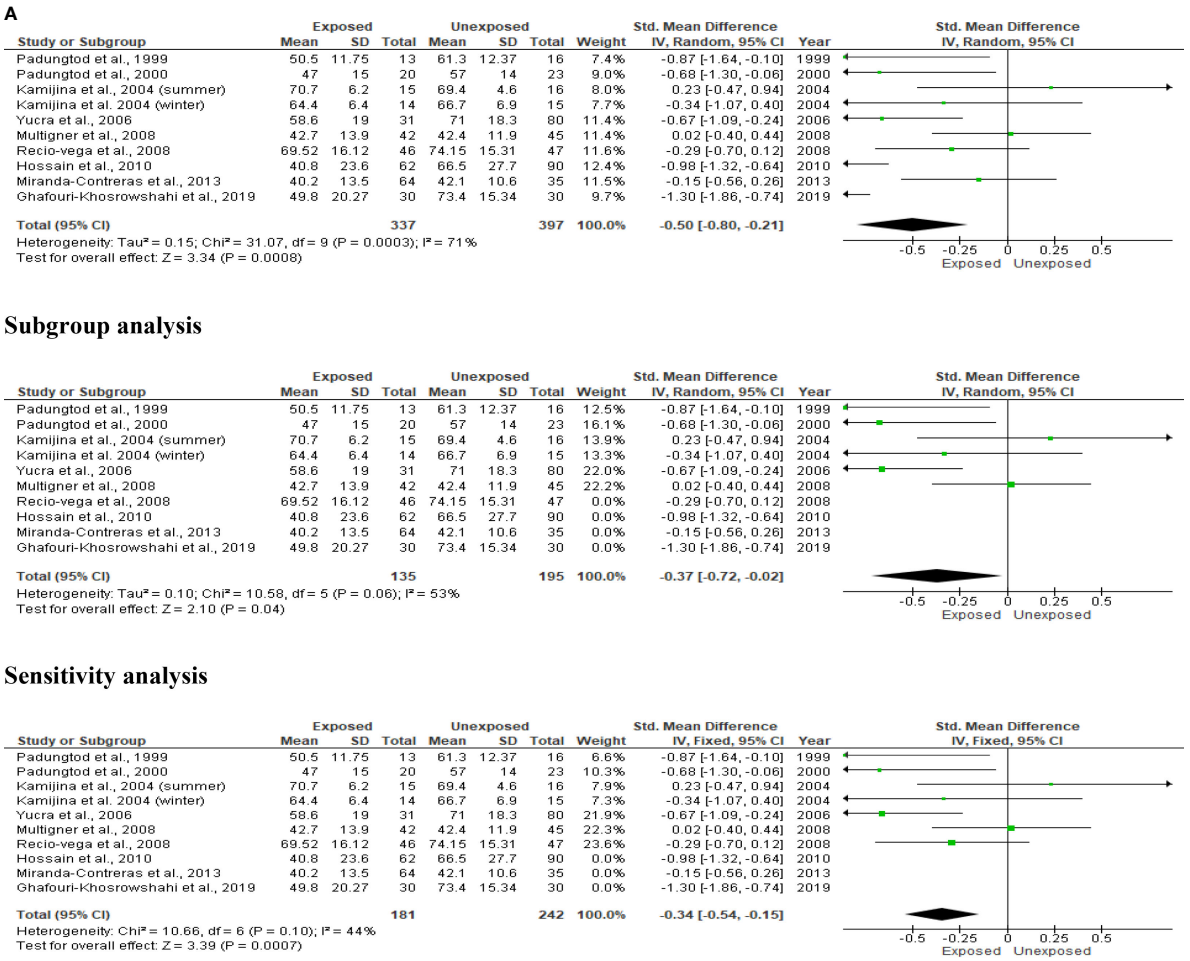

FIGURE 7
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on total sperm motility (%).

sperm motility in OP pesticide-exposed individuals was significantly lower than in controls (SMD -0.50 [95% CI: -0.80, -0.21] p=0.0008). Inter-study heterogeneity was significant (I^2 = 71%; $\chi 2$ p=0.0003). The asymmetry of the Funnel plot indicated significant publication bias. The observed significant reduction in total sperm motility persisted even after subtype and sensitivity analyses (Figure 7).

Sperm morphology

The sperm morphology analysis comprised eight studies with a total of 641 men (291 exposed and 350 unexposed controls). Kamijina et al. (28) examined winter and summer variations of this. The exposed subjects had significantly fewer sperm with normal morphology (SMD -0.49 [95% CI: -0.93, 0.06] p=0.03) than the unexposed subjects. Highly significant inter-study heterogeneity was noted (I^2 = 85%; $\chi 2$ p= 0.00001). Asymmetry in the funnel plot, which was discovered, is yet another indication of publication bias. This sperm morphology result was not influenced by subtype and sensitivity analyses (Figure 8).

Sperm multiple anomaly index

The analysis of the sperm multiple anomaly index only included two studies, totaling 106 subjects exposed to OP pesticides and 80

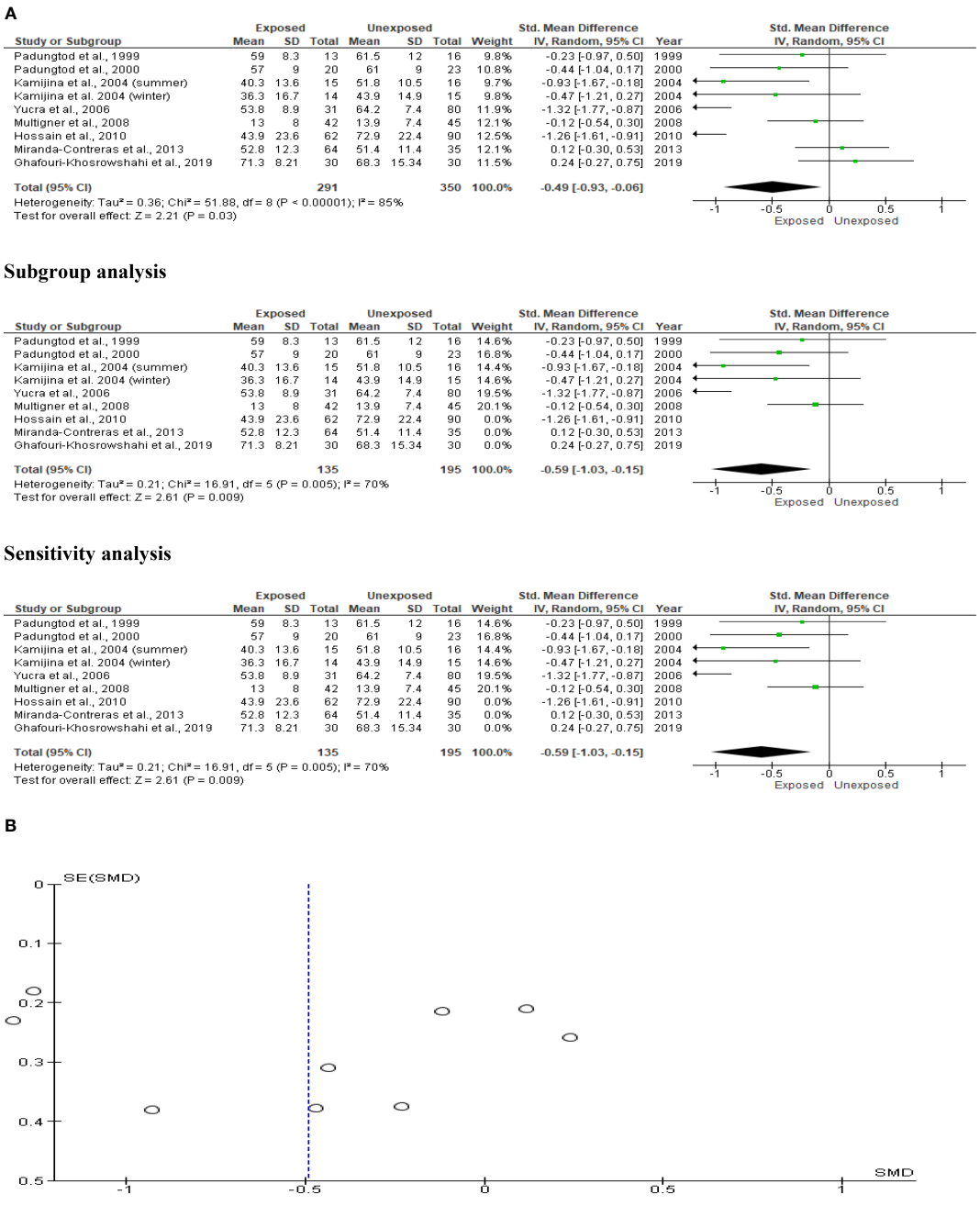

FIGURE 8
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on normal sperm morphology (%).

unexposed controls. When compared to the unexposed control, there was no discernible difference between exposure to OP pesticides and the sperm multiple anomaly index (SMD -0.01 [95% CI: -0.31, 0.28]; p=0.92). Additionally, there was no discernible inter-study heterogeneity (I^2 = 0%; $\chi 2$ p =0.57). Confirming the absence of publication bias, funnel plot symmetry was also discovered (Figure 9).

Sperm viability

Sperm viability was examined in five studies involving a total of 212 exposed participants and 238 controls. Kamijina et al. (28) conducted an evaluation of this during the summer and the winter. When compared to unexposed controls, exposure to OP pesticides did not significantly affect sperm viability (SMD -0.23 [95% CI: -0.56, 0.11]; p=0.19). Inter-study heterogeneity was significantly evident in the analysis (I^2 = 65%; $\chi 2$ p =0.01). Furthermore, funnel plot asymmetry was found, which is consistent with the presence of publication bias. This observation in sperm viability did not change after subtype and sensitivity analyses (Figure 10).

Leukocyte level

Only two studies with a total of 263 subjects (93 exposed and 170 unexposed controls) were included in the analysis of sperm leukocytes. Increased, but marginal, leukocyte levels were observed in OP pesticides-exposed subjects compared to the unexposed controls (SMD 0.98 [95% CI: 0.02, 1.95] p=0.05). Significant inter-study heterogeneity was observed (I^2 = 91%; $\chi 2$ p=0.0006). The observed symmetry of the Funnel plot denoted no publication bias (Figure 11).

Serum FSH

The effect of OP pesticide exposure on serum FSH was studied in four studies, with Kamijina et al. (28) reporting results in both the summer and winter seasons. There were 318 subjects in total (132 exposed subjects and 186 unexposed controls). OP pesticides exposure did not significantly affect serum FSH concentrations in subjects who were exposed to OP pesticides compared to their unexposed counterparts (SMD -0.07 [95% CI: -0.30, 0.16] p=0.55). There was no significant inter-study heterogeneity observed (I^2 = 8%; $\chi 2$ p=0.38). The Funnel plot was asymmetrical denoting the presence of publication bias. This observation in serum FSH did not change after subtype and sensitivity analyses (Figure 12).

Serum LH

Kamijina et al. (28) evaluated this in two seasons, summer and winter, allowing analysis of this outcome in a total of 318 subjects (132 exposed subjects and 186 unexposed controls). OP pesticides exposure did not significantly alter circulating LH levels in OP pesticides-

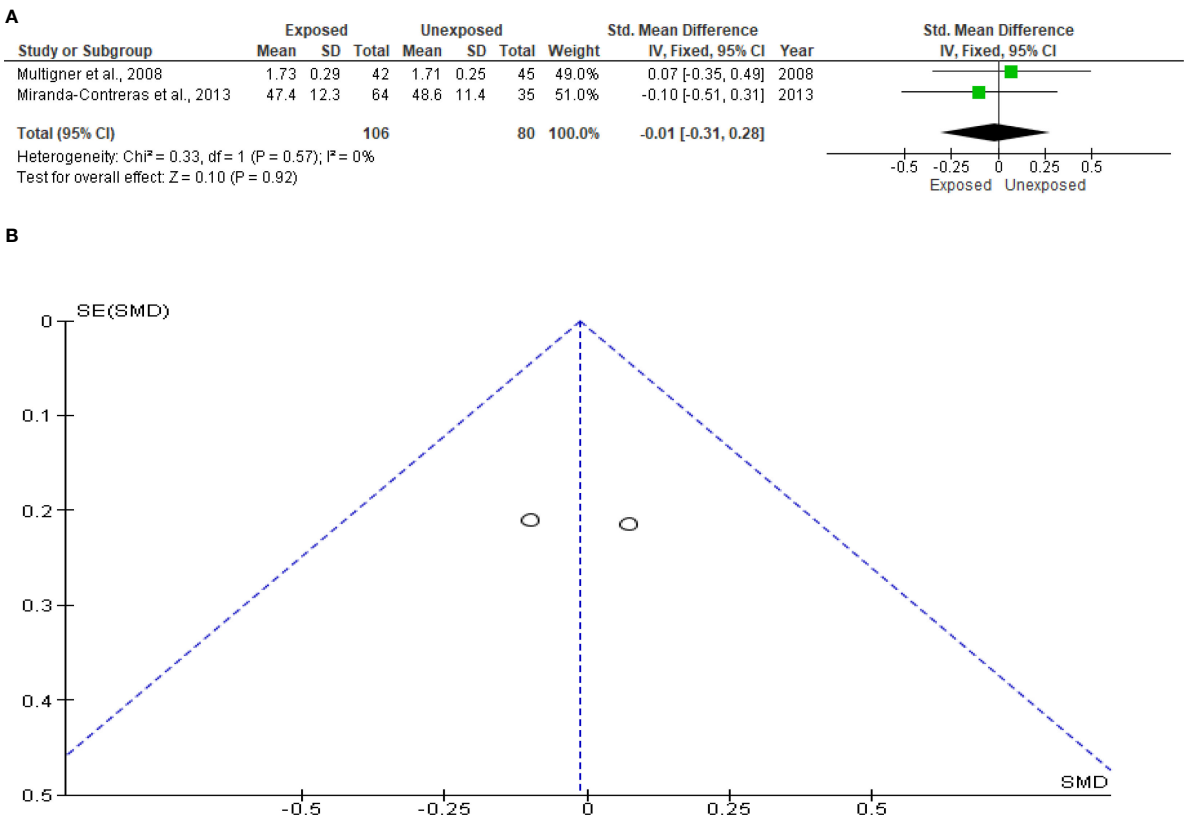

FIGURE 9
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on sperm multiple anomaly index.

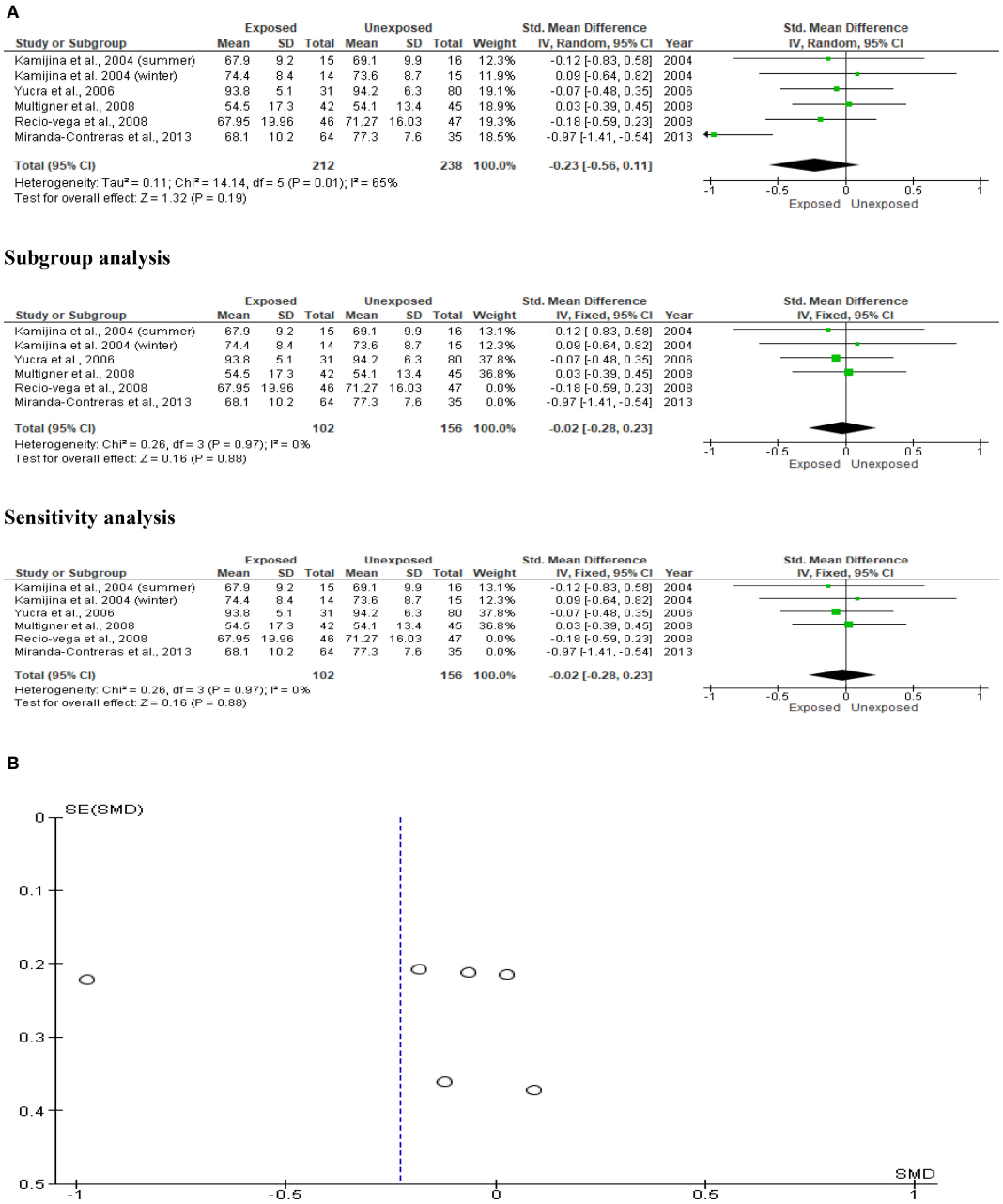

FIGURE 10
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on sperm viability (%).

exposed subjects compared to the unexposed (SMD -0.24 [95% CI: -0.90, 0.41] p=0.47), with the presence of significant inter-study heterogeneity (I^2 = 86%; $\chi 2$ p< 0.00001). The Funnel plot was asymmetrical, depicting publication bias. This observation in serum LH did not change after subtype and sensitivity analyses (Figure 13).

Serum testosterone

The impact of OP pesticides on serum testosterone was examined in four studies, with Kamijina et al. (28) reporting findings from both the summer and winter seasons. In total, 318 subjects (132 exposed and 186 unexposed controls) were used. The analysis revealed that there was no significant difference in the circulating testosterone levels between the OP pesticides-exposed subjects and unexposed controls (SMD 0.23 [95% CI: -0.46, 0.93] p=0.51). Significant inter-study heterogeneity was observed (I^2 = 88%; $\chi 2$ p<0.00001). The Funnel plot was asymmetrical denoting the presence of publication bias. This observation in serum testosterone did not change after subtype and sensitivity analyses (Figure 14).

Discussion

Key findings

This study reports a significant decline in sperm count, concentration, progressive and total motility, and normal

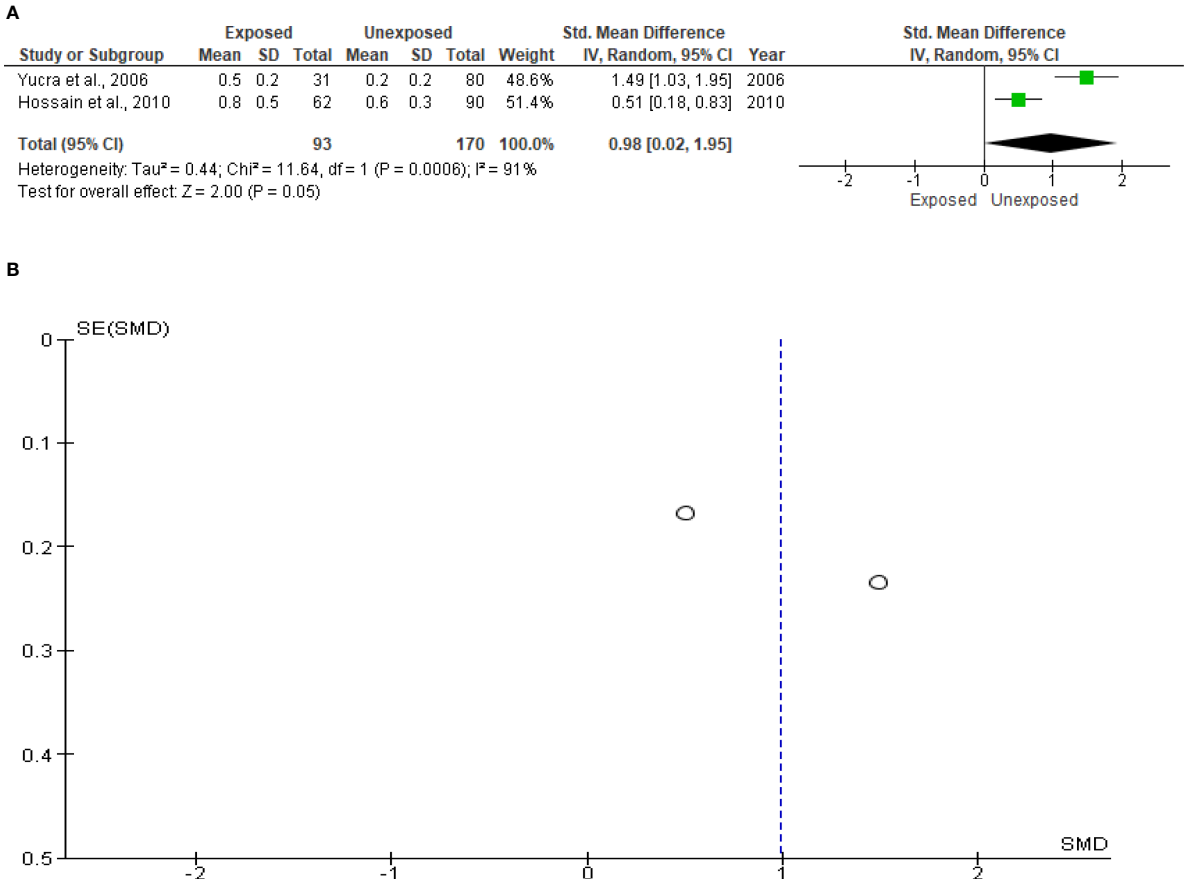

FIGURE 11
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on leukocytes (x 10^6/mL).

morphology in individuals who were exposed to OP pesticides compared with unexposed controls. Although seminal fluid leukocyte levels were higher in OP pesticides-exposed individuals compared with unexposed controls using both studies that were included in this study (26), this was not significant when the studies were pooled together. In addition, it was observed that the circulating levels of LH, FSH, and testosterone were comparable between the OP pesticides-exposed and unexposed groups; this suggests that OP pesticides-induced low semen quality is testosterone-independent. Therefore, the data presented in this study provide a robust indication and strengthens available evidence that OP pesticides exposure lowers semen quality by reducing sperm count, concentration, motility, and normal morphology.

Comparison to previous studies

The decline in sperm count observed in OP pesticides-exposed men is consistent with the findings of Padungtod et al. (24) and Recio-vega et al. (25), while our finding that OP pesticides-exposure cause reduced sperm concentration is also in agreement with the findings of Padungtod et al. (24) and Ghaouri-khosrowshahi et al. (27). In addition, these findings agree with observational cross-sectional studies that reported a negative association between OP pesticides and sperm count (22, 39–41). Sperm count is a measure of spermatogenesis, while sperm concentration is the most important parameter of testicular toxicity (42). Thus, based on the results on sperm count and concentration presented here, our data support the claim that OP pesticides impair spermatogenesis and exert toxic effects on testicular cells, especially germ cells. This forms an extension of the reports of Perez-Herrera et al. (43) that cells at all stages of spermatogenesis are a target of OP pesticides, and this effect may be mediated by paraoxonase (PON1) polymorphism.

In addition, our findings that OP pesticides significantly reduce sperm motility and normal sperm morphology align with some previous reports (24, 28, 37, 26, 27). These findings also agree with observational cross-sectional studies that documented a negative association between OP pesticides and sperm motility (22, 39, 41, 44, 45) and normal morphology (22, 40, 44–47). Since sperm function requires sperm motility, especially progressive motility (42), and sperm morphology is an important predictor of exposure to toxic substances and male factor infertility (48, 49). Our findings that OP pesticides reduces sperm motility and normal morphology confirm the spermo-toxic effect of OP, suggest that OP impairs sperm function, and also implicate OP in the incident male factor infertility. Although most of the human studies did not assess the likely mechanisms of action of the effect of OP pesticides on semen

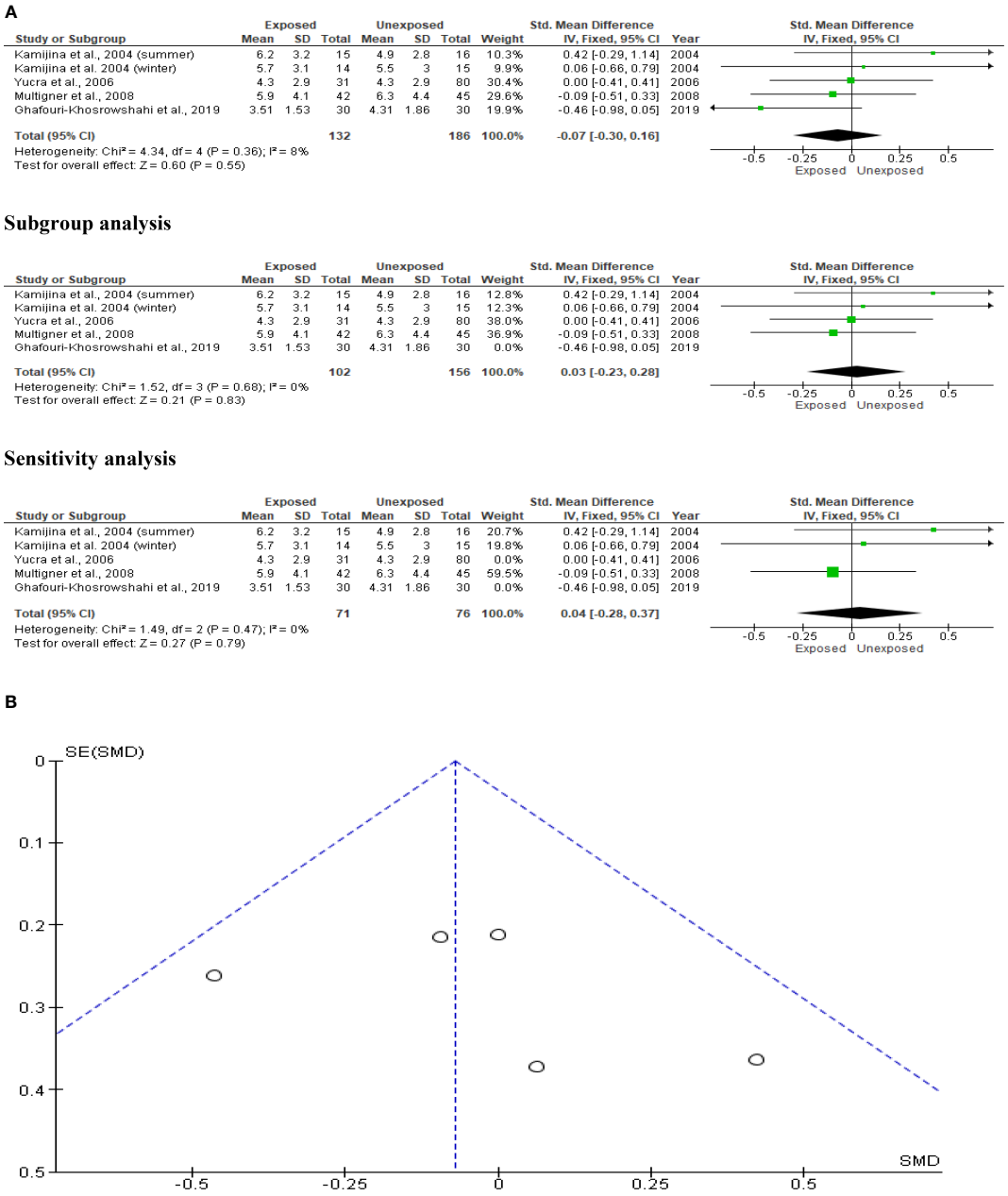

FIGURE 12
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on serum FSH (IU/L).

quality, GhafouriKhosrowshahi et al. (27) demonstrated that the impact of OP pesticides on semen quality may be due to its ability to increase nitric oxide, reduce total antioxidant capacity, and induce lipid peroxidation in the serum and seminal fluid.

Previous studies using animal models revealed that dichlorvos and diazinon, commonly used OP pesticides, exert spermotoxicity such as broken spermatozoa and reduced sperm motility (50, 51) as well as testicular toxicity (52). Suzuki et al. (53) demonstrated that OP pesticides-induced testicular and sperm toxicity was mediated via fatty acid amide hydrolase (FAAH), which plays key roles in spermatogenesis and sperm motility acquirement. Inhibition or downregulation of FAAH stimulates the cannabinoid signal, resulting in apoptosis of testicular cells like the Sertoli and Leydig cells by depriving the developing germ cells nutrients and hormonal signals needed for optimal development (54, 55). Exposure to Fenitrithion, an OP pesticides, induces testicular and sperm toxicity by inhibiting FAAH, although testicular AEA levels, which are usually modulated by FAAH inhibition, were not altered (53).

Studies have reported the direct testicular toxic effects of parathion, an OP pesticides, (56, 57), with an associated increase in abnormal sperm morphology, reduced chromatin quality, and increased apoptosis of germ cells. Parathion and its metabolite, paraoxon, also inhibit spermatogonial proliferation (38).

The toxic effects of OP pesticides have been linked with excessive generation of free radical (58; (59–61), which may alter the normal physiological function of the blood-testis barrier (62)

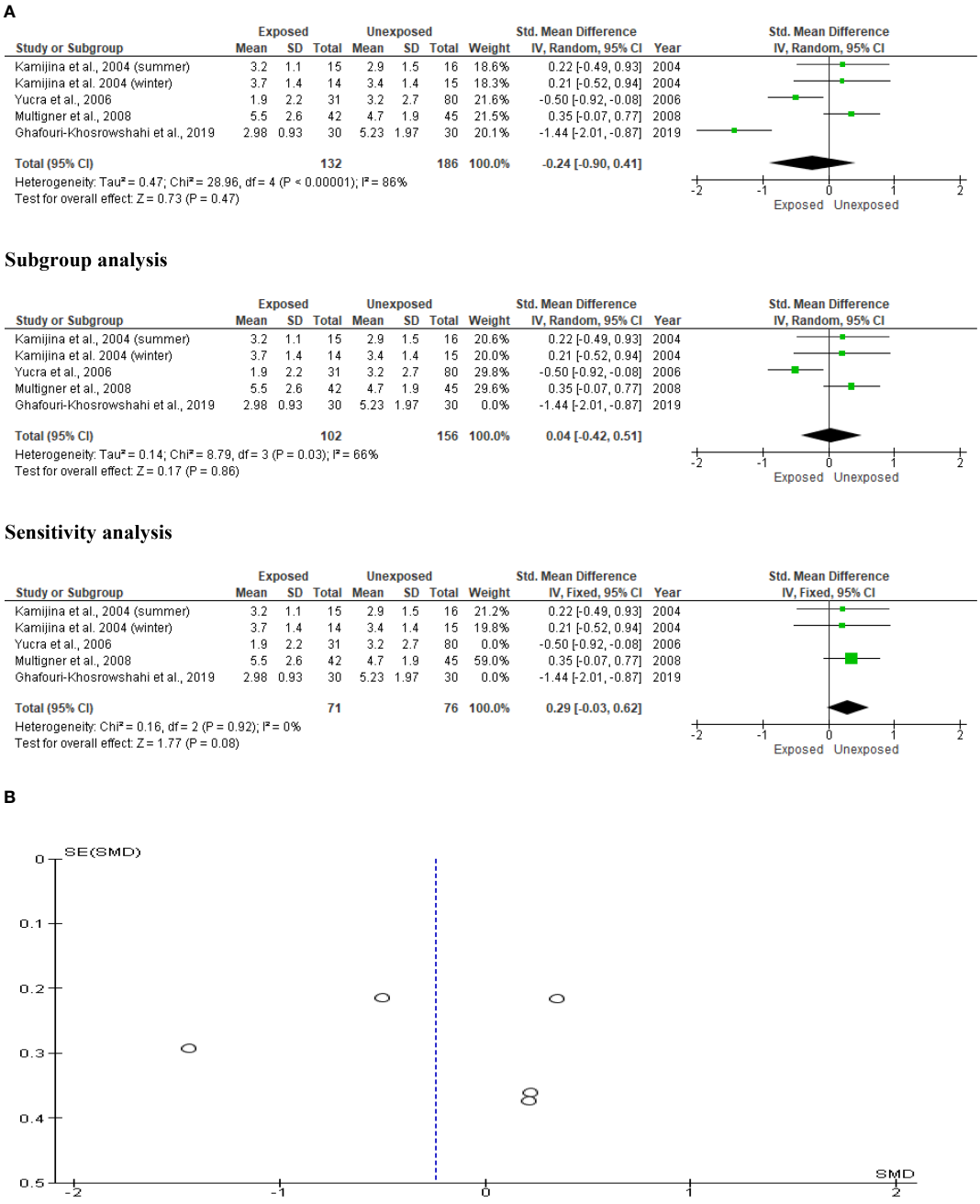

FIGURE 13
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on serum LH (IU/L).

producing covalent bonds with the occludens zone 2 (ZO2) (63) with multiple effects. This leads to lipid peroxidation of the sperm cell membrane, which is rich in polyunsaturated fatty acids (64), which exposed the protein content to denaturation and increases the susceptibility of the DNA in the nucleus to oxidative injury (65).

In the nucleus, OP chemicals modify the levels of mRNA encoding *Nrf2* and *OGG1*, which are important in the antioxidant buffering system and DNA repair (66–68). This may contribute, at least in part, to the observed reduction in the total antioxidant capacity of the seminal fluid in OP pesticides-exposed individuals (27), resulting in germ cell damage and consequent low sperm count and concentration. This may also promote ultrastructural abnormalities such as vacuolization, nuclear pyknosis, lipid droplets (50, 51, 66, 69), and increased DNA fragmentation (70). These may also explain the observed OP-induced sperm dysmotility and reduced normal sperm morphology.

Limitations and strengths

This study has some limitations. First, it is likely, that the non-inclusion of non-English publications in the present meta-analysis and the scarcity of well-designed studies to be included might have limited the pooled sample size. This may inadequately explore the

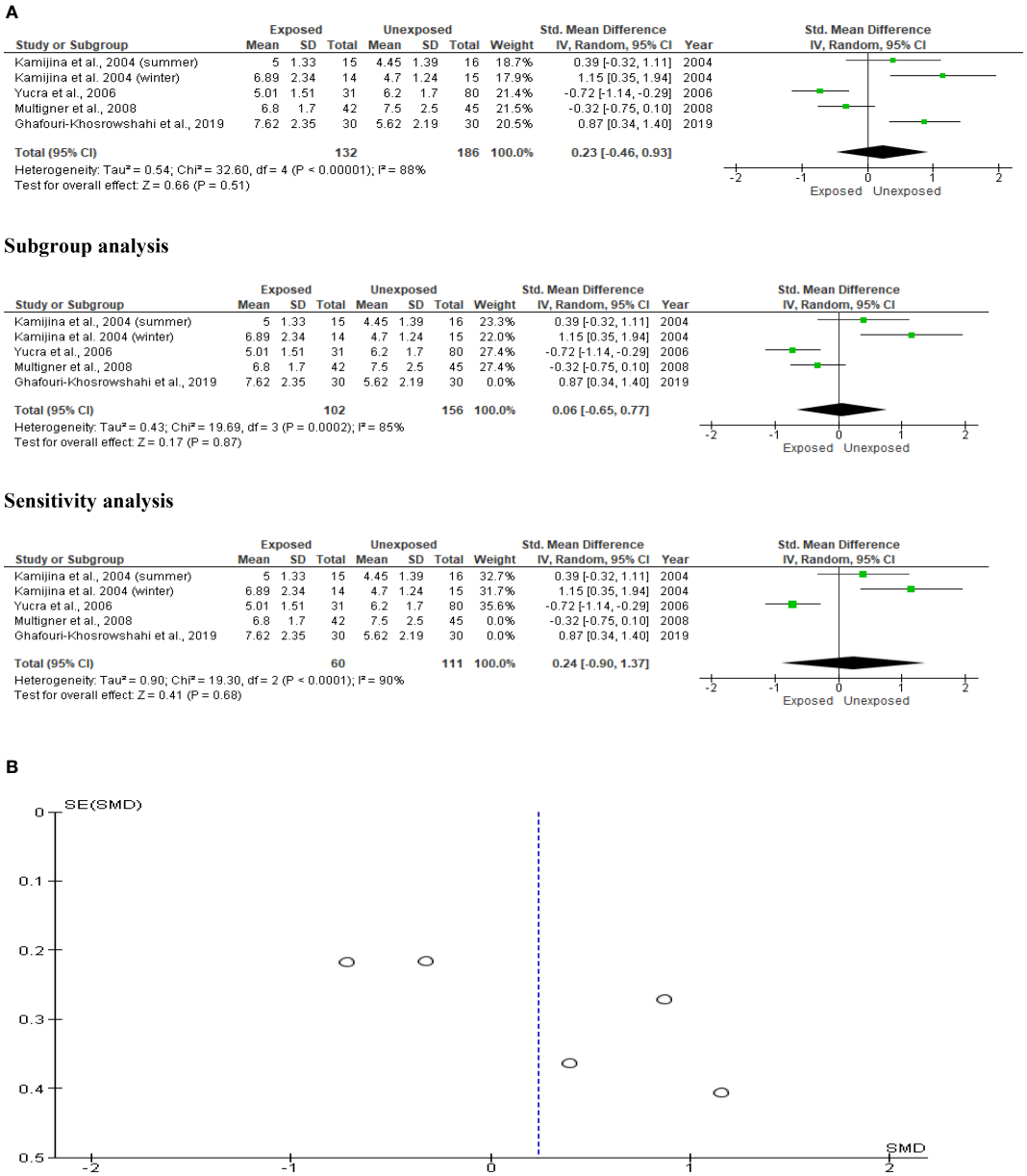

FIGURE 14
Forest plot **(A)** and publication bias funnel plot **(B)** of the effect of organophosphate pesticide exposure on serum testosterone (ng/mL).

impacts of OP pesticides on semen quality and testosterone levels. In addition, the included studies are from a few countries, which may not necessarily be a good global representative. Also, the included studies did not report the exposure level of the studied population, which may affect the study outcome. Furthermore, the heterogeneity in the included studies resulted in the presence of outliers in the present meta-analysis; however, we were able to adjust for this with the statistical approach used. Nonetheless, owing to the completeness of our search, the present study seems to be the first robust study including all available case-control human studies reporting data on OP pesticides and semen quality and/or testosterone levels, avoiding many limitations of previous related studies. The present study also provides an extensive review of possible mechanisms using existing published data.

Wider implications of our findings

OPs are pesticides, but also used as flame retardants and plasticizers, hence exposure to OPs is a common and global phenomenon. This rigorous and comprehensive meta-analysis reveals that OP pesticides exposure causes a significant decline in sperm count, concentration, total and progressive motility, and normal sperm morphology, which is consistent with direct suppressive and toxic effects of OP pesticides on spermatogenesis and sperm cells respectively, potentially affecting male fertility. However, testosterone levels remain unaltered despite a previous report by that OP pesticides significantly reduced testosterone levels.

The observed decline in sperm quality has wider implications beyond male fertility. Studies have linked low semen quality with

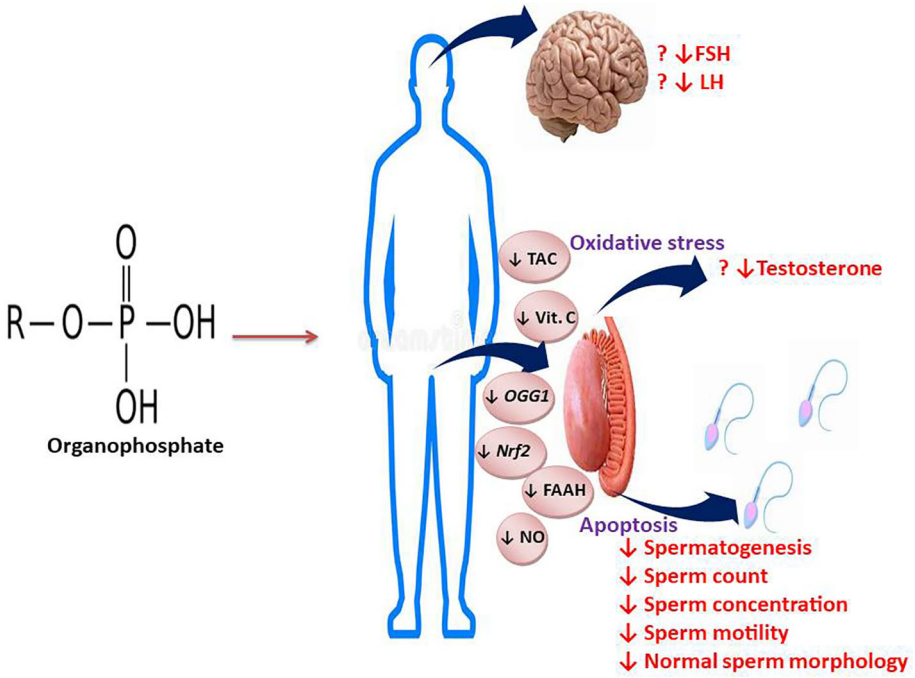

FIGURE 15
Graphical abstract illustrating the effect and mechanisms of action of OP on semen quality and male reproductive hormones.

socio-economic challenges (3, 14) and overall morbidity and mortality. Thus the observed decline in semen quality may exert ripple effects across the male lifespan. Our findings should, therefore drive a search for possible measures to prevent and ameliorate the impacts of OP pesticides on male fertility.

Conclusion and future perspective

The present comprehensive meta-analysis clearly demonstrates that exposure to OP pesticides causes reduced sperm count, concentration, total and progressive motility, and normal sperm morphology, possibly via a testosterone-independent mechanism (Figure 15). These findings strengthen existing evidence in the literature on the negative impacts of OP pesticides exposure on semen quality. Well-designed large case-control studies evaluating the effect and possible associated mechanisms of OP pesticides on semen quality are needed to reach more definitive conclusions. Also, possible measures that may prevent and/or ameliorate OP-induced low semen quality should be researched.

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

Conceptualization and design: MAH, TMA, and REA. Data curation: MAH, TMA, AEA, OBA, and ARE. Funding acquisition: MAH, TMA, AEA, OBA, and ARE. Investigation: MAH, TMA, AEA, OBA, and ARE. Methodology: MAH, TMA, AEA, OBA, and ARE. Project administration: MAH, TMA, AEA, OBA, and ARE. Supervision: MAH and REA. Validation: MAH, TMA, AEA, OBA, and ARE. Writing-original draft: MAH, TMA, and REA. Writing-review and editing and final approval: MAH, TMA, AEA, OBA, and ARE. 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.

Publisher's note

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The influence of lifestyle interventions and overweight on infertility: a systematic review, meta-analysis, and meta-regression of randomized controlled trials

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This study aimed to investigate the effect of lifestyle intervention (LSI) on diagnosed infertility in overweight and obese women. A systematic review and meta-analysis were conducted. A literature search was performed on the following databases from September 2022 to December 2022: PubMed, Web of Science, and SPORTDiscus. The inclusion criteria were the following: women between 18 and 45 years of age, BMI over 25.0 kg/m^2, diagnosed with infertility, a weight loss intervention, and control group part of RCTs. In total, 15 studies were identified and included. The meta-analysis shows a beneficial effect of LSI on reducing weight, waist circumference, and BMI and increasing infertility. A significantly beneficial effect of lifestyle intervention on weight reduction was observed for participants who initially had a higher BMI, while a non-significant effect was observed for individuals with a BMI above 35 kg/m^2. The meta-analysis showed a beneficial effect of lifestyle intervention on ovulation incidence and sex hormone-binding globulin. The lifestyle intervention group had 11.23 times more ovulatory incidence than the control group, which in turn increased the ability to conceive. As robust evidence for the effect of lifestyle interventions on infertility in obese and overweight women was found, it is advised to integrate similar interventions into future infertility treatment processes.

KEYWORDS

physical activity, infertility, intervention, overweight, pregnancy, ovulation

Introduction

Infertility is a medical state generally defined as a failure to conceive after 12 months of regular intercourse. Infertility is a rising problem in human society, and although the prevalence worldwide has been difficult to ascertain with limited population-based studies and inconsistent clinical definitions, it is estimated to affect between 8 and 12% of reproductive-age couples (1).

There are no exact data for Slovenia, but it is estimated that the situation is comparable to that in other European countries, which means that every 8 out of 12 couples face fertility problems (2). Primary infertility means that a couple has never achieved pregnancy, whereas secondary infertility means that a couple has had at least one prior successful conception (3).

Causes of infertility can be found in both female and male partners of reproductive-age couples. In 40–50% of the cases, the cause of infertility can be found in the reproductive system of the female partner, while in 30–40%, the cause of infertility is found in that of the male partner, and in 10% of cases, the cause is found in that of both partners. However, in 10% of couples, the cause of infertility remains unknown—idiopathic infertility (4). In women, as much as 80% of infertility can be attributed to three causes: endometriosis (5), tubal factor infertility, and polycystic ovary syndrome (PCOS) (6). Besides, a couple's lifestyle (inactivity, stress, unsuitable diet), smoking habits (6–8), excessive consumption of alcohol (6, 7) and coffee (7), environmental pollutants (9), and psychological factors (6) can play major roles in human fertility. Excessive body weight is also an important cause of infertility (6, 10–12) and may trigger certain factors that negatively affect infertility (abnormal metabolism, hormonal disorders, menstrual and ovulary disorders, PCOS, hyperinsulinemia, hyperandrogenism, etc.) (11, 12).

The prevalence of obesity and overweight is rising worldwide and has a detrimental effect on different functions of the human body, including reproduction. In particular, obese women suffer from hormone disorders, which lead to menstrual dysfunction, anovulation, and, consequently, infertility. In women with PCOS, hormone disorders and subfertility are common, while with additional obesity, the adipocytes begin to function as endocrine organs (13). A higher BMI is associated with a poorer fertility prognosis and simultaneously shows poorer reproductive results, regardless of the method of conception. Furthermore, a high BMI leads to a higher miscarriage rate, poor pregnancy outcomes, a higher risk of complications during pregnancy, and impaired fetal wellbeing (14). It was found that weight reduction in obese and overweight women improves reproductive outcomes by ameliorating fertility, regularizing menstrual cycles, and increasing the chance of spontaneous ovulation and conception in anovulation (11, 12, 15).

Various approaches are used to reduce weight, including interventions that change lifestyle habits such as applying regular sports activities as well as nutritional and psychological counseling, while drugs that can contribute to weight loss have been used less frequently. Various studies have shown that weight-loss lifestyle-changing interventions in overweight and obese women have a positive effect on hormonal and metabolic factors. These interventions affect the levels of fasting glucose, insulin, androstenedione, testosterone, anti-Mullerian hormone, estrogen, the homeostasis model assessment of insulin resistance (HOMA-IR), and sex hormone-binding globulin (SHBG) (16–18). Lifestyle interventions (LSI) also increase the rate of spontaneous as well as *in vitro* fertilization (IVF) pregnancies and the number of live births (19–21).

Abbreviations: BMI, body mass index; PCOS, polycystic ovary syndrome; LSI, lifestyle interventions; IVF, *in vitro* fertilization; WC, waist circumference; PA, physical activity; CI, confidence intervals; OR, odds ratio.

Physical activity (PA) has an important role during preconception, pregnancy, and postpartum. Well-balanced PA and energy state have fundamentally been related to an optimal reproductive system and good general health (22). It is necessary to consider the intensity and frequency of exercise because excessive exercise can have a negative effect on fertility. However, several studies have confirmed the positive effect of regular and moderate PA on fertility in women. A systematic review by Hakimi and Cameron has shown that exercise, with or without diet, can lead to a resumption of ovulation in overweight/obese women suffering from PCOS or anovulatory infertility (23). A prospective cohort study investigated the relationship between PA and time to pregnancy. In this study, moderate PA was associated with a small increase in fecundability, regardless of BMI. These findings indicate that PA of any type might improve fertility among overweight and obese women, a subgroup at higher risk of infertility (24).

In a recent meta-analysis, the effect of PA on the reproductive health of young women was analyzed (15). However, in that particular study, there were no data on which intervention and PA might have the best results or the greatest effect of the included components. Moreover, no data on PA frequency, intensity, or duration were reported. PA is an important factor in weight loss, which is sometimes underestimated, but it is necessary to realize that not all forms of PA are suitable for obese people. Thus, to establish more detailed associations between weight-loss lifestyle-changing interventions and infertility, the present study performed a meta-analysis with the inclusion of recent studies that clearly stated the abovementioned relevant LSI parameters. Potential results from effective detailed PA interventions would be very relevant to integrate as evidence-based recommended LSI within the health system, specifically in the treatment of infertility in overweight and obese women.

Materials and methods

Search strategy and study selection

The literature search was conducted from September 2022 to December 2022. The following databases were examined: PubMed, Web of Science, and SPORTDiscus. The word AND was used between the main groups of keywords related to infertility ("infertility", "sterility", "subfertility", "*in vitro* fertilization", "IVF"), gender ("female", "women"), weight ("obesity", "overweight"), and intervention ("weight reduction", "lifestyle", "healthy lifestyle", "lifestyle intervention", "intervention", "physical activity" or "training"), and the word OR was used between the keywords within the group.

The first review of study titles and abstracts was conducted by the first reviewer (AS), and the final review and selection were conducted by another reviewer (AP). Eligible studies that passed the selection process were included according to the determined inclusion and exclusion criteria. The inclusion criteria were the following: women between 18 and 45 years of age, BMI over 25.0 kg/m^2, diagnosed with infertility or PCOS or both, a weight loss intervention, a control group, availability in the full-text English language, and research design RCTs. In the final review of the selected articles, studies that did not have results for further and definitive analysis were excluded. The study selection process is illustrated in Figure 1 following

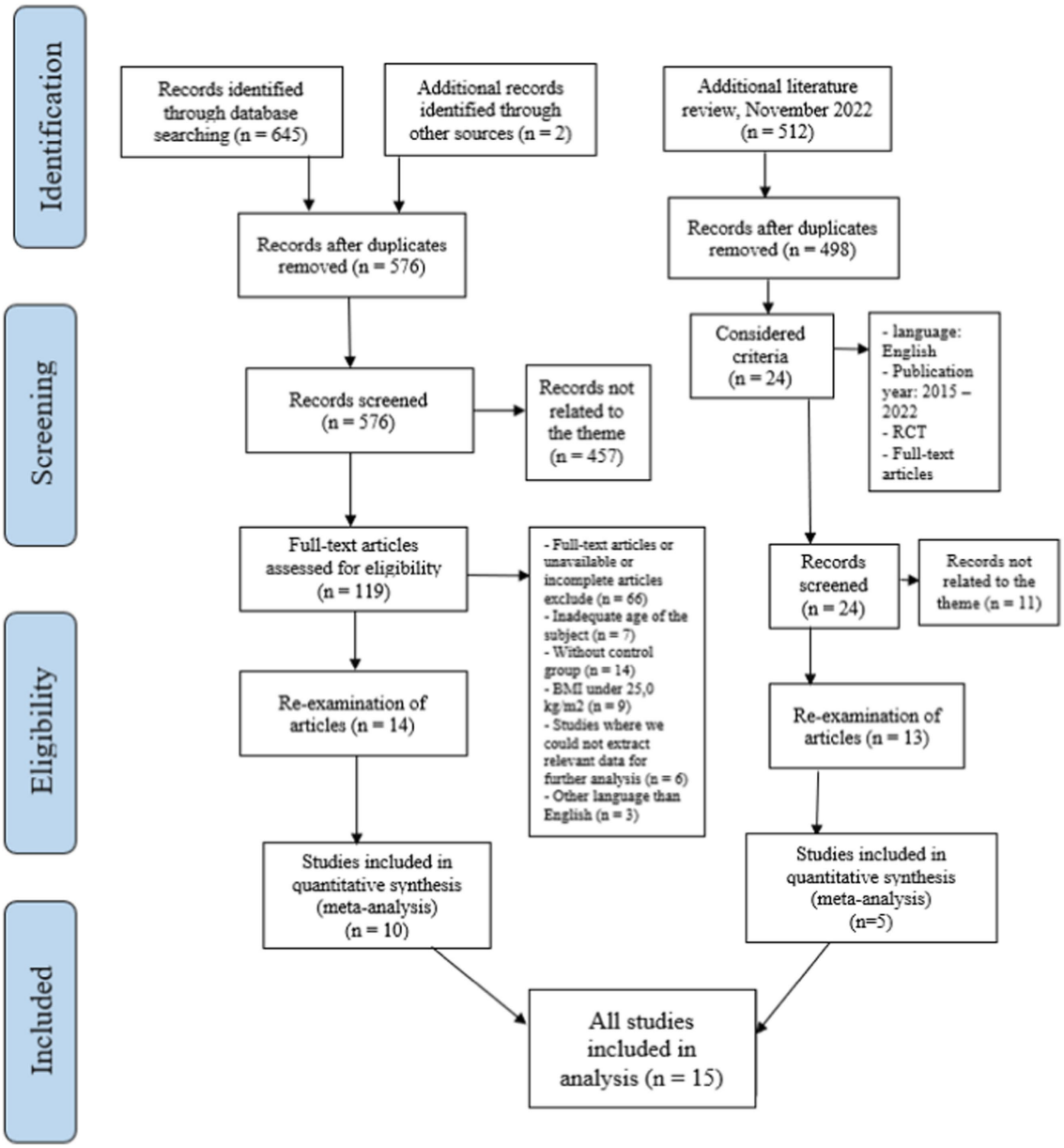

FIGURE 1
PRISMA flow diagram of the study of the first and second selection processes.

Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (25).

The primary outcome measures included ovulation improvement, pregnancy rates, and live birth rates, while the secondary outcome measures included changes in weight, BMI, waist circumference, and hormonal and blood factors.

Data extraction

The methodological quality of the included studies was assessed using the PEDro scale (26) by two reviewers independently (EA and AP). The PEDro scale comprises 11 items designed to rate methodological quality (26). Each satisfied item contributes 1 point to the overall PEDro score (range 0–10 points). However, item 1 (indicate briefly pertaining to external validity) was not included as part of the study quality rating for this review because it pertains to external validity, which was beyond the scope of the current review questions. Additionally, the Template for Intervention Description and Replication (TIDieR) checklist was used to assess the completeness of the intervention descriptions for both the experimental and control groups (27). The quality of evidence was assessed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system, where classifications were made as follows: "high quality," "moderate quality," "low quality," and "very low quality" (28). However, several reasons might lead to the degradation of the quality of the evidence (28). Thus, in the current study, the following criteria were considered when assessing confidence in evidence: design

limitation (if the majority of studies in the meta-analysis had a PEDro score of <6); imprecision based on small sample size [< 300 for each pooled outcome (29)]; and inconsistency of the results (substantial heterogeneity within effect estimates, I2 ≥ 50%). This review did not consider the indirectness criterion because the eligibility criteria ensured a specific population with relevant outcomes.

Statistical analysis

The meta-analyses were performed using Comprehensive Meta-analysis software (version 2.0; Biostat Inc., Englewood, NJ, United States). Except for ovulation and pregnancy, for all reported outcome measures, the difference in means (DM) and 95% CIs were calculated and presented in their respective units. Thus, weight was presented in kilograms (kg), BMI in kg/m^2, waist circumference in centimeters (cm), blood glucose in mmol/l, blood insulin in milli mass units per liter (mU/L), SHBG and testosterone (nmol/L), and FAI index (no unit). HOMA-IR was calculated by multiplying fasting serum insulin (μU/ml) and fasting plasma glucose (mmol/L) in arbitrary units. The odds ratio (OR) was reported for ovulation, pregnancy, and live births. The random-effects model of the meta-analysis was applied in all comparisons to determine the effect of the intervention on measures of interest. To investigate the effects of BMI on weight management and waist circumference, a subgroup analysis was performed by comparing groups with initially lower (i.e., <35 kg/m^2) and greater (i.e., ≥35 kg/m^2) BMI, respectively.

Furthermore, a random-effects meta-regression was performed to examine whether the effects of LSI on weight and pregnancy were moderated by the initial age and BMI of the participants, as well as different training variables. Training variables were grouped according to the following: training volume (i.e., period, weekly frequency, and the total number of training sessions) and time spent in training (i.e., duration of a single training session). To minimize the risk of overfitting, a meta-regression was performed when a minimum of 10 studies were eligible per examined covariate (30).

The publication bias was assessed by examining the asymmetry of the funnel plots using Egger's test, and a significant publication bias was considered if the value of p was <0.10. The I^2 statistic was used to investigate between-study heterogeneity, where values of 25, 50, and 75% represent low, moderate, and high statistical heterogeneity, respectively (31). Statistical significance was set at the level of a value of p of <0.05.

Results

Egger's test was performed to provide statistical evidence of funnel plot asymmetry. The results indicated no publication bias for the following meta-analysis: weight management (p = 0.497), waist circumference (p = 0.777), glucose (p = 0.732), insulin (p = 0.804), HOMA-R (p = 0.901), SHBG (p = 0.106), and FAI (p = 0.246), respectively. For all other analyses, the results indicated publication bias (p < 0.10).

Study selection and characteristics

Following a systematic literature search in different databases, 15 studies were identified and included (Table 1). The trials included a mix of three study design types: seven RCTs, three randomized comparison trials, and five RCT pilot studies. The research covered different LSIs: diet, PA, pharmacological treatment, and psychological help.

Quality and completeness of reporting

The reported completeness of intervention reporting was higher for the experimental conditions (mean: 73%; range from 27 to 100%) than for the control groups (mean: 57%; range from 18 to 91%). Compared to previously published data about the completeness of intervention reporting in interventional studies (32), the current meta-analysis included studies with sufficiently detailed exercise program descriptions. Table 2 shows the summarized results of the GRADE system and the PEDro scale, both used for assessing the quality of evidence.

The TIDieR checklist (Figure 2) provides a systematic way to describe the intervention, including rationale, materials used, procedures, how, where, when, and by whom the training was provided, and how the training was tailored and modified.

Effect of LSI on the anthropometric measures

Weight

The current meta-analysis of twelve studies with a total of 1,205 patients showed a beneficial effect (DM = −3.52 kg, 95% CI -6.57 to −0.47, df = 11; p = 0.024) of weight management on infertility (Figure 3). The evidence was downgraded from high to moderate due to high heterogeneity (I^2 = 78%; p < 0.001). Owing to this substantial heterogeneity, sub-analysis and meta-regression analyses were performed. Sub-group analysis revealed that the effects of the interventions were not moderated by BMI (Q = 0.001; p = 0.980). In brief, a significantly beneficial effect was observed only for participants who had initially high BMI (DM = −3.69 kg, 95% CI -6.76 to −0.61, n = 6, p = 0.019) and not for those with less than 35 kg/m^2 of BMI (DM = −3.62 kg, 95% CI -8.05 to 0.81, n = 6, p = 0.109).

BMI

The meta-analysis of nine studies with a total of 977 patients showed a beneficial effect (DM = −1.75 kg/m^2, 95% CI -2.60 to −0.90, df = 8; p < 0.001) on BMI management. The evidence was downgraded from high to moderate due to moderate to high heterogeneity (I^2 = 65%; p = 0.004). Hence, sub-analysis and meta-regression analyses were performed. Sub-group analysis revealed that the effects of the interventions were not moderated by BMI (Q = 0.081; p = 0.776). In brief, LSI significantly had a notably positive impact on reducing BMI for the participants with an initial BMI of less than 35.0 kg/m^2 (DM = −1.82 kg/m^2, 95% CI -2.91 to −0.72, n = 5, p = 0.001). This beneficial effect was also observed for more obese individuals with a BMI over 35.0 kg/m^2 (DM = −1.56 kg/m^2, 95% CI -2.93 to −0.19, n = 4, p = 0.025).

Waist circumference

The meta-analysis of nine studies with a total of 749 patients showed a beneficial effect (DM = −3.34 cm, 95% CI -5.06 to −1.63,

TABLE 1 Characteristics of the included studies.

Study	Study design	Population (initial weight, BMI, sample size, A, PCOS, ART)	Duration	Intervention (PA, diet, PBA, PT)	PA intervention	Outcome measure (W, BMI, O, P, M, LB)	Results
Gallety et al. (45)	RCT	EX: 102.4 ± 18.9; NR; 32 CON: 101.9 ± 18.1; NR; 32 A: 26–36	24 weeks	EX: PA, diet, PBA CON: PA, diet, PBA, PT	60 min of group exercise, 1 t/w	W	EX: 97.5 ± 5.2 CON: 96.7 ± 5.2
Thomson et al. (39)	Randomized comparison trial	EX: 97.6 ± 18.4; NR; 31 CON: 102.1 ± 18.3; NR; 33 *3rd: 100.5 ± 18.1; 30* A: 18–41 PCOS	20 weeks	EX: PA, diet CON: PA, diet, *3rd: diet*	EX: aerobic training of 25–45 min of walking or jogging 5 t/w, intensity 60–80% HR max CON: aerobic training as EX, resistance training: 3×12, 2 t/w, 5 consisted resistance exercises: bench press, lat pulldown, leg press, knee extension, and sit-ups, progressive intensity (training load of 50–60% 1RM in the first 2 weeks and increased to 60–75% 1RM for the following weeks)	W, O, P, M	EX: W 87.5 ± 18.4, O 50.0% (3/6), P 3.2% (1/31), M 42.9% (9/21) CON: W 93.5 ± 18.4, O 42.9% (3/7), P 3.0% (1/33), M 44.4% (8/18) *3rd: 91.9 ± 18.6, O 50.0% (6/12), P 3.3% (1/30), M 21.4% (3/14)*
Palomba et al. (20)	Randomized comparison trial	EX: 85.3 ± 6.4; 31.3 ± 2.6; 32 CON: 86.2 ± 6.9; 32.3 ± 3.7; 32 *3rd: 87.0 ± 6.9; 31.1* ± 2.9; *32* A: 18–35 PCOS	6 weeks	EX: PA, diet CON: PA, diet, PT *3rd: PT*	Structured exercise training (30 min on a bicycle ergometer, 3 t/w), the intensity increased gradually until a target of 60–70% VO2max consumption was achieved (according to an initial cardiopulmonary exercise test)	W, BMI, O, P	EX: W 86.2 ± 6.9, BMI 28.9 ± 2.3, O 12.5% (4/32), P 0% (0/32) CON: W 81.8 ± 6.0, BMI 32.3 ± 3.5, O 37.5% (12/32), P 3.1% (1/32) *3rd: 86.3 ± 6.4, BMI 28.4* ± 2.5, *O 9.4% (3/32), P 0% (0/32)*
Moran et al. (51)	RCT, pilot study	EX: 93.0 ± 16.0; 34.0 ± 4.5; 18 CON: 92.1 ± 13.8; 33.9 ± 4.4; 20 A: 18–41 ART	5–9 weeks	EX: PA, diet CON: they got advice but no active follow-up	Progressive walking program (20–45 min, 3 t/w), resistance training (1–2, 8–10 reps, 2 t/w), moderate intensity	W, BMI, P, LB	EX: W 89.2 ± 3.0, BMI 32.6 ± 1.1, P 66.7% (12/18), LB 38.9% (7/18) CON: W 91.6 ± 1.2, BMI 33.7 ± 0.4, P 40.0% (8/20), LB 25.0% (5/20)

(Continued)

TABLE 1 (Continued)

Study	Study design	Population (initial weight, BMI, sample size, A, PCOS, ART)	Duration	Intervention (PA, diet, PBA, PT)	PA intervention	Outcome measure (W, BMI, O, P, M, LB)	Results
Sim et al. (48)	RCT	EX: 95.8 ± 12,7; 35.1 ± 3.8; 27 CON: 104.0 ± 16.1; 38.0 ± 5.2; 22 A: 18–37 ART	12 weeks	EX: PA, diet, PBA CON: they got advice but no active follow-up	Unsupervised increasing walking to a target of 10,000 steps over 6 weeks (measured by a pedometer) and then 6 weeks 10,000 steps; intensity light to moderate	W, BMI, LB	EX: W 89.2 ± 4.6, BMI 32.7 ± 1.6, LB 44.4% (12/27) CON: W 102.4 ± 3.6, 37.4 ± 1.3, LB 13,6% (3/22)
Legro et al. (18)	Randomized comparison trial	EX: 96.0 ± 15.8; 35.1 ± 4.6; 50 CON: 95.2 ± 14.5; 35.1 ± 4.6; 50 *3rd: 94.6 ± 14.4; 35.5* ± 4.4; *49* A: 18–40 PCOS	16 weeks	EX: PA, diet CON: PA, diet, PT *3rd: PT – control group*	Aerobic exercise (10 min/day of bris walking or similar aerobic activity for the first 5 days and gradually increased over 4 months to 30–35 min/day), 5 t/w, the goal is 150 min/week activity	W, BMI, P, LB	EX: W 89.9 ± 6.1, BMI 32.9 ± NR, P 26.0% (13/50), LB 26.0% (13/50) CON: W 89.1 ± 6.1, BMI 34.8 ± NR, P 26.0% (13/50), LB 24.0% (12/50) *3rd: W 93.5 ± 1.1, P 14.3% (7/49), LB 14.3% (7/49)*
Dokras et al. (47)	RCT	EX: 97.0 ± 15.5; 35.4 ± 4.6; 44 CON: 94.6 ± 15.0; 35.3 ± 4.2; 43 *3rd: 95.1 ± 1.4; 35.3* ± 4.4; *45* A: 27–42 PCOS	16 weeks	EX: PA, diet, PT (for losing weight) CON: PA, diet, PT (to improve infertility) *3rd: PT*	Aerobic exercise (10 min/day of bris walking or similar aerobic activity, began at 10 min for the first 5 days and gradually increased over 16 weeks to 30–35 min), 5 t/w, the goal is 150 min/week activity	W, BMI	EX: W 90.6 ± 6.4, BMI 33.3 ± 2.4 CON: W 88.2 ± 6.4, BMI 33.2 ± 2.4 3rd: 93.9 ± 1.2
Einarsson et al. (34)	RCT	EX: 92.4 ± 8.0; 33.1 ± 1.3; 152 CON: 91.0 ± 8.4; 32.9 ± 1.4; 153 A: 18–38 PCOS, ART	16 weeks	EX: PA, diet, PT CON: PT	All patients had scheduled individual visits with a health professional at weeks 0 (baseline), 2, 5, 8, and 12, where weight was recorded. Not reported type, frequency, and intensity of PA.	W, BMI, P, LB	EX: W 83.3 ± 6.8, BMI 29.8 ± 2.4, P 10.5% (16/152), LB 29.6% (45/152) CON: W 92.9 ± 1.9, BMI 32.5 ± 0.7, P 2.6% (4/153), LB 27.5% (45/153)

(Continued)

TABLE 1 (Continued)

Study	Study design	Population (initial weight, BMI, sample size, A, PCOS, ART)	Duration	Intervention (PA, diet, PBA, PT)	PA intervention	Outcome measure (W, BMI, O, P, M, LB)	Results
Becker et al. (40)	RCT	EX: 77.0 ± 2.0; 28.7 ± 0.6; 14 CON: 74.4 ± 2.7; 28.8 ± 1.0 ,11 A: 18–35 ART	12 weeks	EX: diet CON: no intervention, advice	Maintain the same level of PA as before the intervention	W, BMI, P, LB	EX: W 72.5 ± 0.8, BMI 26.7 ± 0.5, P 21.4%, LB 21.4% CON: W 73.7 ± 0.7, BMI 29.1 ± 0.3, P 0.0%, LB 0.0%
Mutsaerts et al. (19)	RCT	EX: NR; NR; 290 CON: NR; NR; 287 A: 18–39 ART	24 weeks	EX: PA, die, PBA CON: no intervention. The patient went directly to IVF	Aerobic exercise (daily PA was stimulated with the use of a pedometer, aimed at 10,000 steps per day), at least 2 or 3 t/w, moderate intensity	P, LB	EX: P 53.6%, LB 27.1% CON: P 58.8%, LB 35.2%
Nagelberg et al. (44)	RCT, pilot study	EX: NR; NR; 10 CON: NR; NR; 11 A: 18–42 PCOS	4 weeks	EX: PA, diet CON: PA	Aerobic exercise (daily PA was stimulated with the use of a pedometer, aimed at 10,000 steps per day); exercise diary	O, P	EX: O 40.0%, P 40.0% CON: O 9.1%, P 27.3%
Espinos et al. (49)	RCT, pilot study	EX: 91.7 ± 11.8; NR; 21 CON: 89.2 ± 1.5; NR, 20 A: 29–37 ART	12 weeks	EX: PA, diet CON: no intervention. The patient went directly to IVF	Aerobic exercise (walking on a treadmill or pedaling stationary bicycles), 3 t/w, 60 min	W, O, P, LB	EX: W 85.3 ± 11.1, O 54.5%, P 57.1%, LB 61.9% CON: W NR, O NR, P 35.0%, LB 30.0%
Rothberg et al. (50)	RCT, pilot study	EX: 108.0 ± 10.0; 41.0 ± 4.0; 6 CON: 107.0 ± 14.0; 41.0 ± 4.0; 5 A: 18–40	12 weeks	EX: PA, diet CON: diet	Aerobic exercise (40 min/day, moderate PA)	W, BMI, O, P, LB	EX: W 94.0 ± 6.0, BMI 36.0 ± 2.0, O 50.0%, P 50.0%, LB 50.0% CON: W 102.0 ± 5.0, BMI 39.0 ± 2.0, O 0.0%, P 0.0%, LB 0.0%
Kiel et al. (41)	RCT, pilot study	EX: 85.7 ± 3.5; 28.9 ± 2.4; 8 CON: 87.9 ± 2.9; 31.2 ± 1.3; 10 A: > 18 ART	10 weeks	EX: PA, diet CON: gift card for the local gym for 85$	Resistance training (3 t/w, 2 times 4×4 min high-intensity training, third 10×1 min high-intensity training, 85–95% HRmax, walking or running on a treadmill)	W, P	EX: W 85.1 ± 4.7, BMI 29.6 ± 1.2, P 50–0% CON: W 87.2 ± 4.7, BMI 30.3 ± 1.2, P 44.0%

(Continued)

TABLE 1 (Continued)

Study	Study design	Population (initial weight, BMI, sample size, A, PCOS, ART)	Duration	Intervention (PA, diet, PBA, PT)	PA intervention	Outcome measure (W, BMI, O, P, M, LB)	Results
Legro et al. (42)	RCT	EX: 108.4 ± 22.7; 39.2 ± 7.0; 187 CON: 107.4 ± 20.8; 39.4 ± 6.9; 191 A: 18–40	16 weeks	EX: PA, diet CON: PA	Aerobic exercise (daily PA was stimulated with the use of a Fitbit activity tracker and a pedometer, aimed at 10,000 steps per day)	W, BMI, P, LB	EX: W 101.1 ± 6.0, BMI 36.6 ± 2.1, P 53.2%, LB 32.4% CON: W 107.3 ± 3.4, BMI 39.3 ± 1.3, P 48.2%, LB 37.2%

RCT, randomized controlled trial; EX, experimental group; CON, control group; BMI, body mass index; A, age; PCOS, women with polycystic ovary syndrome; ART, Assisted reproductive technology; NR, not reported; PA, physical activity; PBA, psychological/behavior advice; PT, pharmacological treatment; t/w, times per week; W, weight; O, ovulation; P, pregnancy; M, menstrual cycle; LB, live birth; 3rd, the third group.

[a]Assisted reproductive tehchnology (ART) includes all fertility treatments in which either eggs or embryos are handled (in vitro fertilization - IVF), intra- cytoplasmic sperm injection – ICSI, cycles or subsequent cryostored embryo transfer cycles). The main type of ART is IVF.

TABLE 2 Grades of recommendation, assessment, development, and evaluation (GRADE) for results summarized.

Outcome	Trials (n)	Participants (n)	Diff in Means	LLCI	HLCI	I^2 (%)	PEDro score	Quality of evidence (GRADE)
Weight	12	1,205	−3.52	−6.57	−0.47	76	7	Moderate quality
BMI	9	977	−1.75	−2.60	−0.90	65	7	Moderate quality
WC	9	749	−3.34	−5.06	−1.63	26	7	High quality
Glucose	5	183	−0.19	−0.29	−0.09	0	7	Moderate quality
Insulin	5	551	−0.98	−2.23	0.28	2	7	Moderate quality
HOMA-IR	5	183	−0.01	−0.45	0.43	59	7	Moderate quality
SHBG	5	551	5.55	1.89	9.21	47	7	Moderate quality
FAI index	3	172	−0.53	−1.91	0.86	0	7	Moderate quality
Testosterone	5	551	0.12	0.02	0.23	0.	7	Moderate quality
Ovulation	4	123	11.23*	2.51	50.23	35	7	Moderate quality
Pregnancy	11	1,567	1.49*	1.04	2.15	44.	7	High quality
Live births	9	1,526	1.51*	0.92	2.47	65	7	Moderate

* - data are presented as odds ratio; LCI – lower limit confidence interval; HLCI – higher limit confidence interval, I^2 – test of heterogeneity.

df = 8; p = 0.001) on WC management. The evidence was graded as high quality.

Effect of LSI on blood-related parameters

The meta-analysis of five studies with a total of 183 patients showed no beneficial effect on blood glucose (DM = −0.19 mmoL/L, 95% CI -0.29 to −0.09, df = 4; $p < 0.001$), insulin (DM = −0.98 mU/L, 95% CI -2.23 to 0.28, df = 4; $p = 0.127$), HOMA-IR (DM = −0.01, 95% CI -0.45 to 0.43, df = 5; $p = 0.974$), testosterone (DM = −0.12 nmoL/L, 95% CI -0.02 to 0.23, df = 4; $p = 0.024$), and FAI index management (DM = −0.53, 95% CI -1.91 to 0.86, df = 2; $p = 0.457$), respectively. However, a beneficial effect was found for SHBG (DM = −5.55 nmoL/L, 95% CI -1.89 to −9.211, df = 4; $p = 0.003$). The quality of evidence for all parameters investigated was downgraded to moderate due to imprecision or moderate to high heterogeneity (Table 2).

Effect of LSI on ovulation, pregnancy, and live birth incidence

The meta-analysis of four studies with a total of 123 patients showed a beneficial effect of LSI on ovulation (OR = 11.23, 95% CI 2.51 to 50.23, df = 3; $p = 0.002$), pregnancy (OR = 1.49, 95% CI 1.04 to 2.15, df = 10; $p = 0.032$, $I^2 = 44\%$), and live births (OR = 1.51, 95% CI 0.92 to 2.47, df = 8; $p = 0.099$; $I^2 = 65\%$), respectively. The evidence for ovulation was downgraded from high to moderate due to the reported imprecision (sample size <300), while data on pregnancy were rated as high-quality evidence.

Table 3 shows the results of the meta-regression analysis for two categories of variables: (a) patient-related (initial age, weight, and BMI) and (b) training variables such as training volume (i.e., period, weekly frequency, total number of training sessions) and time spent in training (i.e., duration of a single training session). No significant predictors were found for weight reduction following LSI.

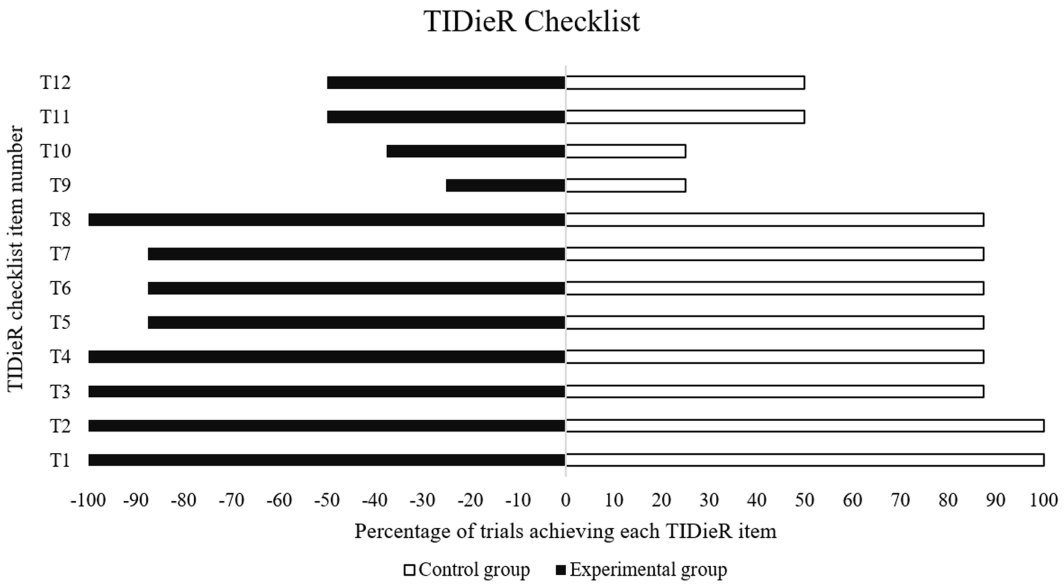

FIGURE 2
TIDieR checklist.

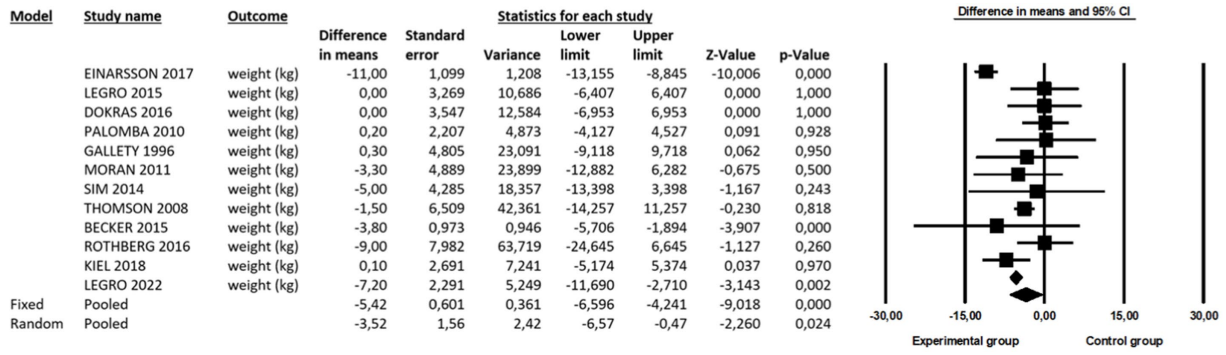

FIGURE 3
A beneficial effect of LSI on weight.

Meta-regression analysis for patient-related and training variables in pregnancy

Table 4 shows the results of the meta-regression analysis for two categories of variables: (a) patient-related (initial age and BMI) and (b) training variables such as training volume (i.e., period, weekly frequency, and total number of training sessions) and time spent in training (i.e., duration of a single training session). It was found that a training period (weeks) is a predictor of successful pregnancy following LSI.

Discussion

In this systematic review and meta-analysis, 576 journals were screened, and 10 articles were selected. During an additional literature review conducted in December 2022, 498 journal articles were screened. Of these, five articles were selected for their qualitative insights on LSI and infertility intervention in overweight and obese women. Only a limited number of articles specifically explored the relationship between LSI, female infertility, and obesity. Furthermore, only a few studies followed the included subjects for a longer period of time so that long-term results on pregnancy and live births were also visible. The results of the present meta-analysis indicate that LSI can be an effective treatment for weight management, as evidenced by decreased weight (DM = −3.52 kg, $p = 0.024$), BMI (DM = −1.75 kg/m^2, $p < 0.001$), and WC (DM = −3.34 cm, $p = 0.001$) after LSI. Moreover, we observed positive effects of LSI on increasing ovulation and pregnancy rates in overweight women with infertility. Furthermore, a meta-regression analysis showed no significant predictors among the related variables for the effect of LSI on weight management.

Our results are in line with previously published studies showing that LSI is efficient in reducing body weight (15, 33): for 12 of the 15 included studies, the reduction ranged from 0.7 to 12.9%, while in the control group, the average weight loss ranged from 0.1 to 8.4%. However, one study also found a weight gain (34). Additional subgroup analysis showed that the initial BMI of the participants was

TABLE 3 Meta-regression for patient-related and training variables of different subscales to predict intervention effect on weight management.

	Coefficient	Standard error	95% lower CI	95% upper CI	*Z* value	*p* value
Patient-related variables						
Age (years)	−0.671	0.571	−1.4546	0.3857	−1.257	0.321
Weight (kg)	−0.0048	0.0137	−0.0316	0.0220	−0.35	0.7268
Body Mass Index (kg/m^2)	−0.3257	0.3962	−1.1022	0.4509	−0.82	0.4111
Training volume						
Training period (weeks)	−0.0190	0.2256	−0.4611	0.4231	−0.08	0.9329
Training frequency (per week)	−0.6487	1.0057	−2.6197	1.3224	−0.65	0.6189
Total number of training sessions (per study)	−0.0194	0.0421	−0.1019	0.0631	−0.46	0.6443
Time spent in training						
Duration of a single training session (min)	0.0092	0.1423	−0.2698	0.2881	0.06	0.9486

Bolded values refer to the statistical significance of the observed results; the CI confidence interval.

TABLE 4 Meta-regression for patient-related and training variables of different subscales to predict intervention effect on pregnancy.

	Coefficient	Standard error	95% lower CI	95% upper CI	*Z* value	*p* value
Patient-related variables						
Age (years)	−0.0341	0.1057	−0.2413	0.1731	0.47	0.6392
Weight (kg)	0.0251	0.0328	−0.0386	0.0952	0.57	0.563
Body Mass Index (kg/m2)	0.0381	0.0544	−0.0685	0.1447	0.70	0.4835
Training volume						
Training period (weeks)	−0.0718	0.0223	−0.1155	−0.0281	−3.22	**0.0013**
Training frequency (per week)	0.1259	0.1478	−0.1638	0.4157	0.85	0.3943
Total number of training sessions (per study)	−0.0029	0.0147	−0.0317	0.0259	−0.20	0.8432
Time spent in training						
Duration of a single training session (min)	0.0253	0.0289	−0.0314	0.0820	0.88	0.3815

Bolded values refer to the statistical significance of the observed results, the CI confidence interval.

not a significant moderator of those effects ($Q = 0.001$; $p = 0.980$). On the contrary, the summarized effects of a pairwise comparison showed that the initial BMI of participants may influence the overall decrease in weight after LSI. Data processing was performed separately for two groups according to the initial BMI level, namely for subjects with a BMI below 35.0 kg / m^2 and a BMI above 35.0 kg / m^2. For subjects with a BMI below 35.0 kg / m^2, the BMI decreased, but not significantly (DM = −3.62 kg, $p = 0.109$), while for the subjects in the group with a BMI above 35.0 kg / m^2, the BMI decreased significantly (DM = −3.69 kg, $p = 0.019$). This raises the question of what kind of intervention and lifestyle change would be most appropriate for women with a BMI below 35.0 kg/m^2. Moreover, the form of exercise (including type, volume, and intensity) and progression should probably be more frequent and/or intense for women with a BMI below 35.0 kg/ m^2 than for women with a BMI above 35.0 kg/ m^2, as tailored exercise could lead to a greater reduction in weight and BMI.

WC was reported in 9 of the 15 studies, and the meta-analytic approach showed a reduction of WC in the experimental group on

average from 1.4 to 12.2%, while in the control group, there was an average reduction between 0.0 and 10.6%. Phy et al. previously showed that an 8-week and 12-week weight loss intervention in overweight and obese women with PCOS has an effect on reduction in WC and consequently improved insulin sensitivity, reduced testosterone, and improved fertility (35, 36).

Regular PA has been found to increase SHBG levels (37), and low serum SHBG levels are considered a relevant biomarker of abnormal metabolism and are related to insulin resistance and abnormalities in glucose and lipid metabolism (38). Previously published meta-analyses showed that LSI does impact SHBG, which was reported in five studies (20, 39–42).

In 4 of the 15 studies in the present meta-analysis, LSI was shown to have a beneficial effect on ovulation, and in 11 of the 15 studies on the pregnancy of overweight and obese women diagnosed with infertility. The LSI group had 11.23 times more ovulatory incidence than the control group, which in turn increased the ability to conceive. Furthermore, it was established that a 2–5% reduction in body weight has been associated with the restoration of ovulation (43). Accordingly, the proportion of pregnancies was higher in the intervention group, as almost a fifth of the subjects became pregnant, that is, 18.40%. In the LSI group, there was a higher proportion of live births: 17.83%.

Our findings are consistent with the systematic reviews already carried out. However, our analysis differs from the previous studies (7, 11, 15) because here we used the meta-analytic approach to identify various LSIs to explore their effects on fertility factors. LSI includes various components, and a variation was observed even in the length of LSI included in the presented MA. The mean length of LSI was 14.2 weeks, with the shortest intervention being 4 weeks (44) and the longest being 24 weeks (19, 45, 46), with 12 and 16 weeks being the most common durations. PA was part of the intervention in the fourteen studies included, but they were very differently defined. Ten LSI involved only aerobic exercises such as walking, brisk walking, jogging, or similar activity (18, 19, 39, 42, 44, 45, 47–50), two interventions involved structured exercise training (20, 41), one intervention included both aerobic and resistance training (51), and two did not specify the type of exercise intervention (34, 40). Three interventions with aerobic exercise were defined as 10,000 steps per day (19, 44, 48).

Pharmacological treatment was used in 2 intervention groups and 6 control groups out of the 15 included studies. In three of the 15 intervention groups and one control group, psychological or behavioral advice was provided to the subjects and performed by a health professional (34). Psychotherapy can be an important intervention that should be recommended for couples suffering from any form of infertility.

In addition to the intervention and control groups, 3 out of 15 studies had a second experimental group (20, 39, 47), and one intervention had an active control group (18). However, the present study's focus was on comparing the LSI and control groups, even though several comparisons between studies were possible. At the same time, this might be one of the limitations of the present meta-analysis.

It should be mentioned that some of the selected studies had additional groups not addressed in the present analysis: 3 out of 15 studies had a third test group (20, 39, 47), and one intervention had a real control group (18). Since the focus of the present study was on comparing the LSI and control groups, it was decided that additional comparisons between studies were not performed. This is one of the limitations of the present meta-analysis.

When it comes to the issue of infertility, it is necessary to mention two aspects that were not known in the past, but their understanding and influence can help in comprehending the problem of infertility in the future. Scientists have determined that COVID-19 has had and continues to have an effect on the reproductive health of both women and men. Angiotensin-converting enzyme 2 (ACE2), a functional receptor for SARS-CoV-2, is a key component of the renin-angiotensin (SRA) system that modulates the cleavage of angiotensin II (Ang II) and Ang (1–7). Upon cell invasion, COVID-19 disrupts SRA by reducing ACE2 expression in host cells, leading to an increased Ang II inflammatory response (52). Ang II, ACE2, and Ang (1–7) regulate basic functions in the female and male reproductive systems. In women, this includes folliculogenesis, steroidogenesis, oocyte maturation, ovulation, and endometrial regeneration (53). According to the researchers, knowing the effect of the virus on fertility also changes and adapts infertility treatment (54), which slowed down a bit during the pandemic as clinics that perform artificial insemination procedures had stopped or limited treatment (55, 56).

Artificial intelligence (AI) has been widely applied in the field of reproductive health to enhance diagnosis, treatment, and overall healthcare delivery. Medenica et al. have found that AI has proven to be a very important and effective tool that will bring great innovation to the field of reproductive medicine. There are many ways in which artificial intelligence affects reproductive health: medical imaging and diagnostics (analyze medical images, MRIs, etc., to assist in detecting conditions), ART (AI can optimize and predict the success rate of IVF), customized and individualized treatment plans (based on patients' medical histories and genetic information), and fertility tracking and predictions (to optimize timing for conception) (57, 58).

Limitations and research recommendations

The advantage of the present research was the narrow and precise inclusion criteria, with which a small number of studies with comparable LSI were obtained. A meta-analysis was performed for each factor, using the PEDro scale to assess the reporting quality of randomized controlled trials and the TIDieR rating for reporting details of the intervention elements of a study.

The present review is limited because the studies and LSI parameters are very diverse. Consequently, the selection presented difficulties for comparison. Moreover, as there was a range of types of PA inside each LSI, it is not obvious which type of PA can improve fertility and better influence reproductive health. Only a few studies have defined PA as FITT (frequency, intensity, time or duration, and type). For future research, it is suggested that the PA with the acronym FITT be precisely defined, allowing other researchers to perform the exercise. In addition, it would be of great interest for research and practice to directly compare the effects of LSI on the body anthropometrics of subjects with BMIs above and below 35.0 kg/m^2, as the results from the present meta-analysis showed inconclusive findings. Thus, these results must be interpreted with caution as the group comparison did not achieve a level of significance ($Q = 0.001$, $p = 0.980$), while pairwise comparisons did for women with BMI $\geq 35\,kg/m^2$ ($DM = -3.69\,kg$, $p = 0.019$) but not for those with less than $35\,kg/m^2$ of the initial BMI ($DM = -3.62\,kg$, $p = 0.109$). Thus, it is necessary to define the form and progression of the exercise, which probably should be different for women with a BMI above and below

35.0 kg/m^2 when weight loss is a primary goal. Moreover, it would be of great interest to assess the adherence to an intervention in relation to LSI effectiveness in weight management. For future research, it is suggested that LSI be defined in greater detail, structured more carefully to suit the participants' characteristics, and conducted over a longer period. The suggested modifications might lead to a greater effect of LSI, which consequently means that, in the case of appropriate findings, they could be implemented in practice and healthcare.

We would like to emphasize that an investigation of motor skills combined with BMI might provide further insights into the LS-fertility association and, in that sense, whether physical fitness parameters might be relevant biomarkers that can describe the risk of infertility.

Conclusion

The findings of the present meta-analysis LSI (PA, diet, pharmacological treatment, or psychological advice) may have beneficial effects on some reproductive health outcomes in overweight and obese women with diagnosed infertility. The present meta-analysis showed that LSI has a beneficial effect on anthropometric measures (weight, BMI, and WC) and no beneficial effect on blood-related parameters, except SHBG. Moreover, the beneficial effects of the LSI were established as improved ovulation, a higher chance of pregnancy, and live births for overweight and obese infertile women.

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

AS: Conceptualization, Data curation, Investigation, Project administration, Writing – original draft, Writing – review & editing. VH: Conceptualization, Writing – review & editing. CJWM: Data curation, Supervision, Validation, Visualization, Writing – review & editing. EA: Data curation, Validation, Writing – review & editing. MV: Funding acquisition, Resources, Validation, Writing – review & editing. TBP: Supervision, Validation, Writing – review & editing. AP: Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing, Data curation, Supervision, Validation.

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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.

Publisher's note

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Associations between urinary phthalate concentrations and antral follicle count among women undergoing *in vitro* fertilization

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Background: Phthalates are ubiquitously used in a variety of products and have an adverse effect on folliculogenesis. However, previous epidemiological studies on the associations between phthalate exposure and antral follicle count (AFC) produced conflicting results. The present study aimed to evaluate the associations between urinary phthalate metabolite concentrations and AFC among women undergoing *in vitro* fertilization (IVF).

Methods: We collected 525 urine samples and measured 8 phthalate metabolites from IVF patients. Poisson regression models were conducted to evaluate the associations between phthalate metabolite concentrations and AFC. In addition, participants were stratified into a younger group (< 35 years) and an older group (≥ 35 years) to explore the potential effect modification by age. We also performed sensitivity analyses by restricting our analyses to only infertile women diagnosed with tubal factor infertility to test the robustness of the results.

Results: Significant positive associations were observed among urinary MBP, MEOHP and ∑PAEs concentrations and AFC after adjusting for age, BMI, year of study and infertility diagnosis. Compared with women in the first tertile, women in the third tertile of MBP and MEOHP had 7.02% (95% CI: 1.18%, 12.9%) and 8.84% (95% CI: 2.83%, 14.9%) higher AFC, respectively, and women in the second and third tertiles of ∑PAEs had 6.19% (95% CI: 0.37%, 12.0%) and 9.09% (95% CI: 3.22%, 15.0%) higher AFC, respectively. In addition, MBP, MEOHP and ∑PAEs also had significant positive associations with AFC in trend tests for dose-response. In the age-stratified analysis, we found a stronger relationship between phthalate metabolite concentrations and AFC among older women and an inverse association among younger women. We observed similar results in the sensitivity analyses.

Conclusion: We found positive associations between phthalate exposure and AFC, which support the idea that phthalate exposure may accelerate primordial

follicle recruitment and lead to higher AFC in women undergoing IVF. More studies are needed to better understand their relationships.

KEYWORDS

phthalate, urine, antral follicle count, infertile women, *in vitro* fertilization

1 Introduction

Infertility is an ongoing reproductive health problem around the world, and exposure to environmental contaminants is an important factor (1, 2). Phthalate esters (PAEs) are a group of synthetic compounds that are abundantly used as plasticizers or solvents in a variety of products, such as polyvinylchloride, building and finishing materials, personal care products, cosmetics, toys, food packages, medications and medical devices (3). Phthalates in products can be released into the environment due to nonchemical bonds, and human exposure occurs through inhalation, ingestion and dermal absorption (4). Phthalates are rapidly metabolized into monoesters once they enter the human body, in which they have stronger biological activity (5). The metabolites are excreted mainly via urine and have a half-life less than 24 hours (6), therefore, urinary metabolite concentrations are generally used to represent body phthalate exposure levels (7).

Phthalates are endocrine disruptors characterized mainly by their reproductive and developmental toxicity (8). According to the results of several animal studies, they can disrupt ovarian development, inhibit follicle growth, and impair oocyte maturation and embryo development (9–14). Epidemiological studies found that phthalate exposure was associated with adverse reproductive outcomes, such as decreased oocyte retrieval, mature oocytes, fertilized oocytes, good-quality embryos, clinical pregnancy rate and live birth rate (15–18).

Antral follicle count (AFC) is a critical value used to evaluate women's fecundity, and is defined as the sum of 2-10 mm follicles in both ovaries as observed on ultrasound in the early follicular phase (menstrual days 2-4) (19). As the earliest acquirable follicle parameter in the reproductive clinic, AFC was routinely measured during the infertility treatment period for ovarian reserve assessment, infertility diagnosis, and treatment strategy determination, and AFC was also found to be associated with reproductive outcomes (20, 21).

Phthalates have been shown to reduce antral follicle number in mice (22) and inhibit antral follicle growth in *in vitro* culture (23). However, phthalate exposure in humans is complicated and continuous, and the results among human studies are controversial. Messerlian et al. reported that urinary phthalate metabolite concentrations were adversely associated with AFC (24), and Li et al. found both positive and negative associations between phthalate metabolite concentrations in serum and AFC (25). Additionally, we observed positive dose-response associations between urinary phthalate metabolites and AFC in our previous study (26). In mammals, the follicle cycle starts from primordial follicle activation and undergoes a series of developments until atresia or ovulation; thus, more AFC may suggest more follicle recruitment from the beginning. It has been reported that di(2-ethylhexyl) phthalate (DEHP) can accelerate primordial follicle recruitment in mice, leading to a lower proportion of primordial follicles and a higher proportion of developing follicles in ovaries (27–29). In the present study, we enlarged the sample size and reanalyzed the association between phthalate exposure and AFC among women undergoing *in vitro* fertilization (IVF). We also performed stratified analyses to explore potential effect modification by age and conducted sensitivity analysis to test the strength of our results.

2 Materials and methods

2.1 Participants

To explore the potential effects of phthalate exposure on reproductive health, women who sought infertility treatment were recruited at two separate times at the Reproductive Medicine Center of Tongji Hospital, Wuhan, China, as described previously: from July to August 2014 (30) and from November to December 2016 (31). Briefly, women aged from 20 to 45 years who were infertile and with indications for IVF or intracytoplasmic sperm injection were eligible. Subjects who had an ovariectomy history, other iatrogenic injuries, or health conditions such as autoimmune diseases, congenital gonadal dysplasia, endocrine diseases and sexually transmitted diseases were excluded. Participants who provided a urine sample for phthalate metabolite detection and had AFC data within a 4-month period before enrollment were included in this study. Information on age, height, weight, smoking

Abbreviations: AFC, Antral follicle count; AMH, Anti-Müllerian hormone; BMI, Body mass index; DEHP, Di(2-ethylhexyl) phthalate; IVF, *In vitro* fertilization; MEHP, Mono(2-ethylhexyl) phthalate; MEHHP, Mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, Mono(2-ethyl-5-oxohexyl) phthalate; MEP, Mono-ethyl phthalate; MBP, Mono-n-butyl phthalate; MOP, Mono-n-octyl phthalate; PAEs, Phthalate esters.

status and ethnicity was collected at enrollment. These studies were approved by the Ethics Board of Tongji Hospital, and informed consent was obtained for all participants.

2.2 Sample collection and measurement

On the day of ovum pick-up surgery, urine samples were collected and transferred to the laboratory immediately, aliquoted and frozen at −80 °C. The concentrations of mono-ethyl phthalate (MEP), mono-methyl phthalate, mono-n-butyl phthalate (MBP), mono(2-ethylhexyl) phthalate (MEHP), mono-benzyl phthalate, mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-n-octyl phthalate (MOP) were analyzed by high-performance liquid chromatography and tandem mass spectrometry as described in our previous study (26, 32). The limits of detection ranged from 0.01-0.04 μg/L. Values for metabolites with concentrations less than the LOD were assigned with LOD/ $\sqrt{2}$. Concentrations of creatinine were measured using clinical chemistry analyzers (17).

2.3 Infertility data and AFC measurement

AFC, which was measured on days 2-4 of the menstrual cycle by transvaginal ultrasound, and other clinical data (e.g., infertility type, duration of infertility and infertility diagnosis) were abstracted from electronic medical records. Infertility types included primary infertility and secondary infertility. Infertility diagnosis was classified as female factor, male factor, mix factor or unexplained reasons. Female factor infertility included diminished ovarian reserve, tubal factor, ovulatory dysfunction, endometriosis and uterine factor.

2.4 Statistical analysis

Descriptive information of the participants is presented as the number (%) or mean ± standard deviation where appropriate. Phthalate metabolite concentrations are presented as quartiles and geometric means. The molar sum of metabolites of DEHP (∑DEHP) was calculated by the sum of MEHP/278.34, MEOHP/292.34 and MEHHP/294.34. ∑PAEs were also calculated by the molar sum of the concentrations of the eight phthalate metabolites detected in this study. Phthalate metabolite concentrations were standardized by creatinine due to urine dilution and categorized into tertiles (numbered 1, 2, 3 from lowest to highest tertile). We used multivariate generalized linear models with Poisson distribution and log-link function to evaluate the association of metabolite concentrations with AFC by comparing the second and third tertiles to the first (reference category). Tertiles of phthalate metabolite concentrations were also used as continuous variables in models to evaluate the dose-response relationships between metabolites concentrations and AFC. Age (continuous), body mass index (BMI, continuous), year of study (2014 or 2016) and infertility diagnosis (female factor, male factor, mix factor or unexplained) were selected as covariates according to biological relevance or prior knowledge. Race and smoking status were not included as covariates due to the low frequencies of non-Han ethnicity (3.4%) and smokers (4.0%). Age was a critical independent impactor of AFC, to explore its potential modification effect, women were stratified into younger group (< 35 years) and older group (≥ 35 years) before analyses (33, 34). To test the strength of our results, we conducted sensitivity analyses by restricting our analyses to only women who sought infertility treatment due to tubal factors or male factors and had a normal BMI (18.5-24.9 kg/m^2). As the results showed that age could modify the associations between phthalate metabolite concentrations and AFC, we further performed re-analyses based on age stratification. Statistical analysis was conducted by SPSS (version 22.0, IBM Co., Armonk, USA).

3 Results

This study comprised 525 women undergoing IVF who were recruited on two separate occasions, as shown in Table 1. A total of

TABLE 1 Description of the study population (N = 525).

Characteristics	Mean ± SD or N (%)
Age (years)	31.1 ± 5.1
BMI (kg/m^2)	21.9 ± 2.7
Ethnicity	
Han	507 (96.6)
Other	18 (3.4)
Smoking status	
Ever	21 (4.0)
Never	504 (96.0)
Year of study	
2014	110 (21.0)
2016	415 (79.0)
AFC	13.4 ± 6.8
Duration of infertility (years)	3.7 ± 2.9
Infertility type	
Primary infertility	286 (54.5)
Secondary infertility	239 (45.5)
Infertility diagnosis	
Female factor	344 (65.5)
Tubal factor	206 (39.2)
Ovulatory dysfunction	45 (8.6)
Diminished ovarian reserve	56 (10.7)
Endometriosis	24 (4.6)

(Continued)

TABLE 1 Continued

Characteristics	Mean ± SD or N (%)
Uterine factor	13 (2.5)
Male factor	76 (14.5)
Mix factor	86 (16.4)
Unexplained	19 (3.6)

110 subjects were enrolled in 2014, and 415 were enrolled in 2016. The average (± SD) age and BMI of the study population were 31.1 ± 5.1 years and 21.9 ± 2.7 kg/m^2, respectively, and most of them were of Han ethnicity (96.6%) and had never smoked (96.0%). The average (± SD) duration of infertility was 3.7 ± 2.9 years, and more than half of the subjects had primary infertility (54.5%) and were diagnosed with female factors (65.6%). The average (± SD) AFC was 13.4 ± 6.8. Most of the tested phthalate metabolites were detected in urine in most of the participants (> 92.6%), except for MOP (26.1%), as shown in Table 2. MBP was the phthalate with the highest level of exposure (median: 187 μg/L), and except for mono-benzyl phthalate (median: 0.16 μg/L), the remaining 5 metabolites had similar concentrations (median: 12.9-18.8 μg/L). MOP was removed from further analyses because of its low detection frequency.

In the Poisson regression models adjusted for age, BMI, year of study and infertility diagnosis, we found that concentrations of MBP, MEOHP and ΣPAEs were positively associated with AFC (Figure 1). Compared with women in the first tertile, women in the third tertile of MBP and MEOHP had a 7.02% (95% CI: 1.18%, 12.9%) and 8.84% (95% CI: 2.83%, 14.9%) increase in AFC, respectively, and women in the second and third tertiles of ΣPAEs had a 6.19% (95% CI: 0.37%, 12.0%) and 9.09% (95% CI: 3.22%, 15.0%) increase in AFC, respectively (Supplementary Table S1). In trend tests by tertile for the dose-response relationship, MBP, MEOHP and ΣPAEs also showed significant positive associations with AFC.

In the age-stratified analysis, we observed a stronger relationship between phthalate metabolite concentrations and AFC among women ≥ 35 years and inverse associations among women less than 35 years (Figure 2). Compared to women in the first tertile, younger women in the second tertile of MEP and ΣDEHP had 6.50% (95% CI: -12.8%, -0.18%) and 7.37% (95% CI: -13.8%, -0.89%) lower AFC, respectively (Supplementary Table S2). However, trend tests for dose-response were not significant. Among older women, subjects in the second tertile of mono-methyl phthalate and ΣPAEs and the third tertile of MEP, MBP, MEHP, MEHHP, MEOHP, ΣDEHP and ΣPAEs had a 15.3% to 39.5% increase in AFC when compared with women in the first tertile (Supplementary Table S3). In addition, MEP, MBP, MEHP, MEHHP, MEOHP, ΣDEHP and ΣPAEs also showed significant trends. To explore whether the discrepancies were caused by differences in phthalate exposure levels, we compared phthalate

TABLE 2 Distributions of urinary phthalate metabolite concentrations (μg/L).

Metabolites	LOD	% > LOD	GM	25th	50th	75th
MMP	0.03	92.6	9.19	5.11	12.9	29.4
MEP	0.02	100	16.5	6.83	14.6	34.2
MBP	0.01	100	161	82.0	187	350
MBzP	0.01	96.4	0.18	0.05	0.16	0.85
MEHP	0.02	98.7	12.2	5.73	13.5	29.7
MEHHP	0.01	100	18.2	10.1	18.5	31.4
MEOHP	0.01	100	13.3	6.96	13.8	23.8
MOP	0.04	26.1	0.05	< LOD	< LOD	0.05
ΣDEHP[a]	–	–	0.17	0.08	0.17	0.30
ΣPAEs[a]	–	–	1.35	0.77	1.42	2.65

LOD, limit of detection; GM, geometric mean.
[a]ΣDEHP and ΣPAEs were expressed in μmol/L.

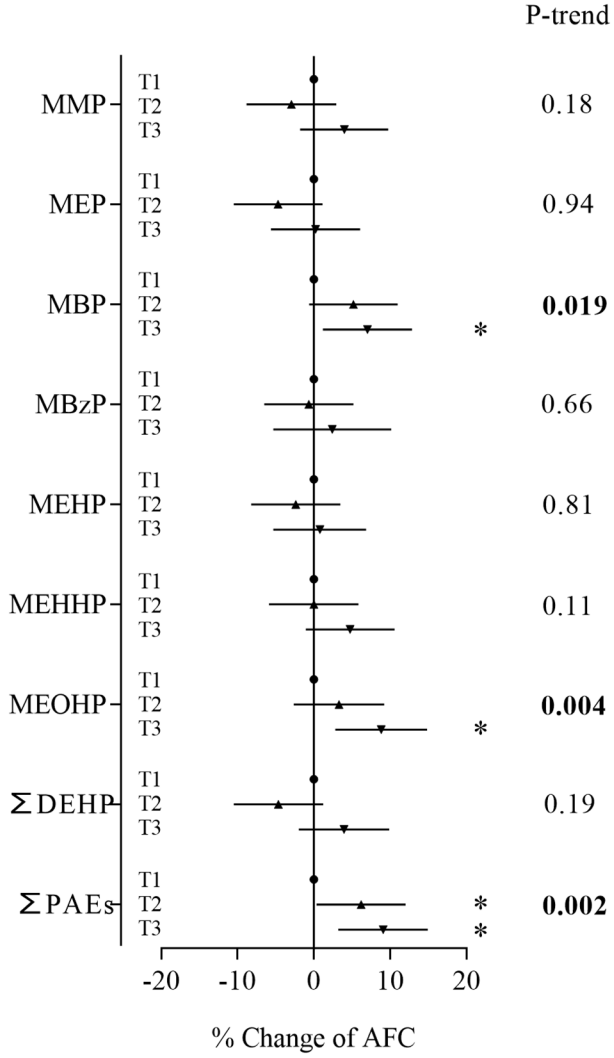

FIGURE 1
Associations between phthalate metabolite concentrations and AFC. Models were adjusted for age, BMI, year of study and infertility diagnosis. *P < 0.05.

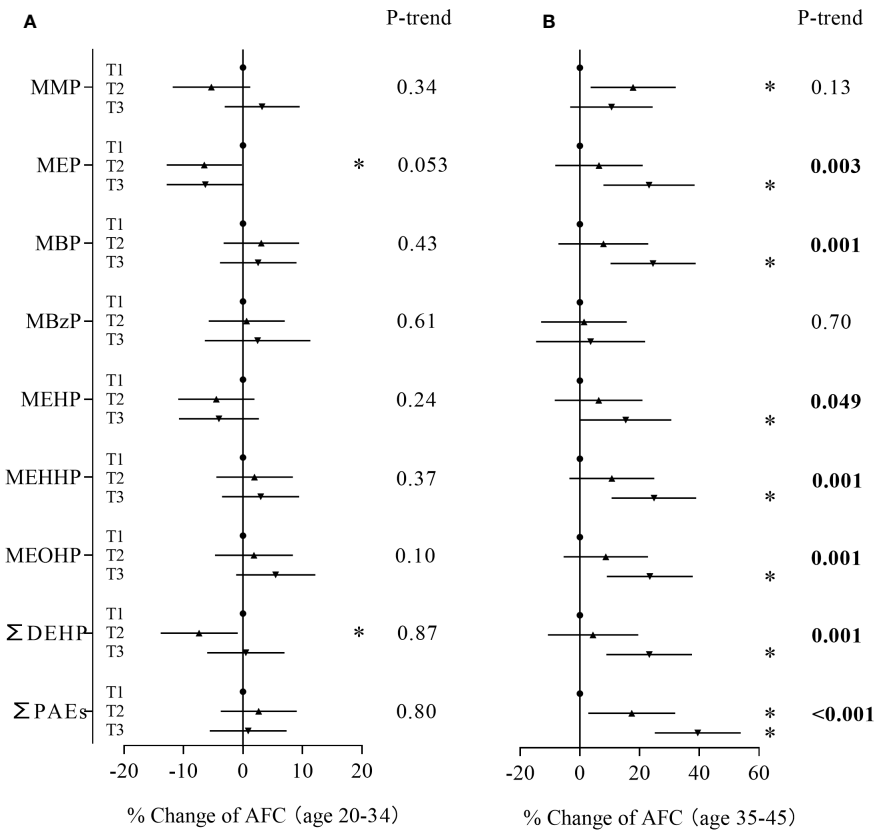

FIGURE 2
Associations between phthalate metabolite concentrations and AFC stratified by age. **(A)**: women < 35 years, **(B)**: women ≥ 35 years. *P < 0.05.

metabolite concentrations between the two groups. The results showed that younger women and older women had similar phthalate exposure levels (Supplementary Table S4).

In the sensitivity analyses based on women who were diagnosed with tubal factor infertility and infertility due to male factor, MBP, the major contaminant, and sum of PAEs showed positive associations with AFC (Figure 3). Women in the second and third tertiles of MBP and third tertile of ΣPAEs had an AFC range from 9.48% to 12.2% higher than women in the first tertile (Supplementary Table S5). These metabolites also showed consistent significant positive associations with AFC in trend tests. In the sensitivity analyses based on women with normal BMI, MBP and the sum of PAEs still showed positive associations with AFC (Figure 4; Supplementary Table S8). Similarly, stronger relationships were observed between phthalate metabolite concentrations and AFC among women ≥ 35 years than women < 35 years in all sensitivity analyses (Supplementary Tables S6, S7, S9, S10).

4 Discussion

In this study, we found that most of the phthalate metabolites were highly detected in urine samples, and MBP had the highest concentration. The European Food Safety Authorities have recommended the tolerable daily intake for several phthalates, as 10 and 50 μg/kg bw/day for DBP and DEHP, respectively (35). Estimated daily intakes for DBP and DEHP were calculated as reported (36), 58.7% and 10.3% of subjects had higher estimated daily intakes than the tolerable daily intake for DBP and DEHP, respectively, in this study. Compared with previous studies based on 125 non-pregnant women (37) and 946 pregnant women (38) in the same region, the detection rate was similar, and MBP uniformly showed the highest concentration, followed by DEHP metabolites, while the values were higher in our study (median: MBP 187 vs. 62.1 and 41.8 μg/L, MEHHP 18.5 vs. 6.34 and 5.05 μg/L, MEOHP 13.8 vs. 4.74 and 3.99 μg/L, MEHP 13.5 vs. 2.15 and 2.11 μg/L, respectively). This result may indicate an association between phthalate exposure and women with infertility.

We found that urinary MBP, MEOHP and ΣPAEs concentrations were positively associated with AFC, and most of the relationships remained in sensitivity analyses, which suggests the robustness of our results. In line with a previous study, Li et al. reported that the serum concentration of MEHHP was associated with an increased AFC among 297 IVF women (25). However, in a study of 215 women who sought infertility care, Messerlian et al. found a nonlinear inverse association between urinary DEHP metabolite concentrations (MEHP, MEOHP, MEHHP and MECPP) and AFC (24). These discrepancies may be attributed to differences in demographic characteristics (e.g., age, BMI, race, etc.). Furthermore, phthalate exposure status varied between studies. In Messerlian's study, MEP showed the highest concentration, while

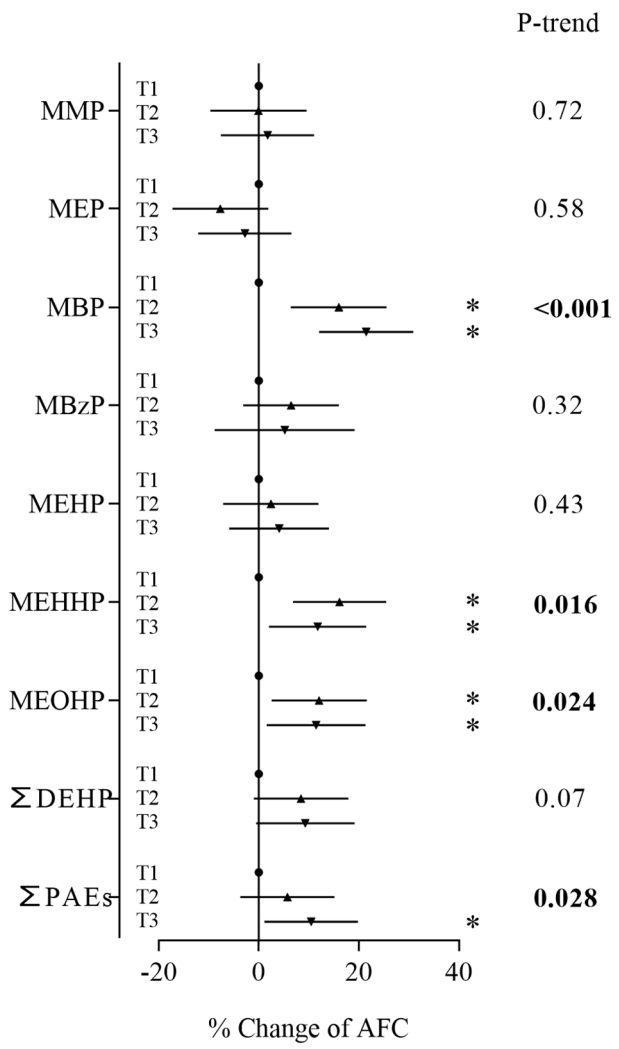

FIGURE 3
Associations between phthalate metabolite concentrations and AFC based on women diagnosed with tubal factor and infertility due to male factor. *$P < 0.05$.

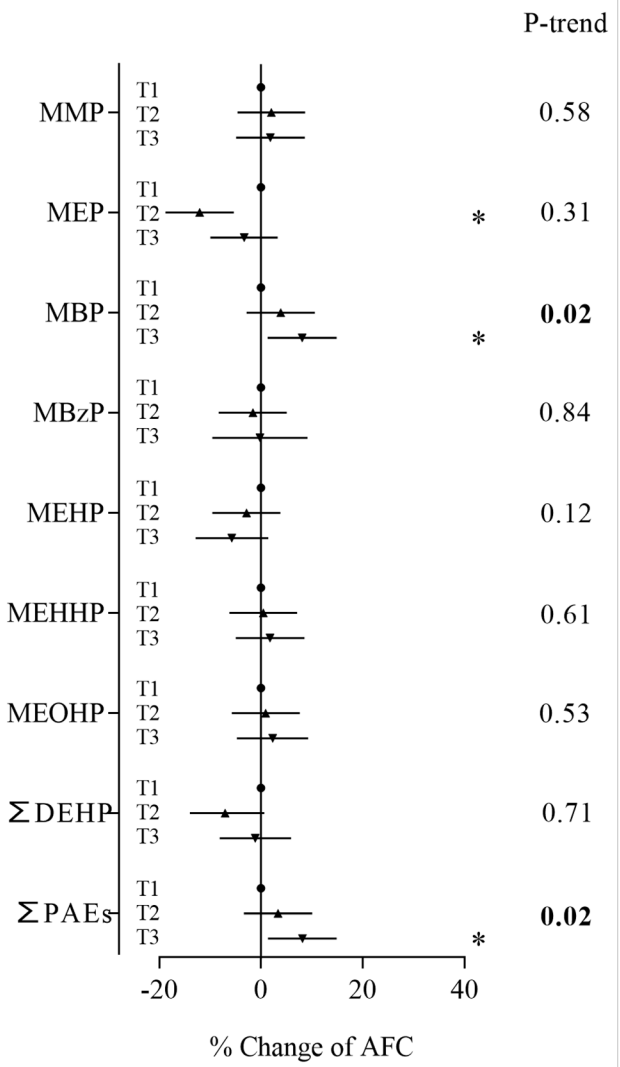

FIGURE 4
Associations between phthalate metabolite concentrations and AFC based on women with normal BMI. *$P < 0.05$.

the concentration of MBP was relatively low (SG-adjusted median: 54.2 and 12.8 μg/L, respectively), which was in contrast with our data (median: 14.6 and 187 μg/L, respectively). The overall metabolite concentrations were low in Li's study (median: MEP 2.19 μg/L, MBP 4.17 μg/L). In addition, phthalate may exert toxicity with a nonmonotonic dose-response effect or even a U-shape effect (39).

Primordial follicles, which are composed of a prophase-arrested oocyte enclosed by a single layer of flattened pre-granulosa cells, form at approximately 15-22 weeks of gestation and are complete by 6 months after birth in the human ovary (40). Their fates were to remain dormant, awaken and develop, or die directly during dormancy (41). Only a few of the dormant primordial follicles are activated under the regulation of intercellular and intracellular signals. Once awakened, primordial follicles join the follicle growing pool; most of them undergo atresia during development, and a small proportion undergo consecutive development through primary follicles, secondary follicles, antral follicles, preovulatory follicles and ovulation (42, 43). Therefore, a greater AFC indicates that more primordial follicles were awakened originally. In support of our findings, it has been reported that DEHP accelerates primordial follicle recruitment by decreasing the percentage of primordial follicles and increasing the percentage of developing follicles in postnatal mice (27) and in adult mice (28). Exposure to di-n-butyl phthalate, the prototype of MBP, promotes the depletion of follicular follicles by accelerating primordial follicle recruitment in rats (29). Additionally, in an *in vitro* culture neonatal ovary model, Hannon et al. revealed that MEHP could directly accelerate primordial follicle recruitment by over activating PI3K signals (44).

The number of primordial follicles in the ovary determines the ovarian reserve (40). Any factors that accelerate primordial follicle recruitment will promote the depletion of ovarian reserve and shorten the reproductive life span of women. It has been reported that phthalate exposure was associated with the risk of premature ovarian failure (45). On the other hand, although a higher AFC was observed in women with higher urinary phthalate metabolite

concentrations in this study, follicle development competence may be affected. Previous studies found that phthalate could increase oocyte oxidative stress, disrupt the cell cycle, impair meiotic competence, induce oocyte and granulosa cell apoptosis, inhibit oocyte maturation, cause epigenetic alterations, disrupt DNA damage repair gene expression, etc., which may contribute to adverse reproductive outcomes (10, 46–50).

Age was a critical independent risk factor for female fecundity and was associated with adverse IVF outcomes (e.g., lower oocyte retrieval, pregnancy rate, delivery rate, etc.) (34, 51). In age stratification analyses based on all subjects and in sensitivity analyses, we found that the associations between phthalate metabolite concentrations and AFC were modified by age, which is in line with a previous study (24). In women with advanced age, the ovary may be more vulnerable to external hazardous factors such as phthalates. The risk resistance ability and repair capacity decline with ovarian ageing, such as mitochondrial DNA instability and dysfunction, disturbance of antioxidant signaling and increase in oxidative damage, DNA damage, aneuploidy, epigenetic alteration, microenvironmental alteration (52–54). Moreover, exposure duration is also an important factor. It has been widely reported that women are exposed to phthalate from the fetal period to childhood and then adulthood (38, 55, 56), and long-term exposure is more likely to lead to adverse consequences.

In summary, our results showed that phthalate metabolite concentrations in urine were positively associated with AFC among women undergoing IVF. However, this study still has some limitations. (1) The AFC was measured when women attended the infertility clinic for ovarian reserve assessment, while urine samples were collected the on day of surgery, which was up to 4 months after AFC measurement, and the median interval between AFC measurement and sample collection was 72 days. Humans are continuously exposed to phthalates, and it has been reported that a spot urine sample was a moderate predictor of the 4-month exposure level of phthalates (57). (2) The study population was enrolled from a reproductive center, and they had higher phthalate exposure levels than the normal population as described above; therefore, these findings may not be applicable to the general population. (3) It is important to note that phthalate exposure is just one of the factors that can influence reproductive health and fertility. Lifestyle, genetics, and other environmental factors also play a role, we cannot rule out the influences from other factors in this study. (4) We found positive associations between phthalate exposure and AFC, and while the causation cannot be determined, more studies are needed to better understand their relationships.

5 Conclusions

The results support the idea that phthalate exposure could accelerate primordial follicle recruitment and promote the depletion of ovarian reserve. Our research fills the gap between animal studies showing that phthalates accelerate primordial follicle recruitment and epidemiological research showing that phthalates are associated with premature ovarian failure. Considering the high exposure frequency and level of phthalates in infertile women, more studies are needed to determine the effect of phthalate on ovarian reserve and folliculogenesis.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Board of Tongji Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YY: Formal Analysis, Investigation, Methodology, Writing – original draft. YD: Data curation, Investigation, Resources, Writing – review & editing. NG: Investigation, Resources, Writing – review & editing. FL: Supervision, Writing – review & editing. TD: Investigation, Methodology, Writing – review & editing. YL: Funding acquisition, Resources, Supervision, Writing – review & editing.

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The association of Life's Simple 7 and infertility among U.S. women

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Background: The Life's Simple 7 (LS7) metric is a comprehensive measure of cardiovascular health (CVH) that encompasses seven distinct risk factors and behaviors associated with cardiovascular disease (CVD). Some studies have shown an association between infertility and CVD. The present study aimed to explore the potential association between the LS7 factors and infertility.

Methods: A cross-sectional study was conducted on a sample of 3537 women aged 18-44 years from the National Health and Nutrition Examination Survey (NHANES) spanning the years 2013-2018. The LS7 metrics encompassed various factors including physical activity, smoking habits, body mass index, blood pressure levels, dietary patterns, blood glucose levels, and total cholesterol levels. We computed a 14-point LS7 score based on participants' baseline data, classifying them as "inadequate" (3-6), "average" (7-10), or "ideal" (11-14). Infertility is defined as an affirmative answer to either of two questions on the NHANES questionnaire: "Have you tried to conceive for at least one year without success?" and "Have you sought medical help for your inability to conceive?" Logistic regression was utilized to estimate odds ratios (O.R.s) and 95% confidence intervals (C.I.s).

Results: In total, 17.66% of participants were classified as individuals who reported experiencing infertility. In the continuous analysis, each one-unit increase in LS7 score was associated with a significantly decreased odds of infertility (OR=0.88 [0.77-0.89]). Analyzing the categorical representation of LS7 score, compared to individuals with poor scores, those with ideal scores exhibited a substantial 58% reduction in the odds of infertility (OR=0.42 [0.26-0.69]). Additionally, the observed interaction suggested that the influence of age on the relationship between LS7 and infertility is not consistent across different age groups (*P* for interaction < 0.001). Among individuals aged 35 or younger, each unit increase in LS7 score was associated with a substantial 18% (OR=0.82 [0.76-0.89]) decrease in the odds of infertility. However, in the older age group (>35), the association was attenuated and non-significant.

Conclusions: Our research suggests a significant inverse association between LS7 scores and infertility. Age demonstrated a varying impact on this relationship, with a more pronounced impact observed among individuals aged 35 or younger.

KEYWORDS

infertility, cardiovascular health (CVH), Life's Simple 7 (LS7), National Health and Nutrition Examination Survey (NHANES), female

1 Introduction

Infertility is a medical condition that has traditionally been defined as the incapacity to achieve a viable pregnancy following a period of 12 months or longer of regular, unprotected sexual intercourse. It is estimated to impact approximately 8.5% of women in the United States between the ages of 15 and 49 (1, 2). Infertility, as highlighted by the Centers for Disease Control and Prevention (CDC) in the United States, extends beyond a mere quality-of-life concern, encompassing substantial public health implications including psychological distress, societal marginalization, financial strain, and marital disharmony (3). Moreover, it should be noted that infertility is not exclusively a distinct ailment of the reproductive system, but rather frequently exhibits physiological or genetic associations with various other diseases and conditions, including cardiovascular disease (4, 5).

Cardiovascular disease (CVD) stands as a prominent contributor to mortality rates within the United States (6). Polycystic ovary syndrome (PCOS), a well-established factor contributing to infertility, has been linked to compromised glucose tolerance and cardiovascular disorders (7, 8). Approximately 25% of cases of female-factor infertility are attributed to anovulation related to PCOS, while a significant proportion remains unexplained. The National Health and Nutrition Examination Survey (NHANES) has found a significant association between the experience of infertility in women and cardiovascular health (9). Nevertheless, the correlation between cardiovascular health metrics and infertility lacks sufficient evidence, making it necessary to investigate further in order to elucidate the impact of cardiovascular disease prevention on women experiencing infertility.

The American Heart Association (AHA) has developed a health guideline known as Life's Simple 7 (LS7), which serves as a metric for cardiovascular (CV) health (10). LS7 categorizes individuals into poor, intermediate, and ideal levels based on seven CVD risk factors and behaviors: smoking, physical activity, body mass index (BMI), total cholesterol, fasting glucose, blood pressure, and diet (11). Multiple studies have demonstrated a correlation between elevated LS7 scores, which serve as a measure of ideal cardiovascular well-being, and a lower risk of developing cardiovascular disease (12) and non-cardiovascular disease outcomes such as heart failure (13, 14), cancer (15), depression, and cognitive impairment (16), To date, there has been a dearth of research investigating the potential association between Ideal cardiovascular health and infertility.

As a result, the present study investigates the association between LS7 and infertility in women. The study utilizes a substantial sample size comprising individuals aged 18 to 44, sourced from the National Health and Nutrition Examination Study (NHANES).

Abbreviations: Cardiovascular disease, (CVD); Life's Simple 7, (LS7); cardiovascular health, (CVH); Polycystic ovary syndrome, (PCOS); National Health and Nutrition Examination Survey, (NHANES); Body mass index, (BMI); American Heart Association, (AHA); Odds ratios, (O.R.s); Confidence intervals, (C.I.s).

2 Methods

2.1 Study population

The NHANES is a comprehensive, multistage, and probabilistic survey of the U.S. national population that offers a wealth of data on the general health and nutrition of the U.S. population (17). The NHANES employs a meticulously structured stratified, multistage probability sampling design to systematically enlist a representative cohort from the U.S. civilian, non-institutionalized populace. This sampling strategy entails the meticulous division of the nation into diverse strata based on nuanced demographic and geographic characteristics. Within each stratum, discrete clusters are identified, and a randomized selection of households ensues. Selected participants undergo a comprehensive health examination encompassing meticulous medical, dental, and physiological assessments. Simultaneously, participants partake in detailed interviews to elicit comprehensive information on various health-related domains, encompassing demographics, lifestyle patterns, and dietary habits. Furthermore, the research protocol includes the collection of biological specimens, such as blood and urine, facilitating the assessment of health indicators, nutritional status, and the determination of exposure levels to environmental factors.

The 2013–2018 continuous cycle of the US NHANES dataset was used for this investigation. A total of 29,400 individuals took part in the three cycles under consideration. After excluding male participants (N=14452), individuals below the age of 18 or above the age of 44 (N=10733), those with any missing data for the LS7 metrics (N=171), and individuals with missing data for any of the variables included in this study (N=467), a total of 3537 participants were included for analysis (see Figure 1).

2.2 Study variables

Information about age, marital status, education, race and ethnicity, poverty level index and smoking status were all self-reported. The measurement of socioeconomic status involved the utilization of the poverty income ratio, which represents the proportion of family income in relation to the federal poverty threshold, taking into account the specific year in which the interview was conducted. Poverty was operationally defined as a ratio equal to or less than one (18).

The LS7 framework encompasses various factors that contribute to overall health, including physical activity, smoking habits, BMI, dietary patterns, blood glucose levels, blood pressure, and total cholesterol levels. The measurement of height and weight was conducted using standardized methodologies. BMI was calculated as weight in kilograms over height in meters squared (kg/m^2), and was categorized using criteria established by the National Institutes of Health as underweight (<18.5 kg/m^2), normal (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m2), and obese (≥30 kg/m2). Due to the relatively small number of respondents in the underweight category, the underweight category was joined with the normal category after a sensitivity

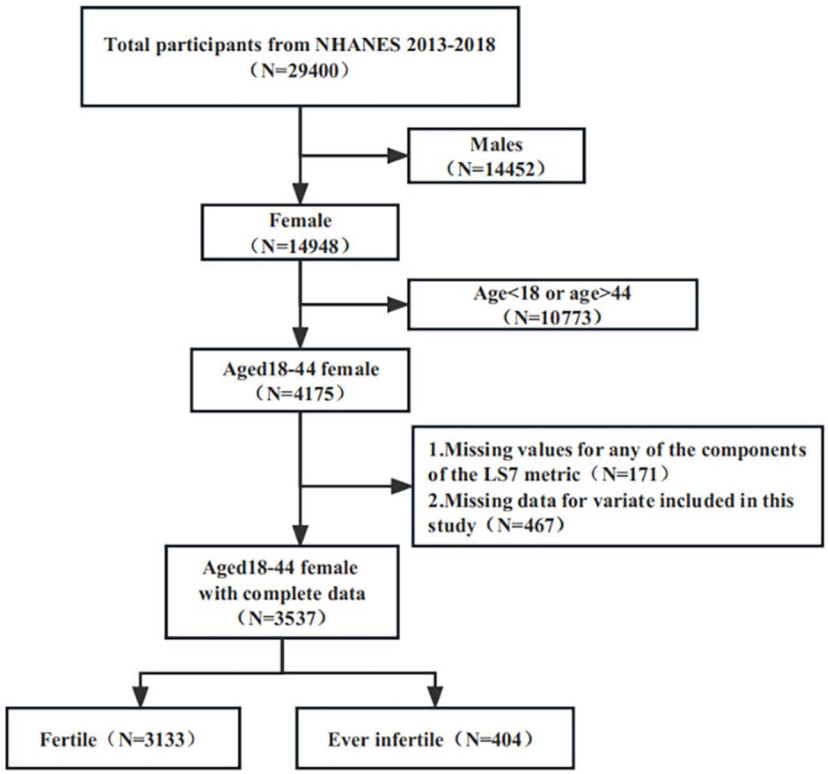

FIGURE 1
Flowchart of participant selection. NHANES, National Health and Nutrition Examination Survey.

analysis showed little difference in the results between excluding the underweight category and including them in the normal weight category (19). Specifically, to measure height accurately, one should remove shoes and bulky clothing, use a flat headpiece to form a right angle with the wall, and measure from the base on the floor to the marked measurement on the wall using a metal tape. To measure weight accurately, one should use a digital scale, avoid using bathroom scales that are spring-loaded, and ensure that the scale rests on a firm, stable table. The assessment of physical activity involved quantifying the frequency of engaging in activities of moderate to vigorous intensity, such as walking, jogging, running, bicycling, swimming, dancing, or yard work, within the preceding 30-day period. The levels of glucose and total cholesterol were assessed using previously established methodologies (20). The assessment of diet intake was conducted by interviewers who had received specialized training. This assessment involved the use of two 24-hour dietary recall questionnaires. The U.S. Department of Agriculture utilized the mean of two recalls pertaining to various dietary components (such as fruits and vegetables, fish, whole grain, sodium, and added sugar) in order to calculate the healthy diet index. The risk factors associated with cardiovascular disease were classified into three levels: "poor," "intermediate," and "ideal." Each level was assigned a score of 0, 1, and 2, respectively (refer to Appendix 1). The scores were aggregated, with a maximum value of 14 representing the ideal level of cardiovascular health. The LS7 lacks validated cut points, and the cut points employed in previous studies have not demonstrated consistency. Nevertheless, numerous studies have demonstrated that individuals with scores of 10 or 11 or higher exhibit a reduced occurrence of both cardiovascular and non-cardiovascular diseases (21, 22). In accordance with earlier studies, the entire LS7 score was therefore categorized as being insufficient (0–7), average (8-10), or ideal (11–14) (23–25).

Infertility was assessed by each woman's response to two questions from the NHANES questionnaire: 1) "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?" and 2) "Have you ever been to a doctor or other medical provider because you have been unable to become pregnant?" Any woman who answered "Yes" to either of these questions was considered to have a history of infertility.

2.3 Statistical analysis

The statistical analysis was conducted utilizing the statistical computing and graphics software R (version 4.3.1). The baseline characteristics of the participants were presented using mean values with standard error (SE) and proportions. Categorical variables were analyzed using the Rao-Scott x2 test, while continuous data were analyzed using analysis of variance. Logistic regression models were employed, taking into account the weighting of the data. The study employed logistic regression models to calculate odds ratios (O.R.s) and 95% confidence intervals (C.I.s) in order to

assess the relationship between ideal cardiovascular health, represented as continuous or categorical variables, and fertility status. The Benjamini-Hochberg (BH) method was used to control the false discovery rate (FDR) for multiple testing. The multivariate test was constructed utilizing three models. Model 1 did not include any adjusted variables. In Model 2, adjustments were made for age, race, and education. Model 3 included adjustments for age, race and ethnicity, education, marital status, education, and poverty level index. The NHANES study has previously established associations between sociodemographic characteristics and infertility (26, 27). No additional adjustments were made for clinical parameters, including diabetes, hypertension, obesity, dyslipidemia, or hypertension. This occurred due to the measurements already accounted for in the estimation of the LS7 scores. Subsequently, the aforementioned statistical study methodologies were implemented for the subgroups pertaining to age, poverty level index, and marital status. Statistical significance was established at a significance level of P<0.05. Weighting approach was used to make the results more reflective of the broader US population.

3 Results

3.1 Baseline characteristics

Figure 1 depicts the study participants' selection process. Following selection, 3537 suitable participants were included for analysis, reflecting a population of 50,982,232 in the United States. 3133 people (82.34%, representing a population of 44,528,032) were fertile, while 404 (17.66%, representing a population of 6,454,200) were ever infertile.

Table 1 presents the differences in the chosen participants' baseline characteristics. Significant differences were observed between the fertile and infertile groups in the context of weighted analyses. The ever-infertile group differs from the fertile group in that they are more likely to be older (34.54 years vs. 30.38 years, P<0.001), to be married (63.81% vs. 41.71%, P< 0.001), and to have lower scores for smoking, blood pressure, body mass index, and glucose in the estimation of the LS7 scores. However, they have a lower proportion of poverty level index (<=1.3). All in all, Infertile individuals had a lower mean LS7 score than those in the fertile group (8.80 ± 0.18 vs 9.52 ± 0.07, P<0.001).

Table 2 presents a comprehensive compilation of the clinical characteristics exhibited by the subjects, with a particular focus on their cardiovascular health status, which is categorized as a column-stratified variable. When comparing the normal group to the group of participants classified as having ideal cardiovascular health, it was observed that a higher percentage of individuals in the latter group were younger (73.56% aged <=35 years), non-Hispanic white (57.55%), living above the poverty threshold (65.09%), and had education beyond high school (74.80%).

Figure 2 displays the distribution of LS7 components among individuals classified as fertile and ever infertile. Among the fertile population, a significant proportion of participants performed well in terms of physical activity (81.94%), non-smoking (70.47%), blood pressure (75.2%), blood glucose (86.95%), and cholesterol indicators (74.49%). There was a higher percentage than in the ever infertile population. A greater proportion of participants who had been infertile were in the poor categories for body mass index (51.49%) and dietary intake (51.82%). Compared to the fertile

TABLE 1 Weighted characteristics of the study population based on selected participants (weighted sample, N= 50,982,232).

Characteristics	Fertile	Ever infertile	*P-value*
	N= 3133	N= 404	
Age (years)	30.38 ± 0.21	34.54 ± 0.50	<0.001
Education lever (%)			0.461
More than high school	65.61	69.32	
High school	21.23	19.87	
Less than high school	13.16	10.80	
Race/Ethnicity (%)			0.130
Mexican American	12.39	10.63	
Non-Hispanic Black	13.64	12.48	
Non-Hispanic White	54.63	61.58	
Other Hispanic	8.26	5.98	
Other Race	11.09	9.34	
Poverty level index (%)			0.007
<=1.3	30.81	24.97	
1.3-1.85	12.08	7.89	
>1.85	57.10	67.14	
Marital status (%)			<0.001
Married	41.71	63.81	
Divorced/ Separated/Widowed	10.15	11.24	
Never married	33.27	13.97	
Living with partner	14.87	10.98	
Life's Simple 7 components			
Physical activity score	1.82 ± 0.01	1.82 ± 0.03	0.980
Smoking score	1.52 ± 0.02	1.40 ± 0.05	0.033
Blood pressure score	1.72 ± 0.01	1.62 ± 0.03	0.019
Body mass index score	1.01 ± 0.03	0.77 ± 0.07	0.003
Glucose score	1.84 ± 0.01	1.71 ± 0.03	<0.001
Cholesterol score	1.69 ± 0.01	1.56 ± 0.05	0.072
Dietary intake score	0.51 ± 0.02	0.50 ± 0.03	0.980
Life's Simple 7 score	9.52 ± 0.07	8.80 ± 0.18	<0.001

Mean ± SE for continuous variables: P-value value was calculated by weighted analysis of variance.
Percentages for categorical variables: P-value was calculated by weighted chi-square test.
The Benjamini-Hochberg method was used to adjust p values for multiple testing.

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TABLE 2 Characteristics of the selected participants According to Cardiovascular Health Status.

Characteristics	Inadequate	Average	Ideal
Age (%)			
<=35	45.64	63.05	73.56
>35	54.36	36.95	26.44
Education lever (%)			
Less than high school	16.84	14.70	8.15
High school	26.24	21.86	17.04
More than high school	56.91	63.43	74.80
Race/Ethnicity (%)			
Mexican American	11.63	12.55	11.96
Non-Hispanic Black	19.94	14.02	9.15
Non-Hispanic White	53.16	54.97	57.55
Other Hispanic	6.27	7.77	9.20
Other Race	9.00	10.69	12.16
Poverty level index (%)			
<=1.3	37.31	31.99	23.35
1.3-1.85	14.24	10.36	11.55
>1.85	48.45	57.65	65.09
Marital status (%)			
Married	36.61	45.80	48.10
Divorced/Separated/Widowed	16.33	10.73	6.02
Never married	30.77	27.98	34.31
Living with partner	16.30	15.49	11.57
Life's Simple 7 components			
Physical activity score (%)			
Poor	0	0	0
Intermediate	34.86	20.79	9.64
Ideal	65.14	79.21	90.36
Smoking score (%)			
Poor	42.64	21.15	2.13
Intermediate	15.10	12.40	8.77
Ideal	42.26	66.45	89.10
Blood pressure score (%)			
Poor	12.73	2.55	0.09
Intermediate	45.22	24.74	7.83
Ideal	42.05	72.71	92.08
Body mass index score (%)			
Poor	76.62	47.36	6.87
Intermediate	15.10	27.66	24.51
Ideal	8.27	24.97	68.62
Glucose score (%)			
Poor	13.44	1.08	0.00
Intermediate	30.42	11.93	2.21
Ideal	56.13	87.00	97.79
Cholesterol score (%)			
Poor	14.95	5.98	0.80
Intermediate	42.31	22.14	9.49
Ideal	42.75	71.88	89.72
Dietary intake score (%)			
Poor	76.10	59.86	29.72
Intermediate	22.82	39.63	63.21
Ideal	1.09	0.51	7.07
Ever infertile			
No	83.02	85.48	92.26
Yes	16.98	14.52	7.74

Life's Simple 7 score (0-14) Inadequate: (0-7); Average: (8-10); Ideal: (11-14).
Differences in characteristics by cardiovascular health status were all statistically significant (P<0.05); Values are survey-weighted percentages.

group, the ever infertile group had a higher proportion of smokers, those with elevated blood pressure, blood glucose, cholesterol levels and body mass index.

3.2 Association between LS7 and fertility status

Table 3 shows the results of the multivariate regression analysis. In the unadjusted model, the OR for glucose score was 0.59 (95% CI: 0.48-0.71), indicating a significant association with the risk of infertility. Upon controlling for age, education level, and race variables, the observed negative correlation remained statistically significant in model 2 [0.68 (0.55-0.85)]. The findings from Model 3, which was fully adjusted, indicate that there is a negative association between an increase of glucose score and the risk of infertility. Specifically, the OR was 0.64, with a 95% CI ranging from 0.51 to 0.80, suggesting an 37% decrease in the risk of infertility.

Table 4 showed that the OR for LS7 was 0.88 (95% CI: 0.83-0.94), indicating a significant association with the risk of infertility. Upon controlling for age, education level, and race variables, the observed negative correlation was statistically significant in model 2 [0.92 (0.86-0.98)]. The findings from Model 3 suggested that there is a negative association between an increase of 1 unit in LS7 metrics and the risk of infertility. Specifically, the OR was 0.89, with a 95% CI ranging from 0.83 to 0.95, suggesting an 11% decrease in the risk of infertility. Participants who achieved ideal scores on LS7 metrics

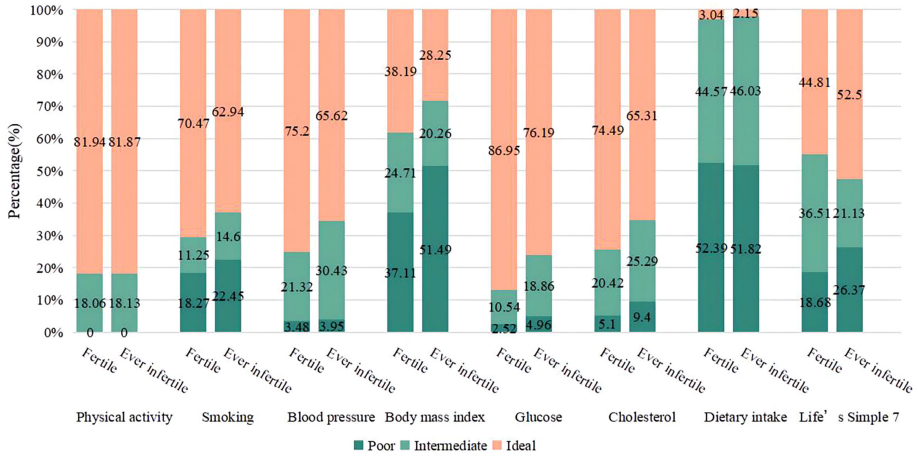

FIGURE 2
Distribution of Life's Simple 7 components in fertile and ever infertile subjects. Differences in poor, intermediate and ideal groups by fertility status were all statistically significant (P<0.01). Life's Simple 7 score Poor: (0); Intermediate: (1); Ideal: (2).

TABLE 3 The association between Life's Simple 7 components score and infertility.

	Model 1	Model 2	Model 3
	OR (95% CI)	OR (95% CI)	OR (95% CI)
	P-value	*P-value*	*P-value*
Physical activity score	0.99 (0.68-1.46)	1.13 (0.74-1.72)	1.11 (0.70-1.75)
	0.980	0.665	0.718
Smoking score	0.84 (0.73-0.96)	0.90 (0.76-1.07)	0.83 (0.68-1.01)
	0.030	0.308	0.126
Blood pressure score	0.72 (0.57-0.91)	0.91 (0.70-1.19)	0.83 (0.63-1.10)
	0.030	0.605	0.270
Body mass index score	0.72 (0.59-0.88)	0.77 (0.62-0.96)	0.75 (0.60-0.94)
	0.008	0.047	0.047
Glucose score	0.59 (0.48-0.71)	0.68 (0.55-0.85)	0.64 (0.51-0.80)
	<0.001	0.007	<0.001
Cholesterol score	0.70 (0.56-0.87)	0.82 (0.64-1.04)	0.83 (0.64-1.07)
	0.008	0.191	0.242
Dietary intake score	0.99 (0.79-1.24)	0.89 (0.71-1.12)	0.85 (0.67-1.07)
	0.977	0.407	0.242

Model 1: no covariates were adjusted.
Model 2: age, education level and race were adjusted.
Model 3: age, educational level, race, poverty ratio and marital status were adjusted.
OR, odds ratios (95% CI) 95% confidence intervals.
The Benjamini-Hochberg method was used to adjust p values for multiple testing.

exhibited a reduced risk of infertility when these metrics were considered as a categorical variable. Similarly, the group with ideal scores had a significantly lower OR for infertility than those with poor scores. Comparable findings were also noted in model 2.

TABLE 4 Association between Life's Simple 7 score and infertility.

	Model 1	Model 2	Model 3
	OR (95% CI)	OR (95% CI)	OR (95% CI)
	P-value	*P-value*	*P-value*
Continuous			
Life's Simple 7 score	0.88 (0.83-0.94)	0.92 (0.86-0.98)	0.89 (0.83-0.95)
	<0.001	0.015	0.004
Categorical			
Poor	ref	ref	ref
Intermediate	0.83 (0.61-1.12)	0.92 (0.68-1.26)	0.81 (0.57-1.13)
	0.248	0.600	0.248
Ideal	0.41 (0.26-0.64)	0.48 (0.30-0.75)	0.42 (0.26-0.69)
	<0.001	0.004	0.003

Model 1: no covariates were adjusted.
Model 2: age, education level and race were adjusted.
Model 3: age, educational level, race, poverty ratio and marital status were adjusted.
OR, odds ratios (95% CI) 95% confidence intervals.
Life's Simple 7 score (0-14) Poor: (0-7); Intermediate: (8-10); Ideal: (11-14).
The Benjamini-Hochberg method was used to adjust p values for multiple testing.

After controlling for all covariates, the ideal group exhibited a 42% lower risk of infertility (OR = 0.42, 95% CI: 0.26-0.69) in comparison to the poor group.

3.3 Subgroup analyses

In the subgroup analyses, the observed interaction suggested that the influence of age on the relationship between LS7 and infertility is not consistent across different age groups (*P* for interaction < 0.001). Among individuals aged 35 or younger, each

unit increase in LS7 score was associated with a substantial 18% (OR=0.82 [0.76-0.89]) decrease in the odds of infertility. However, in women older than 35 years, this association did not reach statistical significance in fully adjusted model. Similarly, subgroups stratified by the poverty level index were used to evaluate the association between the LS7 and the eternally infertile group. Using the fertile group as the reference group, the results revealed a substantial negative connection in fully adjusted model (Table 5). Using marital status as a subgroup for analysis, the relationship between LS7 and the risk of infertility was negatively associated only in those who were married and living with partner.

4 Discussion

Our study is the first to explore the relationship between the American Heart Association's LS7 metrics and fertility status between fertile and ever-infertile subjects in a U.S. population-based sample of women aged 20 to 44. The weighted study found that infertility was prevalent among women aged 18 to 44 at 17.66%, which aligns with the anticipated nationwide prevalence of 12 to 18% (28). The main finding of the study indicates a positive correlation between fertility and the LS7 Score, which reflects adherence to the American Heart Association's LS7 metrics. This suggests that individuals who adhere more closely to these metrics are less likely to experience infertility. Notably, the odds of infertility decrease significantly as ideal cardiovascular health levels increase. This association remains significant even after adjusting for potential confounding factors. The reference group used in the study was individuals with poor LS7 metrics, chosen to highlight the importance of addressing unhealthy lifestyles. Importantly, the results emphasize the need to prioritize LS7 metrics in the general population, not only for reducing the risk of cardiovascular disease but also for mitigating the risk of infertility.

TABLE 5 The association between Life's Simple 7 and infertility stratified by age, poverty level index and marital status.

	OR (95% CI)	*P-value*	*P for interaction*
Age			<0.001
<=35	0.82 (0.76-0.89)	<0.001	
>35	0.93 (0.85-1.02)	0.147	
Poverty level index			0.513
<=1.3	0.86 (0.78-0.95)	0.012	
1.3-1.85	0.83 (0.69-0.98)	0.048	
>1.85	0.90 (0.82-0.99)	0.048	
Marital status			0.240
Married	0.87 (0.79-0.96)	0.024	
Divorced/ Separated/Widowed	1.00 (0.83-1.20)	0.970	
Never married	0.90 (0.81-1.00)	0.090	
Living with partner	0.78 (0.67-0.91)	0.038	

Analyses were adjusted for covariates age, educational level, race, poverty ratio, and marital status when they were not the strata variables.
OR: odds ratios (95% CI) 95% confidence intervals.
The Benjamini-Hochberg method was used to adjust p values for multiple testing.

This study represents the inaugural attempt to examine the comprehensive array of ideal cardiovascular health metrics concerning infertility. Previous research has documented correlations between specific indicators of the LS7 framework, such as BMI (27), smoking (26), and dietary fiber (29)with self-reported infertility. Previous reports also observed older age (30), race (31, 32), and poverty (33) to be associated with higher odds of infertility. The fertility of individuals can be negatively impacted by various lifestyle and environmental factors, including but not limited to smoking and obesity (2). For instance, smoking has been associated with a reduced sperm count and quality in men, as well as an increased risk of infertility in women (34); excessive alcohol intake and caffeine consumption have been linked to infertility in both men and women (35); obesity, particularly in women, has been associated with an increased risk of infertility (36). However, being underweight can also negatively impact fertility in women; a diet high in trans fats, refined carbohydrates, and added sugars can negatively affect fertility in women, while a diet rich in dietary fiber, omega-3 fatty acids, and plant-based protein has been associated with improved fertility (37). Besides, some vitamins and minerals, such as folic acid, B12, and omega-3 fatty acids, have been suggested to improve fertility in women. Improving lifestyle factors can potentially improve fertility outcomes in patients with conditions like endometriosis and PCOS, which are associated with both cardiovascular disease and infertility. However, more research is needed to evaluate the potential benefits of managing cardiovascular risks using the LS7 metrics in patients with these conditions on their fertility outcomes (35).

The previous investigation indicated an elevated susceptibility to cardiovascular morbidity in subfertile women, albeit with a restricted sample size consisting solely of women who ultimately achieved childbirth (38). Previous studies have reported that women with endometriosis are at an increased risk of experiencing coronary heart disease later in life (39). The mechanisms that could explain the elevated risk of CVD in patients with endometriosis include inflammation, oxidative stress, and endothelial dysfunction (40). More studies of the cardiovascular-endometriosis interaction are needed to fully understand the underlying pathophysiology, possible means of early diagnosis, and prevention. Endometriosis is also associated with infertility, and the pathophysiology of both conditions shares some common biological pathways (40). Once again, it is important to note that these studies possess certain limitations regarding their scope, as they do not encompass cases of infertility that are idiopathic or undiagnosed.

The study conducted in LS7 found a correlation between smoking and infertility. The results indicated that individuals who had experienced infertility at any point in their lives had a noticeably higher prevalence of smoking compared to those who were fertile. According to a committee opinion, there is substantial evidence supporting a correlation between cigarette smoking and infertility. Researchers have identified several known toxins in the

ovary and follicular fluid of individuals who smoke cigarettes. There exists a correlation between smoking and a shortened duration of the menstrual cycle, specifically those lasting 24 days or less. This association has been observed to potentially lead to a decrease in fertility (41). The present study has identified correlations between body mass index and infertility. The BMI is a fundamental metric for assessing obesity. Consistent with prior research, there is evidence indicating that obese women experience impaired stromal decidualization. This phenomenon could potentially elucidate the causes of infertility resulting from compromised receptivity and subsequently contribute to the development of placental abnormalities. In addition, it has been observed that women who are obese are at a higher risk of experiencing ovulatory dysfunction as a result of the dysregulation of the hypothalamic-pituitary-ovarian axis (42, 43). PCOS is a prominent etiological factor contributing to infertility. The development of PCOS frequently involves the occurrence of insulin resistance, which subsequently gives rise to various cardiometabolic abnormalities such as dyslipidemia, hypertension, glucose intolerance, diabetes, and metabolic syndrome (MetS). Consequently, women with PCOS face an elevated susceptibility to cardiovascular disease (44). Consistently, our results also implied a negative association between an increase of glucose score and the risk of infertility. Observational evidence has indicated a shared etiology between impaired glucose tolerance, cardiovascular risk, and fertility problems. Elevated sugar levels have been associated with pregnancy complications, and poorly controlled sugar levels may lead to an increased risk of infertility and miscarriage (45). The results indicate a convergence in the underlying mechanisms that contribute to both infertility and cardiovascular disease, which are complex multifactorial syndromes (9). For instance, the activation of the hypothalamic-pituitary-adrenal (HPA) axis has been implicated in the pathogenesis of both conditions (46, 47). The activation of neuroendocrine pathways is associated with stress, which has been independently linked to the development of MetS, cardiovascular disease, and infertility (48). Therefore, infertility may serve as an indicator of cardiometabolic disorders that may be initiated by neuroendocrine or other common mechanisms, and that could potentially be mitigated through timely intervention. An additional research investigation revealed that the adverse cardiovascular characteristics observed in women with PCOS during their reproductive years could potentially impact the well-being of their offspring, in addition to being influenced by genetic factors (49). The primary practical implication of our study is that adhering to the American Heart Association's LS7 cardiovascular health metrics may play a crucial role in the prevention of infertility. Ideally, implement a multifactorial intervention targeting the key LS7 metrics to establish a more comprehensive understanding of the subject matter. The LS7 tool is a straightforward and economically efficient instrument for assessing CVH, with potential applications in monitoring and advancing a novel approach centered on fertility health. The LS7 metrics frequently exhibit interdependent interactions that can collectively contribute to the manifestation of infertility. The utilization of a composite score has the potential to offer valuable insights into the prediction of infertility rates. However, further research should be conducted in the form of prospective studies to substantiate and validate this claim. One of the important ways of clinical treatment for infertility continues to be lifestyle modifications. Given this consideration, when women seek medical assistance for infertility, healthcare providers are presented with a distinctive opportunity to offer guidance to women in their reproductive years regarding behavioral modifications that could potentially reduce the likelihood of developing chronic diseases in the future. It is crucial to address these matters while women are still capable of implementing such changes (50).

According to the present studies, although several studies have suggested an association between endometriosis, cardiovascular disease, and infertility. Women with infertility, particularly related to ovulation disorders and endometriosis, may be at an increased risk of experiencing coronary heart disease later in life (39). Additionally, endometriosis is associated with a higher risk of cardiovascular outcomes, potentially due to factors such as chronic inflammation, oxidative stress, and atherogenic lipid profile (51, 52). As for the impact of controlling cardiovascular risks on fertility outcomes in patients with endometriosis, the research is still limited. However, given the potential interplay between endometriosis, CVD, and infertility, managing cardiovascular risks in these patients could be beneficial for both cardiovascular health and fertility outcomes. Further research is needed to fully understand the underlying mechanisms and the potential benefits of managing cardiovascular risks in patients with endometriosis on their fertility outcomes.

The clinical significance of the observed association between LS7 scores and infertility in our study is underscored by the implications for reproductive health and cardiovascular well-being. Our findings reveal a noteworthy 58% reduction in the odds of infertility among individuals with ideal LS7 scores compared to those with poor scores. From a clinical standpoint, these findings have several noteworthy implications. Firstly, they emphasize the interconnectedness of cardiovascular health and reproductive function (5, 9, 39), supporting the concept of a shared pathophysiological basis. Secondly, the observed reduction in infertility odds among individuals with ideal LS7 scores implies that interventions aimed at improving cardiovascular health may potentially exert a positive influence on fertility outcomes (2, 26, 35–37). Furthermore, the identification of modifiable risk factors within the LS7 framework provides clinicians with actionable targets for intervention and risk reduction. Strategies aimed at optimizing lifestyle factors encompassed by the LS7, such as smoking cessation, regular physical activity, and a heart-healthy diet, may not only contribute to cardiovascular health but also hold promise in the realm of reproductive medicine. In conclusion, the observed association between LS7 scores and infertility suggests a potential avenue for preventive interventions that target cardiovascular health, offering a nuanced perspective on the broader implications of maintaining ideal cardiovascular well-being for reproductive outcomes.

This study utilized data from the NHANES, a comprehensive dataset obtained through population-based sampling techniques that were implemented consistently throughout the United States. The study samples exhibited greater representativeness due to the inclusion of appropriate NHANES sampling weights in all analyses.

However, there are certain limitations on our study. Firstly, the self-report measure of infertility has certain limitations. Specifically, women may experience difficulties in accurately recalling the duration of their attempts to conceive, leading to potential misclassification of the length of time dedicated to conception efforts. Similarly, women clinically diagnosed with infertility prior to trying to conceive for 12 months or who had not tried to conceive may not have been included in our measure of infertility. This category encompasses women who have been diagnosed with endometriosis or PCOS, as well as women who are above the age of 35 and have been unable to conceive after six months of unsuccessful attempts. The underlying mechanism still needs to be determined. Secondly, due to the cross-sectional character of our research, we were unable to establish a causal link between LS7 and infertility. Thirdly, multiple other factors such as occupational exposure and genetic variants may also contribute to the pathology of infertility, further study needs to be done in this research field. Fourthly, the group with infertility may vary, and the LS7 may change with age, these confounding factors may lead to bias to some extent. Lastly, our study lacks specific data on PCOS, limiting our ability to discern whether the observed association between LS7 scores and infertility is independent of PCOS. While we acknowledge the potential relevance of PCOS, our focus was on the general relationship between LS7 scores and infertility, without specific subgroup analyses for PCOS. This study calls attention to the need for targeted research exploring nuanced associations between LS7 scores, infertility, and specific conditions such as PCOS.

5 Conclusion

In the population of women aged 18-44 in the United States, our research suggests a correlation between higher scores on the LS7 metric and a reduced likelihood of experiencing infertility. Additional research is warranted as LS7 metrics represent an ideal state of cardiovascular health, serving as a reliable indicator of a healthy lifestyle. Moreover, these metrics hold potential as a novel approach to addressing infertility concerns. The results of this study provide a new insight that the measurements of preventing cardiovascular diseases may also be associated with a lower prevalence of infertility.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: www.cdc.gov/nchs/nhanes/.

Ethics statement

The studies involving humans were approved by NCHS Ethics Review Board (ERB). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

LW: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. GC: Data curation, Formal Analysis, Methodology, Writing – original draft. SC: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. XZ: Data curation, Formal Analysis, Methodology, Writing – review & editing. MQ: Data curation, Formal Analysis, Methodology, Writing – original draft. YT: Data curation, Formal Analysis, Methodology, Writing – review & editing.

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Conflict of interest

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

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Appendix

APPENDIX 1 Definition of Life's Simple 7 Cardiovascular Health Metrics.

Metric	Life's Simple 7 Cardiovascular Health		
	Poor	Intermediate	Ideal
Blood pressure	Treated BP≥140/90 mm Hg, and BP ≥140/90 mm Hg	SBP 120-139 mm Hg or DBP 80-89 mm Hg or treated to <120/80 mm Hg	<120/80 mm Hg, without BP-lowering meds
Cholesterol	≥240 mg/dL	200-239 mg/dL or treated to <200 mg/dL	<200 mg/dL, without lipid-lowering medication
Glucose	HbA1c >6.4%	HbA1c 5.7%-6.4% or treated with insulin or oral meds to HbA1C <5.7%	HbA1c <5.7%, without meds
Smoking	Current smoker	Former smoker	Never smoker
Body mass index	BMI≥30 kg/m^2	25-29.9 kg/m^2	<25 kg/m^2
Physical activity	No activity	1-149 minutes moderate/ vigorous per week	≥150 minutes moderate/ vigorous per week
Dietary intake	HEI<50	HEI 50-80	HEI>80

AHA indicates American Heart Association; FPG, fasting plasma glucose; BMI, body mass index; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, hemoglobin A1c; HEI, Healthy Eating Index;
The AHA definitions for poor, intermediate, and ideal health were used for blood pressure, cholesterol, BMI, and physical activity; however, modified definitions were used for FPG, smoking, and health diet score.
Glucose, AHA defined poor health as FPG ≥126 mg/dL or HgbA1C ≥7%, intermediate health as FPG 100 to 125 mg/dL or HgbA1C <7%, and ideal health as FPG <100 mg/dL.
Smoking, AHA defined poor health as current smoker, intermediate health as quit smoking <12 months, and ideal health as never smoker or quit smoking ≥12 months.
HEI score includes 3 of 5 primary criteria included in the AHA healthy dietary score: fruits and vegetables, whole grains, and sodium. Not included are sugar-sweetened beverages and fish consumption.

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Phase angle at bioelectric impedance analysis is associated with detrimental sperm quality in idiopathic male infertility: a preliminary clinical study

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Background: In 2020, 38% of adults were affected by obesity, while infertility globally affected 1 in 6 people at some stage of their lives.Body mass index (BMI) provides an easy but occasionally inaccurate estimation of body composition. To achieve a more precise assessment, bioelectric impedance analysis serves as a validated tool that administers electrical energy through surface electrodes. Phase angle as a function of the relationship between tissues resistance and reactance, is a trustworthy predictor of body composition and cell membrane integrity.

Objectives: We aim to assess whether there is an association between phase angle and seminal parameters, as well as sperm DNA fragmentation percentage.

Design: Semen samples of 520 idiopathic infertile patients were analyzed according to 2021 World Health Organization guidelines and evaluated for sperm DNA fragmentation rate. Each participants underwent bioelectric impedance analysis.

Results: Median age was 40 years old, median BMI was 26.3 kg/m2, median phase angle was 6.2°. In the logistic regression analysis adjusted for age and total intracorporeal water, phase angle (continuous) was significantly associated with oligozoospermia (odds ratio [OR]:0.4; p<0.01) and sperm morphology (OR: 0.65; p=0.05) and slightly with sperm DNA fragmentation (OR: 0.98; p=0.07). In subgroup analysis, the logistic regression analysis adjusted for the mentioned parameters showed that a phase angle between 6.2 and 7 (°) (OR: 0.63; p=0.02) and >7 (°) (OR: 0.12; p<0.01) were associated with a reduced risk of oligozoospermia compared to values <6.2 (°). Similarly, a phase angle between

6.2 and 7 (°) (OR: 0.57; p< 0.01 and OR: 0.58; p= 0.01) and PA > 7 (°) (OR: 0.12; p= 0.03 and OR: 0.21; p< 0.01) were associated with a reduced risk of lower sperm concentration and lower total sperm count, respectively, compared to a phase angle < 6.2 (°).

Conclusion: Our study suggests a negative association between phase angle and detrimental sperm parameters in male idiopathic infertility.

KEYWORDS

bioelectric impedance analysis, male infertility, phase angle, semen analysis, sperm DNA fragmentation

1 Introduction

In 2020, obesity was a global condition affecting 38% of adults aged 18 years and older (1). The expected global prevalence is projected to increase from 2.6 billion in 2020 to 4 billion by 2035 (1). Furthermore, predictions indicate that the prevalence of overweight in male and female adolescents will rise from 9% in 2020 to 19% in 2035, underscoring the importance of the issue of overweight (1). Similarly, infertility, defined as the inability to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse, has been acknowledged as a significantly widespread global condition, affecting 1 in 6 people at some point in their lives (2). According to the data, up to 50% of infertility in couples can be attributed to male factors (3). Obesity has been demonstrated to be a relevant risk factor for male infertility through various mechanisms including hypogonadotropic-hyperestrogenic hypogonadism, increased testicular inflammation events, resulting from augmented adipokines, raised testicular temperature, sexual dysfunctions and epigenetic induced alteration (4). Altered environment and the subsequent increase in reactive oxygen species (ROS) lead to elevated DNA damage through direct or indirect interaction with the DNA strand (5, 6). For this reason, several studies extensively tested sperm DNA fragmentation (SDF) as a marker of male infertility such as Chavarro and Kort found a statistically significant correlation between augmented and SDF percentage (7) (8),. Their findings were subsequently confirmed by Fariello et al., who observed a higher percentage of damaged DNA in obese men compared to normal-weight and overweight patients (9). Conversely, a recent meta-analysis published in 2020, involving a total cohort of 8255 patients, concluded that the data were insufficient to demonstrate a positive association between overweight and SDF values (10).

A study involving a large group of participants found that male partner obesity increases the risk of infertility for the couple (11, 12). Moreover, when both partners are obese, the risk of infertility is further elevated (13). Despite documented evidence, the relationship between obesity and seminal parameters remains a subject of ongoing debate.

Several studies reported a negative association between overweight and quality of conventional seminal parameters. Belloc et al. found that overweight negatively affects sperm count, sperm concentration, total sperm volume and sperm motility (14). Conversely, according to Aggerholm et al., obesity only affects semen volume but has no effect on other seminal parameters (15–17). To address this endless debate, Guo conducted a meta-analysis involving 26,814 participants and observed that obese patients had no alteration in sperm motility but experienced statistically significant decreases in total sperm count, sperm concentration, and semen volume (18). Since sedentary lifestyle and consequent augmented BMI seems to contribute to male infertility, it is easy to suggest that physical activity has a potentially significant impact on seminal parameters. Indeed, case-control studies have indicated that individuals in the physically active group exhibit enhanced semen parameters, including semen volume, viability, progressive motility, total motility, and morphology, when compared to the sedentary group (19). However, it has been widely reported that, while physical activity is generally associated with improved seminal parameters, excessively intense and prolonged training may have a negative impact on male fertility. Overtraining and intense physical stress may result in hormonal imbalances, elevated testicular temperature, and subsequent oxidative stress, potentially diminishing the quality of semen parameters (20).

Hakonsen reported that obesity-related oligozoospermia can be improved with weight loss, along with enhancements in reproductive hormonal profile and SDF percentage (21, 22) while Andersen observed that increased sperm concentration and total sperm count persist if weight loss is maintained (23).

While BMI is a convenient and easily calculable metric, its indirect estimation of body composition may be prone to inaccuracies. For instance, it can overestimate fat percentage in persons with higher lean muscle mass, such as athletes, and underestimate adiposity in individuals with lower muscular mass (24). To achieve a more accurate evaluation of body composition,

various alternative technologies have been developed, with one of the most promising being bioelectric impedance analysis (BIA).

BIA is a validated alternative tool that administers electrical energy through surface electrodes and records tissue responses by measuring parameters such as resistance and reactance (25).The working principle underlying BIA is Ohm's law, which establishes that the voltage across a conductor is directly related to the resistance to current flow (26). When an electrical current is introduced to biological tissue, its components facilitate the passage of the charge. The predominant charge carriers include mobile ions in water, while the dipolar components, categorized into positive and negative charges, consist of proteins and the lipidic cell membrane. Substantially, the measurement of those electric features gives information about tissues property and composition This permits the prediction of total body water (TBW), fat mass (FM), lean body mass (LBM), and the percentage of body fat (%BF), providing an accurate estimation of adiposity (27, 28). Phase angle (PA) is a function of the relationship between resistance and reactance representing a measure of extra- and intracellular water content; since electricity flows more easily through hydrated tissue, such as muscle, PA seems the most trustworthy predictor of body composition (25, 29). Phase angle in the context of bioimpedance refers to the phase shift between the voltage and current in an electrical circuit that passes through biological tissues. BIA is a method used to measure the impedance of biological tissues to alternating electrical currents. This impedance includes both resistive (real) and capacitive/reactive (imaginary) components. Moreover, PA represents a reliable marker of membrane integrity and cell mass (30). Essentially, BIA relies on models that predict TBW as a linear function of the resistance index, considering factors such as weight, age, and gender (31). However, literature data are missing regarding the potential association between PA and sperm parameters in patients with infertility. For these reasons, our study aims to assess the existence of an association between PA and seminal parameters, including total sperm count, sperm concentration, total motility, morphology, and SDF percentage in patients with idiopathic male infertility.

2 Materials and methods

A total of 520 consecutive male patients, seeking assistance at the Unit of Reproductive Medicine in the clinic 'Centro HERA' for primary couple infertility, participated in this prospective study (from January 2023 to June 2023. Our study included patients aged 18 years or older who were affected by idiopathic infertility. We collected information on the patients' age and conducted a physical examination, documenting measurements of height, weight, and BMI. Each patient performed sperm analysis, evaluation of SDF and BIA before starting any other treatment. Patients with varicocele, male accessory gland infection, genetic alterations, and hormonal diseases were excluded from the study. The current study protocol obtained approval from the Institutional Review Board at Centro HERA - UMR (Approval No. 1/2023). All subjects provided informed consent upon enrollment in the study.

2.1 Sperm analysis

Semen samples were collected through masturbation into a sterile container following 2–7 days of sexual abstinence. Analysis was conducted immediately after liquefaction. Each sample was assessed for seminal volume, sperm count, progressive motility, and morphology, in accordance with the 2021 WHO guidelines (3).

2.2 Sperm DNA fragmentation

Sperm samples underwent terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) staining using a commercially available kit (Dead End Fluorimetric TUNEL System; Promega, USA) following the manufacturer's instructions. Briefly, sperm were fixed in 4% paraformaldehyde at 4°C and permeabilized with 0.2% Triton X-100 (Promega) in PBS (Nutricell). After permeabilization, the samples were incubated in 100 ml drops with a reagent mix containing terminal deoxynucleotide transferase enzyme solution and 90% staining solution (dUTP fluorescein conjugate) for 1 h at 37°C in a dark humid chamber. Subsequently, the sperm were stained with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA), plus 4',6-diamidino-2-phenylindole (DAPI) and mounted on slides for evaluation using fluorescence microscopy (Olympus BX51). The TUNEL assay results were reported as the percentage of sperm DNA fragmentation, indicating the proportion of cells with DNA damage (32).

2.3 Bioelectric impedance analysis

Participant body composition was assessed during a single-day visit (<1 hour). Individuals were assessed on a direct segmental octopolar multi-frequency device (InBody, Model 770, Cerritos, California, USA), standing with feet apart and elbows extended to avoid body contact for approximately 1 min. The bare feet made positive contact with the base electrodes at the heels and forefeet and subjects grasped two handle electrodes for direct contact with two more electrodes for each hand at thumbs and forefingers. The segmental analysis was computed with proprietary algorithms. Data obtained from the InBody 720 device were processed using the Lookin Body 3.0 program. By data analysis, biometric information for each patient were collected, including:

- Fat mass (FM).
- Lean mass (LM).
- Muscular mass (MM).
- Percentage of body mass (%BF).
- Waist to hip ratio (WHR).
- Abdominal circumference (AR).
- PA (33).

Subjects reported to the laboratory for a single testing session after a minimum of 08 hours of fasting from food, caloric beverages, caffeine, alcohol, and tobacco. Additionally, subjects refrained from strenuous exercise for a minimum of twelve hours before testing. Height (cm) and weight (Kg) were measured upon arrival at the laboratory using a calibrated scale. For all measurements, subjects were instructed to be free from metal (e.g., zippers, jewelry, hard plastic) to avoid interference with data collection accuracy. Multi-frequency bioelectrical impedance analysis (MF-BIA) using the InBody 770 device (Biospace Co.) estimated total body composition, including fat percentage, FM and LM. Subjects stood barefoot on the device's scale for 5 minutes, with the soles of their feet positioned on four corresponding electrodes and holding the handles in both hands to contact corresponding electrodes on the thumbs and palms. Height, sex, and age were entered into the MF-BIA software, and the device collected weight. Subjects remained still for the duration of the assessment.

2.4 Statistical analysis

All statistical analyses were conducted using Stata (Stata Statistical Software: College Station, TX: Stata Corp LP). For all statistical comparisons, results were considered significant when $p < 0.05$. Normally distributed continuous variables were presented as median (interquartile range, IQR), and differences between groups were tested by Student's independent t-test or Mann–Whitney U-test, depending on their normal or non-normal distribution (normality of variables' distribution was tested by Kolmogorov–Smirnov test).

Age-adjusted linear regression models were performed to verify factors correlated with abnormal sperm parameters, expressed as beta-coefficient. The beta-coefficient represents the magnitude of the variation in the independent variable for each increase in the dependent value.

Multivariable logistic regression models were constructed to identify predictive factors of:

- Oligozoospermia, defined as < 39 million or < 15 million/ml of spermatozoa.
- Asthenospermia, defined as motility lower than 32%.
- Teratozoospermia, defined as normal morphology lower than 4%.
- Oligoasthenoteratozoospermia (OAT), defined as the coexistence of these abnormalities.

PA (reference value from 1 to 10) has been categorized into three sub-groups according to the resulting tertiles:

1) PA <6.2;
2) PA between 6.2 and 7;
3) PA >7.

A cut off of 20% has been considered for SDF according to Agarwal (34). Area under the curve (AUC) has been performed to verify accuracy of phase angle in diagnosing OAT.

3 Results

The median age was 40 years old (interquartile range [IQR]: 37.0-45.0), and the median BMI was 26.3 kg/m2 (IQR: 24.2 – 29.3). Additionally, patients' biometric parameters were collected: median FM was 20.15 kg (IQR: 13.1 – 24.8), median percentage of fat mass was 22.95% (IQR: 17.7-27.9), median LM was 62.3 kg (IQR: 57.3 – 67.5), median MM was 36.25 kg (IQR: 33.1 – 38.8), median AC was 96.35 cm (IQR: 86.3 – 103.6), median WHR was 0.945 (IQR: 0.89-0.98), and median PA was 6.2°(IQR: 5.8 – 6.5). Baseline characteristics of the entire cohort are listed in Table 1.

Classification of the analyzed cohort according to BMI in presented in Supplementary Table 1.

All semen analyses were conducted on sperm samples obtained after a median day of ejaculatory abstinence of 4 days (IQR: 3- 4). The semen analysis reported a median SDF of 22.0% (IQR: 16.0-29.0), median sperm concentration of 25.0 million/ml (IQR: 9.2-48.0), median total sperm count of 67.16 million (IQR: 30.8-130.63), median progressive motility of 15.5% (IQR: 5.0-26.0), and median morphology of 6.0% (IQR: 4.0-8.0). A total of 116

TABLE 1 Baseline characteristics of the study cohort (*n=520*).

Age (years old), median (IQR)	40 (37 – 45)
Weight (Kg), median (IQR)	83.1 (74.4 – 92.1)
Height (cm), median (IQR)	177 (173 – 180)
BMI (Kg/m^2), median (IQR)	26.3 (24.2 – 29.3)
FM (Kg), median (IQR)	20.15 (13.1 – 24.8)
LM (Kg), median (IQR)	62.3 (57.3 – 67.5)
MM (Kg), median (IQR)	36.25 (33.1 – 38.8)
%BF (%), median (IQR)	22.95 (17.7 – 27.9)
WL (cm), median (IQR)	88.85 (57.9 – 109.2)
AC (cm), median (IQR)	96.35 (86.3 – 103.6)
WHR, median (IQR)	0.945 (0.89 - 0.98)
PA (°), median (IQR)	6.2 (5.8 – 6.5)
Total sperm count (Mil), median (IQR)	67.16 (30.8 – 130.63)
Sperm concentration (Mil/ml), median (IQR)	25 (9.2 – 48.0)
Total motility (%), median (IQR)	15.5 (5 – 26)
Morphology (%), median (IQR)	6 (4 – 8)
SDF (%), median (IWR)	22 (16 – 29)

BMI, Body mass index; FM, Fat mass; LM, Lean mass; MM, Muscular mass; %BF, Percentage of Body Fat; WL, Waistline; AC, Abdominal circumference; WHR, Waist to hip ratio; PA, Phase angle; SDF, Sperm DNA fragmentation.

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TABLE 2 Anthropometric characteristics and sperm parameters according to the phase angle.

	PA > 6.2	PA ≤ 6.2	P-value
Age (years), median (IQR)	38 (35.0 – 42.0)	42 (38.0 – 46.0)	<0.01
Weight (Kg), median (IQR)	86.9 (77.6 – 94.6)	79.2 (71.7 - 90)	<0.01
Height (cm), median (IQR)	177 (173 -180)	178 (173 – 180)	0.48
BMI (Kg/m^2), median (IQR)	27.7 (25.6 – 29.7)	25.7 (22.4 – 28.4)	<0.01
FM (Kg), median (IQR)	20.4 (14.9 – 25.3)	18 (11.7 – 24.5)	0.08
LM (Kg), median (IQR)	65.1 (60.4 – 70.7)	60.2 (54.7 – 65.9)	<0.01
MM (Kg), median (IQR)	37.6 (35.3 – 40.6)	34.0 (31.3 – 37.3)	<0.01
%BF (%), median (IQR)	23.0 (18.7 – 27.5)	22.8 (17.1 – 30.2)	0.74
WL (cm), median (IQR)	91.2 (63.5 – 110-0)	82.7 (53.6 – 109.2)	0.50
AC (cm), median (IQR)	99.2 (90.1 – 106.0)	92.8 (84.5 – 102.1)	<0.01
WHR, median (IQR)	0.95 (0.9 - 0.99)	0.94 (0.88 - 0.98)	0.10
Sperm volume (ml), median (IQR)	7.42 (3.54 – 14.52)	6.06 (2.76 – 12.62)	0.04
Sperm concentration (million/ml), median (IQR)	28.05 (12.6 – 48.0)	21.45 (8.25 – 49.5)	0.20
Progressive motility (%), median (IQR)	15.0 (6.0 – 27.0)	16 (5.0 – 26.0)	0.81
Morphology (%), median (IQR)	5.0 (4.0 – 8.0)	6.0 (4.0 – 8.0)	0.37
SDF (%), median (IQR)	24.0 (14.0 – 29.0)	22 (17.0 – 29.0)	0.22

PA, Phase angle; BMI, Body mass index; FM, Fat mass; LM, Lean mass; MM, Muscular mass; %BF, Percentage of Body Fat; WL, Waistline; AC, Abdominal circumference; WHR, Waist to hip ratio; SDF, Sperm DNA fragmentation.

patients (22.3%) suffered from OAT. The prevalence of the remaining semen alterations among the cohort is listed in Supplementary Table 2.

Patients with PA ≤ 6.2 had higher median age (42 vs 40; p<0.01), lower median BMI (25.7 vs 26.3; p< 0.01), lower median lean mass (60.2 vs 62.3; p<0.01), lower median muscular mass (34.0 vs 36.2; p<0.01), lower median abdominal circumference (92.8 vs 96.35; p<0.01). Other biometric data did not show significant differences between the two groups. Moreover, patients with PA ≤ 6.2 had a significantly lower total sperm count (60.6 vs 67.16; p<0.05). The other sperm parameters were not significantly influenced by PA variation (Table 2).

Table 3 reports the correlation analysis between all parameters. PA was correlated with SDF (r = - 0.09; p< 0.05) and total sperm count (r = 0.12; p< 0.01). Figures 1A, B shows the scatter plot of the association between phase angle and SDF (Figure 1A) and TSC (Figure 1B).

The age-adjusted linear regression analysis demonstrated that PA was positively associated with sperm count (r=0.01; p=0.01) and morphology (r=0.02; p<0.01) but not with SDF (p=0.81), sperm concentration (p=0.06) and total motility (p=0.58).

In the logistic regression analysis adjusted for age and total intracorporeal water, PA (continuous) (OR [odds ratio]: 0.4; 95%CI 0.27-0.59; p<0.01) was significantly associated with oligozoospermia but not with the SDF (OR: 0.98; p=0.07) and with sperm morphology (OR: 0.65; p=0.05).

Logistic regression analysis of PA sub-groups, adjusted for age, total intracorporeal water, and SDF showed that a PA between 6.2 and 7 (°)(OR: 0.63; 95%CI 0.42-0.94; p=0.02) and PA >7 (°)(OR: 0.12; 95%CI 0.04-0.37; p<0.01) were associated with reduced risk of oligozoospermia compared to PA <6.2 (°). Similarly, PA between 6.2 and 7 (°)(OR: 0.57; 95% CI 0.37- 0.86; p< 0.01 and OR:0.58 95% CI 0.38- 0.88; p= 0.01) and PA > 7 (°)(OR: 0.12; 95% CI 0.0.4- 0.36; p= 0.03 and OR: 0.21; 95% CI 0.07- 0.63; p< 0.01) were associated with reduced risk of lower sperm concentration and of lower total sperm count respectively, compared to PA < 6.2 (°).

PA sub-groups logistic regression analysis adjusted for age and total intracorporeal water did not show any relation with sperm morphology (p=0.98 and p=0.09).

The AUC for phase in angle in diagnosing OAT was 0.61.

4 Discussion

Herein our data suggests that patients with lower PA (°) (≤ 6.2) had detrimental sperm parameters in particular lower sperm concentration and total sperm count. Our study is the first investigating the potential association between PA and low sperm quality in patients with idiopathic male infertility.

Although the exact mechanism by which PA influences sperm parameters remains unclear, several hypotheses have been proposed. First, PA is employed to assess nutritional status and body composition based on the electrical properties of different tissues (35). Indeed, since electricity flows more easily through hydrated tissue, such as muscle, it is foreseeable that suitable PA values are predictors of better body composition (29). On the other hand, some studies claim that BIA has a limited accuracy in predicting body composition (36). BIA appears to be significantly influenced by environmental factors, ethnicity, and medical conditions. Therefore, the development of an appropriate calibration equation is necessary for different groups of participants (37).

The relationship between phase angle from BIA and sperm DNA fragmentation has not been extensively studied or validated. While BIA has been investigated in various clinical contexts, it's utility in predicting sperm DNA fragmentation remains largely unexplored. Sperm quality and DNA integrity are influenced by multiple factors beyond cellular health, including oxidative stress, exposure to toxins, lifestyle factors, and genetic factors. While BIA may provide some insights into overall health status, it may not capture all the determinants of sperm quality and DNA fragmentation.

TABLE 3 Correlation analysis between parameters.

	Age	BMI	FM	LM	MM	WL	AC	WHR	Wtot	Wic	Wec	PA	SDF	Sperm concen-tration	Total sperm count	Motility	Morphology
Age	1.00	-0.01	0.09*	0.13**	-0.09*	0.10*	0.05	0.11*	-0.13**	-0.14**	-0.09	-0.33**	0.25**	0.08	-0.07	-0.14**	0.04
BMI	-0.01	1.00	0.91**	0.58**	-0.09*	0.9**	0.9**	0.79**	0.55**	0.52**	0.53**	0.33**	-0.02	-0.03	-0.01	-0.03	-0.01
FM	0.09*	0.91**	1.00	0.44**	0.42**	0.99**	0.96**	0.91**	0.42**	0.43**	0.42**	0.11**	0.02	0.13	0.01	-0.02	0.01
LM	0.13**	0.58**	0.44**	1.00	0.91**	0.44**	0.56**	0.39**	0.97**	0.89**	0.96**	0.27**	0.05	-0.15**	-0.05	-0.01	-0.07
MM	-0.09*	0.55**	0.42**	0.91**	1.00	0.42**	0.55**	0.38**	0.93**	0.89**	0.92**	0.35**	0.06	-0.14**	-0.05	0.01	-0.02
WL	0.10*	0.9**	0.99**	0.44**	0.42**	1.00	0.96**	0.92**	0.42**	0.43**	0.42**	0.06	-0.01	0.01	0.01	-0.01	-0.01
AC	0.05	0.9**	0.96**	0.56**	0.55**	0.96**	1.00	0.95**	0.56**	0.57**	0.54**	0.22**	-0.02	-0.01	0.01	-0.01	0.01
WHR	0.11*	0.79**	0.91**	0.39**	0.38**	0.92**	0.95**	1.00	0.39	0.42**	0.37**	0.12**	-0.03	0.04	0.03	0.02	0.02
Wtot	-0.13**	0.55**	0.42**	0.97**	0.93**	0.42**	0.56**	0.39	1.00	0.94**	0.98**	0.3**	0.06	-0.14**	-0.04	0.01	0.04
Wic	-0.14**	0.52**	0.43**	0.89**	0.89**	0.43**	0.57**	0.42**	0.94**	1.00	0.92**	0.35**	0.04	-0.09*	0.01	0.07	0.02
Wec	-0.09	0.53**	0.42**	0.96**	0.92**	0.42**	0.54**	0.37**	0.98**	0.92**	1.00	0.18**	0.08*	-0.16**	-0.06	0.01	-0.05
PA	-0.33**	0.33**	0.11**	0.27**	0.35**	0.06	0.22**	0.12**	0.3**	0.35**	0.18**	1.00	-0.09*	0.04	0.12**	0.02	0.1*
SDF	0.25**	-0.02	0.02	0.05	0.06	-0.01	-0.02	-0.03	0.06	0.04	0.08*	-0.09*	1.00	-0.04	-0.1*	-0.07	-0.08
Sperm Concentration	0.08	-0.03	0.13	-0.15**	-0.14**	0.01	-0.01	0.04	-0.14**	-0.09*	-0.16**	0.04	-0.04	1.00	0.76**	0.47**	0.63**
Total sperm count	-0.07	-0.01	0.01	-0.05	-0.05	0.01	0.01	0.03	-0.04	0.01	-0.06	0.12**	-0.1*	0.76**	1.00	0.54**	0.58**
Motility	-0.14**	-0.03	-0.02	-0.01	0.01	-0.01	-0.01	0.02	0.01	0.07	0.01	0.02	-0.07	0.47**	0.54**	1.00	0.56**
Morphology	0.04	-0.01	0.01	-0.07	-0.02	-0.01	0.01	0.02	0.04	0.02	-0.05	0.1*	-0.08	0.63**	0.58**	0.56**	1.00

BMI, Body Mass Index; FM, Fat mass; LM, Lean mass; MM, Muscular mass; WL, Waistline; AC, Abdominal circumference; WHR, Waist to hip ratio; Wtot, Total water; Wic, Intracellular water; Wec, Extracellular water; PA, Phase angle; SDF, Sperm DNA fragmentation.
*p-value <0.05.
**p value <0.01.

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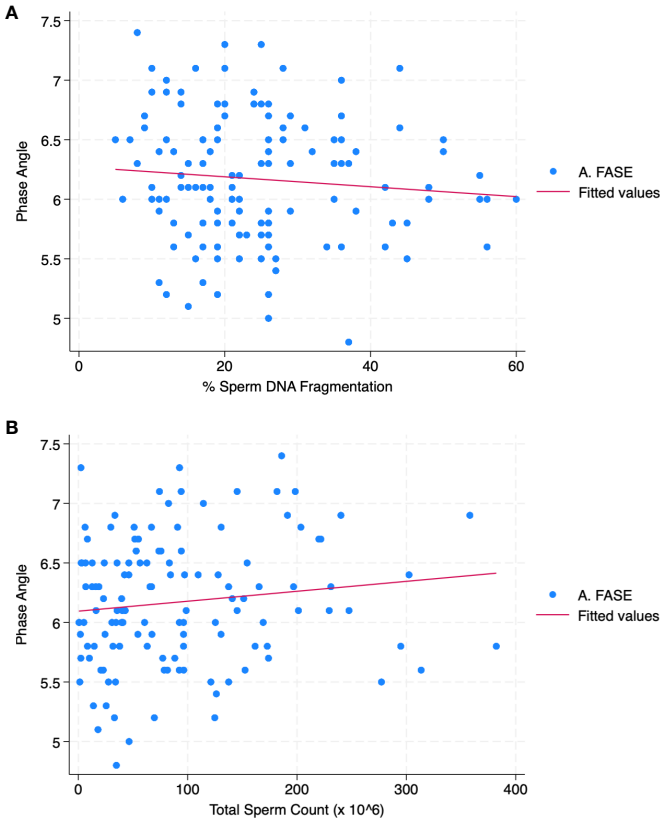

FIGURE 1
Scatter plot graph between phase angle and SDF **(A)** and TSC **(B)**.

However, since PA is a measure of extra and intracellular water content, it serves as a direct indicator of cell membrane integrity; in fact, the smaller the PA, the weaker the cell structure, and the higher the probability of cell death (38). Various studies have reported an association between PA and biochemical markers involved in monitoring chronic diseases as well as cancer prognosis (39, 40). Given that chronic diseases promote inflammation and oxidative stress, which may be responsible for cell damage, PA can serve as an early predictor of inflammation (41). Among chronic disorders, obesity is reported to be a noteworthy promoter of inflammatory status, characterized by an increase in tumor necrosis factor-α (TNF-α), interleukin 6 (IL6), and interleukin 10 (IL10) production (42). Building upon this previous statement, patients with a higher BMI are more likely to exhibit cell membrane damage, contributing to cell fluid imbalance and lower PA values (43).

Based on these premises and considering that chronic oxidative stress is known to affect sperm quality by damaging sperm DNA, PA appears to be a promising predictor of semen quality (44).

To establish phase angle as a strong predictor of male fertility, a multifaceted approach is necessary. Firstly, extensive research is paramount. Investigating the relationship between phase angle and male fertility demands thorough exploration. This entails collecting data from diverse populations to ensure the reliability and universality of findings. Collaboration with experts across various disciplines such as nutrition, physiology, endocrinology, and reproductive medicine is essential. Their insights can illuminate the underlying mechanisms linking phase angle with male fertility, enriching our understanding. Developing diagnostic tools or algorithms that integrate phase angle measurements with other pertinent biomarkers is pivotal. These tools should accurately assess male fertility status, enhancing diagnostic precision. Validation and standardization efforts are indispensable. Validating phase angle's predictive capacity across diverse populations and settings ensures its reliability. Standardizing measurement protocols and interpretation criteria promotes consistency and reproducibility of results. By pursuing these comprehensive steps, phase angle can emerge as a robust predictor of male fertility, facilitating more accurate diagnosis and management of male infertility issues.

Limitations of the study are important to be defined. Infertility often results from a combination of factors, including hormonal imbalances affecting ovulation or sperm production, structural issues impacting the reproductive organs, and systemic health conditions affecting fertility. Phase angle may not capture the multifaceted aspects contributing to infertility. The relationship between phase angle and infertility lacks extensive study and validation. While phase angle has been explored in various clinical contexts such as nutritional status, muscle health, and disease prognosis, its utility in predicting infertility remains largely unexplored. BIA measurements, including phase angle, can be influenced by external factors like hydration status, body temperature, skin integrity, and electrode placement. Fluctuations in these factors can affect the accuracy and reliability of BIA measurements, potentially complicating the interpretation of phase

angle in predicting infertility. Individuals exhibit significant variability in phase angle values based on factors such as age, sex, body composition, and overall health status. Additionally, reference ranges for phase angle may vary across different populations and measurement techniques, making it challenging to establish universal thresholds for predicting infertility based on phase angle alone.

In summary, our data suggest an association between PA and semen parameters; however, further research is needed to fully understand the underlying mechanisms. Furthermore, a limitation of our study was the lack of data regarding the lifestyle habits and comorbidities of our patients, which may be of great relevance when evaluating fertility potential and alterations in semen parameters.

5 Conclusion

In conclusion, further research is needed to fully understand the association between semen parameters and PA. Nevertheless, this preliminary study suggests that the phase angle assessed at BIA may be associated with poor sperm quality in males affected by idiopathic infertility. Our preliminary data may support further studies that can reveal the impact of phase angle with other aspects of couple infertility in the context of assisted reproductive technology. Furthermore, these results highlight the detrimental relationship between abnormal body composition and sperm quality. Clinicians may consider these results when developing strategies to increase the phase angle and improve semen quality.

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 humans were approved by Institutional review board of Centro HERA - UMR "Unità di Medicina della Riproduzione". The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

GR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. AL: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing – review & editing. FG: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing – review & editing. DL: Investigation, Validation, Visualization, Writing – review & editing. MA: Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. AA: Writing – review & editing, Formal analysis. SCi: Investigation, Validation, Visualization, Writing – review & editing. AG: Investigation, Validation, Visualization, Writing – review & editing. SCh: Investigation, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1354733/full#supplementary-material

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The impact and inflammatory characteristics of SARS-CoV-2 infection during ovarian stimulation on the outcomes of assisted reproductive treatment

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Introduction: Despite the global prevalence of coronavirus disease 2019 (COVID-19), limited research has been conducted on the effects of SARS-CoV-2 infection on human reproduction. The aims of this study were to investigate the impact of SARS-CoV-2 infection during controlled ovarian stimulation (COS) on the outcomes of assisted reproductive treatment (ART) and the cytokine status of patients.

Methods: This retrospective cohort study included 202 couples who received ART treatment, 101 couples infected with SARS-CoV-2 during COS and 101 matched uninfected couples. The parameters of ovarian stimulation and pregnancy outcomes were compared between the two groups. The All-Human Inflammation Array Q3 kit was utilized to measure cytokine levels in both blood and follicular fluid.

Results: No difference was found in the number of good-quality embryos (3.3 ± 3.1 vs. 3.0 ± 2.2, P = 0.553) between the infected and uninfected groups. Among couples who received fresh embryo transfers, no difference was observed in clinical pregnancy rate (53.3% vs. 51.5%, P = 0.907). The rates of fertilization, implantation, miscarriage, ectopic pregnancy and live birth were also comparable between the two groups. After adjustments were made for confounders, regression models indicated that the quality of embryos (B = 0.16, P = 0.605) and clinical pregnancy rate (P = 0.206) remained unaffected by SARS-CoV-2 infection. The serum levels of MCP-1, TIMP-1, I-309, TNF-RI and TNF-RII were increased, while that of eotaxin-2 was decreased in COVID-19 patients. No significant difference was found in the levels of cytokines in follicular fluid between the two groups.

Conclusion: Asymptomatic or mild COVID-19 during COS had no adverse effects on ART outcomes. Although mild inflammation was present in the serum, it was not detected in the follicular fluid of these patients. The subsequent immune response needs further investigation.

KEYWORDS

COVID-19, ovarian stimulation, assisted reproductive, embryo transfer, pregnancy, cytokines

Introduction

Coronavirus disease 2019 (COVID-19), is a respiratory illness that spreads easily. It is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is highly contagious (1). COVID-19 spread rapidly worldwide after the virus is identified (2). Approximately 80% of infected patients do not show any symptoms or are asymptomatic, while 15% to 20% have severe pneumonia or multiple organ failure (3). Due to the unpredictable impact of SARS-CoV-2 on reproduction, it was recommended to delay assisted reproductive treatment (ART) during the initial phase of the pandemic (4). As a result of China's relaxation of COVID-19 pandemic control measures since December 2022, more infected patients are during ART treatment being observed (5).

Theoretically, any tissue that expresses the cellular receptor angiotensin-converting enzyme 2 (ACE2) has the potential to be the target for SARS-CoV-2, since it enters host cells primarily via ACE2 (6, 7). Damage is likely to occur in sertoli and leydig cells in the testis, ovarian tissue, oocytes, embryos and endometrium due to the presence of the ACE2 receptor (4, 8–11). The immune system's response to SARS-CoV-2 infection relies on the alterations of cytokines (12). Patients with severe COVID-19 have elevated levels of proinflammatory cytokines (12–14). Cytokines play important roles in folliculogenesis, oocyte maturation, ovulation, fertilization, embryo development and pregnancy establishment (15, 16). They are also involved in the regulation of steroidogenesis (17). These findings suggest that aberrant inflammary status might impair fertility. Therefore, it is crucial to understand whether the virus affects reproduction. However, despite the global prevalence of this disease, limited research has been conducted on the impacts of SARS-CoV-2 infection on human reproduction. More research is required to address this concern.

The aims of this study were to evaluate the effect of SARS-CoV-2 infection on the embryos quality and pregnancy rates in patients undergoing ART treatment and to explore whether specific cytokines can affect the ART treatment outcomes of patients with SARS-CoV-2 infection to also explored.

Materials and methods

Study population

This retrospective study included patients who visited our assisted reproductive unit due to infertility for various reasons and underwent ART treatment from November 1, 2022 to January 31, 2023. Following the onset of the pandemic, all couples undergoing ART treatment were tested for SARS-CoV-2 infection using nasopharyngeal swabs prior to commencing the treatment and the day before oocyte retrieval. Nasopharyngeal swab screening was also conducted on couples who exhibited signs of SARS-CoV-2 infection while undergoing ovarian stimulation or who were exposed to infected individuals and had a high risk of SARS-CoV-2 infection. Nucleic acid or antigen tests were used to diagnose SARS-CoV-2 infection. If one partner who was asymptomatic or mildly symptomatic tested positive for SARS-CoV-2 before oocyte retrieval, the couples decided whether to proceed with the planned oocyte retrieval and/or embryo transfer (ET) or cancel the cycle. COVID-19 patients with moderate or severe infections were advised to cancel their ART treatment cycles. The diagnosing and treating individuals with COVID-19 relied on trial version 9 of the Pneumonia Diagnosis and Management Plan for Novel Coronavirus Infection. Mild COVID-19 was characterized by mild fever, cough, body aches, and other symptoms with no pneumonia; the moderate disease presented with mild pneumonia and other symptoms; and severe COVID-19 was characterized by severe pneumonia and a lack of oxygen. During the study period, none of the couples presented with the severe COVID-19 or required hospitalization. All *in vitro* fertilization (IVF) procedures were conducted in a separate area for individuals who had a positive SARS-CoV-2 RNA test. Additionally, the embryos were stored in a distinct tank filled with liquid nitrogen for cryopreservation. The study group (the SARS-CoV-2-positive group) consisted of couples with at least one SARS-CoV-2-positive partner. The infected group was matched 1:1 by age and cause of infertility to the uninfected group of patients who underwent ART treatment during the same period (SARS-CoV-2-negative group); among these patients neither partner was infected with SARS-CoV-2. A diminished ovarian reserve (DOR) was indicated when the anti-Müllerian hormone (AMH) level was below 1.1 ng/ml (18).

Treatment protocols

The ovarian stimulation protocols were performed as previously described and categorized into antagonist protocols, long protocols and other protocols, including progestin-primed ovarian stimulation and mild stimulation protocols (19). The stimulation protocols and fertilization methods were chosen based on patient characteristics and past cycle performance. The retrieval of oocytes was performed between 36 and 38 hours following the injection of human chorionic gonadotropin (hCG). The peripheral blood and follicular fluid of females were collected on the day of oocyte retrieval. The follicular fluids were obtained during oocyte retrieval as previously described (20). Most fresh ETs were cancelled due to SARS-CoV-2 infection, and a few asymptomatic patients underwent fresh ET with thorough medical counselling. Fresh ET was performed on Day 3 with the best 1 or 2 embryos. Otherwise, fresh ET was cancelled due to fluid accumulation in the cavity, unfavourable endometrium, the risk of ovarian hyperstimulation, hydrosalpinx, and the need for genetic analysis or surrogacy. On Day 3, embryos that had at least 6 blastomeres with ≤25% fragmentation were categorized as good-quality embryos according to the Istanbul consensus workshop (21). Data on the baseline characteristics and treatment outcome information of the patients were retrieved from the ART data system.

The research was carried out in accordance with the principles of the Helsinki Declaration. The Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (IRB-20230204-R) granted approval for this study, and all subjects provided informed consent.

Outcome measures

The primary measures were the quantity of good-quality embryos and the rate of clinical pregnancy. The secondary outcome measures were the number of retrieved oocytes, and the rates of fertilization, implantation, miscarriage and live birth. To study the effect of SARS-CoV-2 infection on male sperm quality, semen parameters before and on the day of oocyte retrieval after SARS-CoV-2 infection were compared.

Clinical pregnancy was defined as the presence of one or more gestational sacs, as visualized by transvaginal ultrasound examination. The fertilization rate by IVF was calculated by dividing the number of fertilized oocytes by the number of retrieved oocytes. The rate of fertilization by intracytoplasmic sperm injection (ICSI) was calculated by dividing the number of fertilized oocytes by the number of metaphase II oocytes. The implantation rate was calculated by dividing the number of gestational sacs by the number of transferred embryos. Miscarriage was defined as the loss of an intrauterine pregnancy before gestational week 28. A live delivery was defined as delivery of a newborn at or after 28 weeks of gestation.

Serum and follicular fluid cytokine measurements

The levels of cytokines in both serum and follicular fluid were measured using the All Human Inflammation Array Q3 kit (RayBiotech Life, GA, USA) following the manufacturer's instructions. The procedures were performed as previously described (5).

Statistical analysis

Statistical analysis was performed by using SPSS version 26.0. The concentrations of the quantified inflammatory factors were processed using R version 4.3.2. Quantitative variables with a normal distribution and homogenous variance are expressed as the mean ± standard deviation, and the means were compared using Student's t test. Categorical variables are summarized using percentages and counts. Differences in the rates were compared by mean of the χ^2 test. Statistical significance was defined as by a two-sided P value less than 0.05.

To determine the factors associated with the quantity of good-quality embryos, a linear regression model was utilized. In the preliminary model, the included variables were age, duration of infertility, cause of infertility, AMH level, the number of retrieved oocytes, and SARS-CoV-2 status. The forward elimination method was utilized to choose the best model, allowing inclusion at $P < 0.05$ and exclusion at $P > 0.15$. The model was compelled to incorporate of SARS-CoV-2 status. The ultimate model included SARS-CoV-2 status, the number of retrieved oocytes, the AMH level, and the duration of infertility.

Multivariate logistic regression analysis was used to compare the pregnancy rate adjusted for confounding factors, such as female age, duration of infertility, type and causes of infertility, AMH level, number of retrieved oocytes, number of high-quality embryos and number of transferred embryos. The model was compelled to incorporate the SARS-CoV-2 status. We used the forward elimination method to select the optimal model, allowing inclusion when $P < 0.05$ and exclusion when $P > 0.15$. The ultimate model incorporated the SARS-CoV-2 status, infertility type, and the quantity of high-quality embryos.

Results

Comparison of baseline characteristics

In total, 101 couples (42 in which the female partner was infected, 23 in which the male partner was infected and 36 in which both partners was infected) met the inclusion criteria and were matched to 101 control couples according to female age and cause of infertility who were not affected by SARS-CoV-2. The mean ages of the females in the study and control groups were comparable, as were the male ages, AMH levels, and BMIs. There were no differences in the type of infertility, infertility cause, number of previous IVF procedures, rate of female vaccination, and rate of male vaccination. The data were shown in Table 1. The interval between the time of the last vaccination and the date of the subsequent IVF treatment cycle was 6-24 months.

Comparison of ovarian stimulation cycle characteristics

The cycle characteristics of patients in both the study and control groups, including the treatment protocols, gonadotrophin dosage, the duration of stimulation, the peak E_2 levels and fertilization methods, were comparable. The quantity of good-quality embryos, the number of oocytes retrieved, the number of fertilized oocytes with 2 pronuclei, the rate of fertilization, and the number of transferred embryos were also comparable between the two groups. The data were presented in Table 2. The effects of SARS-CoV-2 infection on semen parameters were further analysed. Among 59 males with SARS-CoV-2 infection, the semen parameters of 50 males whose semen samples were freshly ejaculated by masturbation were compared before and on the day of oocyte retrieval after infection. The semen parameters of the males were summarized in Supplementary Table S1. After infection, the progressive motility and complete motility of the sperm did not significantly differ ($P > 0.05$). Although the semen volume, sperm concentration and total sperm count were decreased ($P < 0.05$ for all), the values were still within the normal reference range, and the fertilization method was not influenced by the infection. A linear regression model demonstrated no effect of SARS-CoV-2 infection on the number of good-quality embryos (B = 0.16, $P = 0.605$), whereas the duration of infertility (B = -0.153, $P = 0.009$) and the number of oocytes retrieved (B = 0.135, $P < 0.001$) remained significant factors (Table 3).

TABLE 1 Baseline characteristics of ART patients in the SARS-CoV-2-positive versus the control group.

Characteristic	SARS-CoV-2 positive (n = 101)	SARS-CoV-2 negative (n = 101)	*P* value
Female age (years)	33.0 ± 4.5	33.0 ± 4.5	0.937
Male age (years)	34.7 ± 5.9	34.1 ± 5.2	0.383
Duration of infertility (years)	3.4 ± 2.8	3.2 ± 2.5	0.565
AMH (IU/L)	3.2 ± 2.7	2.8 ± 2.1	0.069
BMI (kg/m^2)	21.4 ± 2.6	21.8 ± 2.6	0.853
Type of infertility			0.067
Primary infertility	46.5% (47/101)	59.4% (60/101)	
Secondary infertility	53.5% (54/101)	40.6% (41/101)	
Causes of infertility			0.998
Tubal	29.7% (30/101)	29.7% (30/101)	
Male	13.9% (14/101)	13.9% (14/101)	
DOR	30.7% (31/101)	29.7% (30/101)	
Others	25.7% (26/101)	26.7% (27/101)	
Previous cycles	1.7 ± 1.2	1.9 ± 1.4	0.557
Female vaccination rate	21.8% (22/101)	19.8% (20/101)	0.729
Male vaccination rate	22.8% (23/101)	20.8% (21/101)	0.733

The data are presented as the means ± standard deviations or percentages and counts. ART, assisted reproductive technology; AMH, anti-Müllerian hormone; BMI, body mass index; DOR, diminished ovarian reserve.

Cycle outcomes after fresh ET

In total, nine asymptomatic women with uninfected partners, four asymptomatic women with infected partners and two uninfected women with infected partners underwent fresh ET after thorough medical counselling. The clinical pregnancy rate did not differ (53.3% vs. 51.5%, $P = 0.907$) between the study and control groups. The two groups had similar rates of implantation, miscarriage, ectopic pregnancy and live birth (Table 4). In control group, a woman with a twin pregnancy delivered at 26 weeks due to an unavoidable miscarriage and a complication of SARS-CoV-2 infection. All of the other women had singleton pregnancies and delivered after 37 weeks without acquiring a new SARS-CoV-2 infection. Except for two term neonates from the control group who were admitted to the neonatal intensive care unit (NICU) for respiratory distress and hypoglycemia, the Apgar scores of all neonates in the infected and control groups at 1 and 5 minutes were 10. The pregnancy rate was not affected by SARS-CoV-2 infection according to the logistic regression model ($P = 0.206$).

TABLE 2 Cycle characteristics and treatment outcomes of ART patients in the SARS-CoV-2-positive group versus the control group.

Variable	SARS-CoV-2 positive (n = 101)	SARS-CoV-2 negative (n = 101)	*P* value
COS protocol			0.787
Long protocol	26.7% (27/101)	22.8% (23/101)	
Antagonist protocol	44.6% (45/101)	45.5% (46/101)	
Other protocol	28.7% (29/101)	31.7% (32/101)	
Dosage of Gn used (IU)	2106.9 ± 1036.4	2245.9 ± 851.3	0.050
Duration of stimulation (d)	9.5 ± 3.3	10.2 ± 4.0	0.460
Peak E_2 level (pmol/L)	9677.7 ± 8030.2	9996.0 ± 7872.9	0.606
Number of oocytes retrieved	9.9 ± 6.8	9.7 ± 6.5	0.391
Fertilization method			0.641
IVF	69.5% (66/95)	66.3% (63/95)	
ICSI	30.5% (29/95)	33.7% (32/95)	
IVF fertilization rate	60.8% (399/656)	62.8% (402/640)	0.461
ICSI fertilization rate	68.4% (128/187)	63.8% (104/163)	0.359
Number of 2PN fertilized oocytes	5.5 ± 5.2	5.4 ± 5.2	0.02
Number of good-quality embryos	3.3 ± 3.1	3.0 ± 2.2	0.553
Freeze all oocytes or embryos	64.4% (65/101)	55.4% (56/101)	0.196
Number of transferred embryos	1.8 ± 0.4	1.8 ± 0.4	0.773

The data are presented as means ± standard deviations or percentages and counts. COS, controlled ovarian stimulation; G, gonadotrophin; E_2, Estradiol; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; 2PN, 2 pronuclei.

Cytokine profiling of asymptomatic or mildly symptomatic COVID-19 patients

To further check whether the changes in the cytokine profiles were associated with asymptomatic or mild COVID-19, we measured the serum and follicular fluid concentrations of 40 inflammatory factors in 13 female COVID-19 patients and 12 female non-COVID-19 patients (the study and control groups). In the infected group, the serum levels of monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinases 1 (TIMP-1), I-309, tumour necrosis factor receptor I (TNF-RI) and TNF-RII were higher than those in the uninfected group, while the serum concentrations of eotaxin-2 were lower (Figures 1A–G, 2A). Further comparisons of the follicular fluid concentrations of 40 inflammatory factors were performed. No significant differences were observed between the two groups (Figure 2B).

TABLE 3 Linear regression model for the number of good-quality embryos.

Variable	Coefficient	95% confidence intervals		*p* value
		Lower limit	Upper limit	
SARS-CoV-2	0.161	-0.451	0.772	0.605
Duration of infertility	-0.153	-0.269	-0.038	0.009
AMH	0.156	-0.019	0.332	0.081
Number of oocytes retrieved	0.207	0.144	0.270	<0.001

Discussion

This study is the first to examine the pregnancy rate and cytokine levels of patients infected with SARS-CoV-2 during controlled ovarian stimulation (COS). Our findings indicated that SARS-CoV-2 infection during COS did not negatively affect the pregnancy rate. SARS-CoV-2 infection did not impact embryo quality, number of retrieved oocytes, fertilization method, or rate of fertilization or live birth. Except for those of serum MCP-1, TIMP-1, I-309, TNF-RI, TNF-RII and eotaxin-2, the cytokine levels of asymptomatic and mildly symptomatic individuals infected with SARS-CoV-2 during COS were not significantly different. In addition, 40 inflammatory factors were compared in follicular fluid. No significant differences were found between the asymptomatic or mildly symptomatic COVID-19 patients and the uninfected controls. These findings indicated that the absence of symptoms or mild symptoms of SARS-CoV-2 infection did not have a negative impact on ART outcomes.

At the onset of the pandemic, the European Society of Human Reproduction Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) advised halting fertility treatments. The purpose of these recommendations was to safeguard the well-being of couples undergoing ART and their newborns (22). Due to the lack of substantial evidences on the safety of SARS-CoV-2 infection during ART and concerns about the potential adverse effects of SARS-CoV-2 infection, women who were infected with SARS-CoV-2 were more likely intended to give up their cycles rather than continue during the early pandemic period (23).

TABLE 4 Pregnancy outcomes of fresh embryo transfer in the SARS-CoV-2-positive group versus the control group.

Variable	SARS-CoV-2 positive (n = 15)	SARS-CoV-2 negative (n = 33)	*P* value
Implantation rate	33.3% (9/27)	33.3% (20/60)	1.00
Clinical pregnancy rate	53.3% (8/15)	51.5% (17/33)	0.907
Miscarriage rate	12.5% (1/8)	25.0% (3/17)	0.743
Ectopic pregnancy	12.5% (2/8)	11.8% (2/17)	0.958
Live birth rate	33.3% (5/15)	39.4% (13/33)	0.688

During the epidemic, however, unplanned SARS-CoV-2-related closures occurred. Barragan et al. reported that oocytes from two women with asymptomatic SARS-CoV-2 infection showed no presence of SARS-CoV-2 RNA (11). The RNA of SARS-CoV-2 virus was not detectable in the follicular fluid, cumulus cells or endometrium of the infected women (24). Furthermore, a recent study revealed that eight individuals who were confirmed to have COVID-19 on the day of oocyte retrieval did not have SARS-CoV-2 RNA in their follicular fluid (25). It is possible that the zona pellucida could serve as a natural barrier for oocytes *in vivo* against SARS-CoV-2 infection (and other pathogens), despite oocytes and ovarian tissue expressing ACE2 and transmembrane serine protease 2 (TMPRSS2), to allow viral entry (26). According to these previous studies, one can be reassured regarding the possibility of contamination in ART laboratories. However, data regarding the impact of SARS-CoV-2 infection on the ART outcomes remains insufficient.

Youngster et al. conducted a retrospective cohort study on SARS-CoV-2-infected females who received ART within a year of infection (27). According to the study, the presence of SARS-CoV-2 infection did not have any impact on oocyte yield, fertilization or maturation, the number of high-quality embryos, or the rate of clinical pregnancy (27). Nevertheless, the duration between SARS-CoV-2 infection and oocyte retrieval ranged from 8 to 348 days. Similarly, Chen et al. reported that COVID-19 infection within a week prior to oocyte retrieval did not affect the development of oocytes and embryos (28). In contrast, Tian et al. discovered that patients with SARS-CoV-2 infection during COS had lower rates of top-quality embryos and blastocysts (29). Unfortunately, the pregnancy rate and severity of COVID-19 were not mentioned in that study. We observed no negative impact of SARS-CoV-2 infection on ART outcomes in asymptomatic or mildly symptomatic COVID-19 patients indicating that SARS-CoV-2 infection has no adverse effects on the development of oocytes/embryos or pregnancy. Previous studies on the effects of SARS-CoV-2 infection on semen quality have focused mostly on men who have recovered from the infection. Some studies have reported that a history of SARS-CoV-2 infection negatively affects semen parameters (30–32). However, semen parameters do not seem impaired after a mild infection (33). Our results indicated decreased semen volume, sperm concentration and total sperm in the infected male partner. However, the fertilization method and fertilization rate were not influenced. Due to the limitations of sample size, more studies with lager sample sizes are needed.

Systemic inflammation, which is commonly linked to acute COVID-19, can indirectly impact reproduction (34). Previous studies have shown that the severity of COVID-19 is linked to the levels of interleukin(IL)-2, IL-6, IL-8, and tumour necrosis factor-α (TNF-α), and alterations in the expression of these inflammatory cytokines may occur in the early stages of SARS-CoV-2 infection (12, 35, 36). Increased production and elevated levels of IL-6 are thought to be central to the development of the cytokine storms (37). At the same time, cytokines and hormones interact in a complex and systemic manner, influencing the development of follicles and pregnancy. Understanding the

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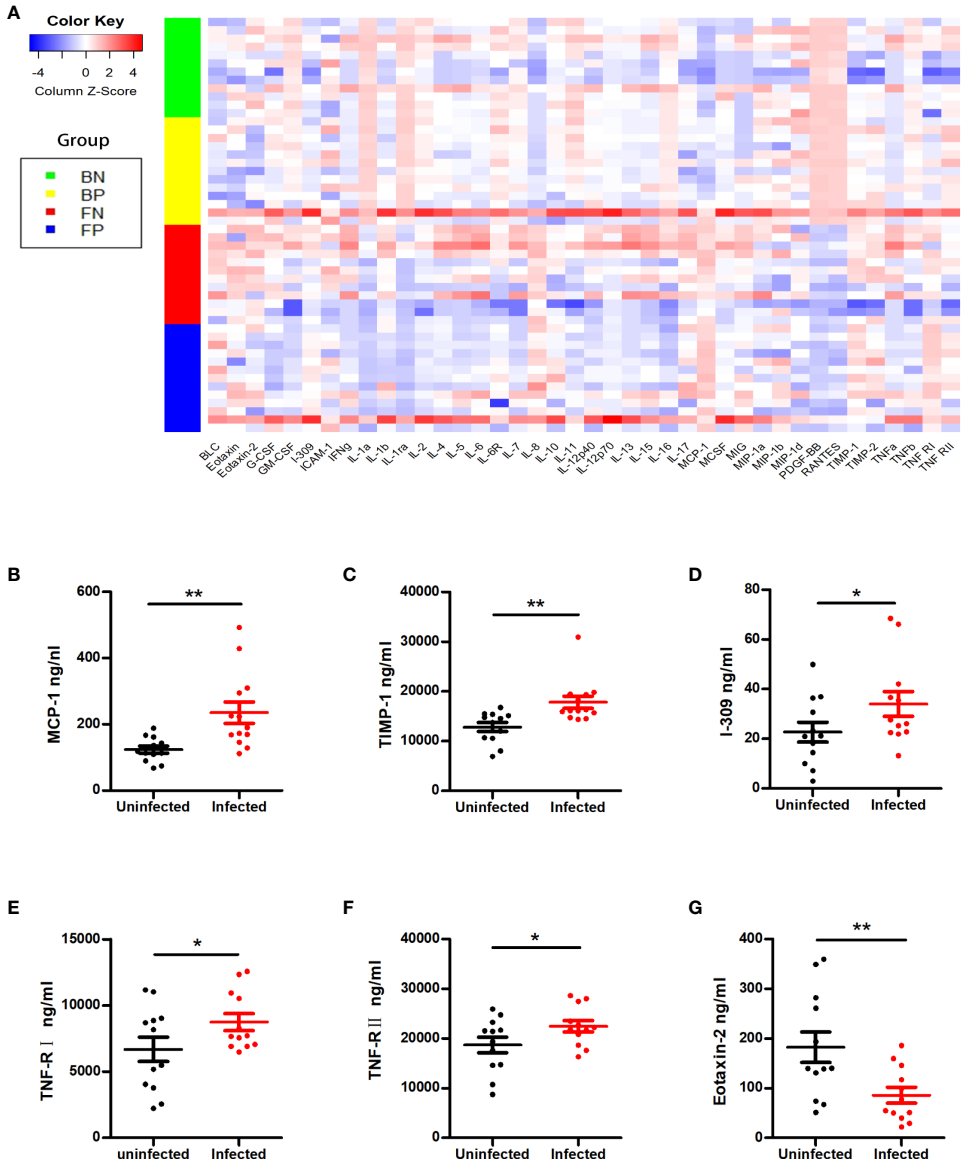

FIGURE 1
Cytokine alterations between the SARS-CoV-2-positive group and the SARS-CoV-2-negative group. **(A)** The Z scores of cytokines in the two groups. BN = the blood of the SARS-CoV-2-negative group; BP = the blood of the SARS-CoV-2-positive group; FN = the follicular fluid of the SARS-CoV-2-negative group; FP = the follicular fluid of the SARS-CoV-2-negative group. **(B–G)** Representative cytokines that showed significant differences between the two groups, including MCP-1 **(B)**, TIMP-1 **(C)**, I-309 **(D)**, TNF-RI **(E)**, TNF-RII **(F)** and eotaxin-2 **(G)**. *$P < 0.05$; **$P < 0.01$.

immune response of the host is essential for assessing the potential reproductive harm caused by SARS-CoV-2 infection. However, the alterations in cytokine levels in the serum and follicular fluid of patients infected with SARS-CoV-2 during ART treatment remain unknown. When we examined markers of overall inflammation in the blood of these COVID-19 patients, we noted that the serum and follicular fluid cytokine levels in both groups were similar, except for a few proinflammatory cytokines in the serum. The levels of the key proinflammatory cytokine MCP-1 were increased in the serum. MCP-1 can induce the luteolysis of the corpus lutea, regulate monocyte differentiation and play a role in cytokine production (17, 38). Since the cytokine storm did not occur at the early stage of infection, the subsequent immune changes induced by MCP-1 might be the key factor associated with the severity of COVID-19. Changes in TNF will alter the local cytokine balance, inhibit steroidogenesis, and result in miscarriage (39). However, except for the high serum TNF-RI and TNF-RII levels, the levels of TNF-α and TNF-β in both the serum and follicular fluid of the infected women did not differ. Since studies indicate the role of TNF receptors as possible blockers of TNF cytokine action (40), the higher receptor level might be related to the limited immune response. As shown in a prior study (41), even a lower level of chemokine eotaxin-2 in infected individuals might also be associated with a milder immune response. Compared to those in systemic immunity, cytokines in follicular fluid act directly on oocytes which is likely more important for modulating the

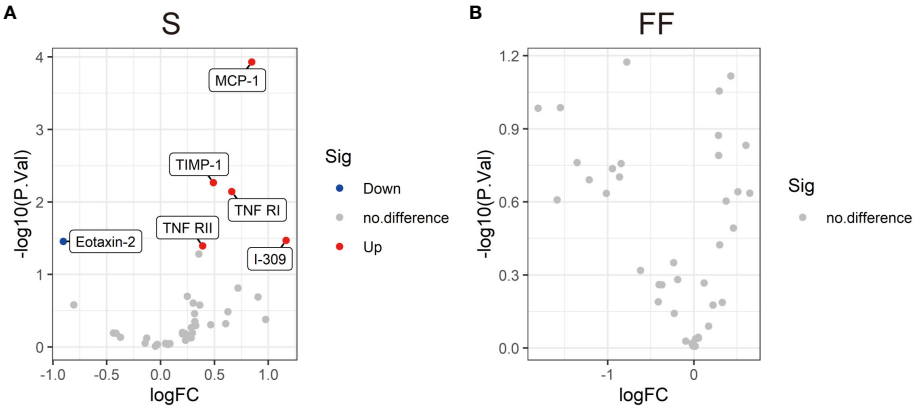

FIGURE 2
Volcano plots showing the differential levels of cytokines (DLCs) in serum **(A)** and follicular fluid **(B)** between the SARS-CoV-2-positive group and the SARS-CoV-2-negative group. Each dot represents a kind of cytokine, and the numbers of DLCs are indicated. Cytokine differential level analysis between the two different groups was performed by R version 4.3.2 software. The cut-offs for the DLCs were $P < 0.05$ and an absolute fold change >1.2.

reproductive processes. Aberrant cytokine level in follicular fluid can lead to abnormalities in folliculogenesis, oocyte quality and embryo developmental capacity (42). Specifically, the cytokine profiles of follicular fluid obtained from asymptomatic or mildly symptomatic COVID-19 patients were similar to those of the individuals in the control group.

The mild immune response may have contributed to the inclusion of patients with only mild clinical symptoms or asymptomatic patients. On the other hand, the COVID-19 vaccine could result in a large reduction in the incidence of symptomatic or severe COVID-19 disease (43). It may also play an important role in the reduced immune response.

The findings of our study indicated that mild or asymptomatic SARS-CoV-2 infection during COS did not have a detrimental impact on ART outcomes. This might be related to the absence of inflammasome activation observed in the follicular fluid of individuals with SARS-CoV-2 infection. In particular, subsequent changes in cytokine levels in infected couples may also be important for pregnancy outcomes and warrant further investigation.

The present study has several limitations. First, our research included only mild or asymptomatic individuals, and COVID-19 patients with moderate or severe symptoms were not included. Second, the analysis did not consider the distinct impacts of male or female infection ART outcomes of due to the small sample size. To ensure the reliability, future studies with larger cohorts and extended follow-up periods will be required for safety validation. Third, the cytokine profiles of pregnancy COVID-19 patients should be monitored. Finally, it was unknown whether COVID-19 infection during COS affects frozen ET outcomes.

Conclusion

In conclusion, asymptomatic or mild COVID-19 during COS had no adverse effects on the outcomes of ART. Although mild inflammation was present in the serum, it was not detected in the follicular fluid of these patients. The subsequent immune response requires further investigation.

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 humans were approved by The Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XH: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Writing – review & editing, Resources, Software, Writing – original draft. GF: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Software, Writing – original draft, Writing – review & editing. QC: Data curation, Project administration, Resources, Writing – original draft, Formal analysis. YS: Data curation, Formal analysis, Project administration, Resources, Writing – original draft. QQC: Data curation, Formal analysis, Project administration, Resources, Writing – original draft, Validation. SW: Data curation, Formal analysis, Project administration, Resources, Validation, Writing – original draft. SL: Data curation, Formal analysis, Project administration, Resources, Writing – original

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1353068/full#supplementary-material

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Association between dietary inflammation index and female infertility from National Health and Nutrition Examination Survey: 2013-2018

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Objective: To investigate the relationship between dietary inflammatory index (DII) scores and infertility in US adults aged 18 to 45.

Methods: Data were gathered from the 2013-2018 National Health and Nutrition Examination Survey (NHANES). In total, 3496 women were included in the study. To examine the relationship between DII, EDII and infertility, a weighted multivariable logistic regression analysis using continuous factors or categorical variables grouped by quartiles was conducted. Using subgroup analysis stratified based on DII and infertility features, the association between DII and infertility has been further studied. In order to determine whether there was a nonlinear relationship between DII and infertility, restricted cubic spline (RCS) analysis was carried out.

Results: For statistical analysis, a total of 3496 individuals — 367 patients with infertility and 3129 persons without infertility — were included. A multivariable logistic regression study revealed a positive relationship between DII and infertility. A significant difference in subgroup analysis was shown in age group and race, although RCS analysis demonstrated nonlinear relationship between the DII and infertility.

Conclusion: For participants aged 18-45 years, higher DII scores were positively correlated with infertility. In addition, anti-inflammatory diets might improve infertility outcomes.

KEYWORDS

infertility, dietary inflammatory index, nutrition, National Health and Nutrition Examination Survey, RCS

Introduction

Infertility is the inability to conceive and reproduce due to a variety of etiologic factors. Infertility is defined as the failure to conceive after at least 12 months of uncontraceptive sexual intercourse (1–4). It is a serious global public health problem that is estimated to affect approximately 15% of the world's population, with nearly 48.5 million (45 million, 52.6 million) couples experiencing infertility globally (5). Infertility is an important component of reproductive health. The inability to have children has a significant impact on the physical and mental health of those women, leading to distress and depression (6, 7), which is also associated with population decline and low fertility rates (8). The factors that lead to infertility are complex — several common diseases may affect female infertility, such as premature ovarian insufficiency (1, 9), polycystic ovary syndrome (10), endometriosis (11, 12), uterine fibroids (13), and endometrial polyps (9). However, in addition to these common diseases, a number of factors related to lifestyle have gained prominence in recent years, such as diet and chronic inflammation, and the diets of modern people also bring about an inflammatory response of the body (14).

Inflammation plays an important role in reproduction. Studies have reported that patients with polycystic ovary syndrome have higher levels of C-reactive protein (CRP), interleukin 18 (IL-18), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), white blood cell counts (WBCs), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein- 1α (MIP-1α) (15–17). In patients with endometriosis, there was a trend of increased inflammatory indices, which confirmed the immunologic alterations in these diseases (18, 19). There was an imbalance between anti-inflammatory and pro-inflammatory cytokines in patients with ovarian failure, so inflammation was closely associated with premature ovarian insufficiency (20, 21). The Dietary Inflammatory Index (DII) is a scoring system that evaluates the inflammatory potential of the diet, with higher scores being more favorable to inflammation (22). The DII was initially developed by Shivappa et al. (22), and it encompasses 45 food parameters including various micro- and macronutrients, spices, and flavonoids. Each of these parameters is assigned a score based on its pro- or anti-inflammatory properties as validated by extensive research studies. However, DII does not account for total energy intake, which can be a confounding factor, as total energy intake may influence the overall inflammatory potential of the diet. To overcome this limitation, Shivappa et al. (23), later proposed the E-DII, which adjusts DII scores for total energy intake using the residual method.Both DII and E-DII have been utilized as tools in numerous epidemiological studies to investigate the relationship between diet-induced inflammation and various health outcomes, including cancer, cardiovascular diseases, metabolic syndrome, and mortality, among others. Previous studies have shown significant associations between DII and risks of obesity and neoplasia (24), but to our knowledge, very little research has reported a correlation between DII and infertility. This represents a significant gap in our knowledge, as understanding the potential association between dietary inflammation and infertility could have important implications for disease prevention and treatment strategies. In our study, we used cross-sectional analyses to investigate women with infertility from 2013-2018 in an attempt to get to the bottom of their relationships.

Materials and methods

The NHANES database is a public program that assesses the health and nutrition of Americans. It is presented through questionnaires, laboratory data, and physical measurements. We selected data from this database for the years 2013-2018. In total, there are 29,400 subjects. We excluded men (14,452), those under 18 years of age (5,630), those over 45 years of age (4,995), women with no information on infertility (656), and those with no DII data (171) were excluded, resulting in 3,496 subjects being included in our study (Figure 1). The formula of sample size calculation:

$$\begin{aligned}\text{Final probability} = (&\Pr(\text{PSU is selected})\\ &\times \Pr(\text{segment of the PSU is selected})\\ &\times \Pr(\text{household is selected})\\ &\times \Pr(\text{individual is selected}))\end{aligned}$$

Measurement of DII and EDII

Individual dietary data were obtained through by the average of the first in-person collection in the Mobile Examination Center (MEC) and a telephone interview 3-8 days later (second 24-hour dietary recall interview). In NHANES, 27 foods were available for DII calculation: 1: carbohydrates; 2: protein; 3: total fat; 4: alcohol; 5: fiber; 6: cholesterol; 7: saturated fat; 8: MUFA; 9: PUFA; 10: n-3 fatty acids eicosapentaenoic (20:5), docosapentaenoic (22:5), docosahexaenoic (22:6); 11: n-6 fatty acids, octadecadienoic (18:2), octadecatrienoic (18:3), octadecatetraenoic (18:4), eicosatetraenoic (20:4); 12: niacin; 13: vitaminA; 14: thiamin (vitamin B1); 15: riboflavin(vitamin B2); 16: vitamin B6; 17: vitamin B12; 18: vitamin C; 19: vitamin E; 20: Fe; 21: Mg; 22: zinc; 23: selenium; 24: folic acid; 25: beta-carotene; 26:caffeine; 27: energy. The calculation of the DII has been reported in the literature (22), and this dietary database has means and standard deviations (SD) for a total of 45 food parameters.DII was calculated by subtracting the mean of their total number from the raw data in the database and dividing by the standard deviation of the parameter to obtain z. z was converted to a percentile score by doubling and subtracting 1 (from -1 to +1, centered on 0). The result was multiplied by the corresponding literature-derived inflammatory effect score for each food parameter. Finally, the overall DII score for each individual is the sum of the DII scores for each specific food parameter. Higher DII scores indicate a more pro-inflammatory diet (25). The procedure for calculating the E-DII was the same as for calculating the DII, which was designed to control the effects of total energy intake, with energy-adjusted DII scores calculated for each 1,000 calories of food consumed (23, 26) (using the energy-standardized version of the World Data Bank). This adjustment is usually done using the residual method, where the residuals from a regression of DII on total energy intake are used to obtain an energy-independent DII score.

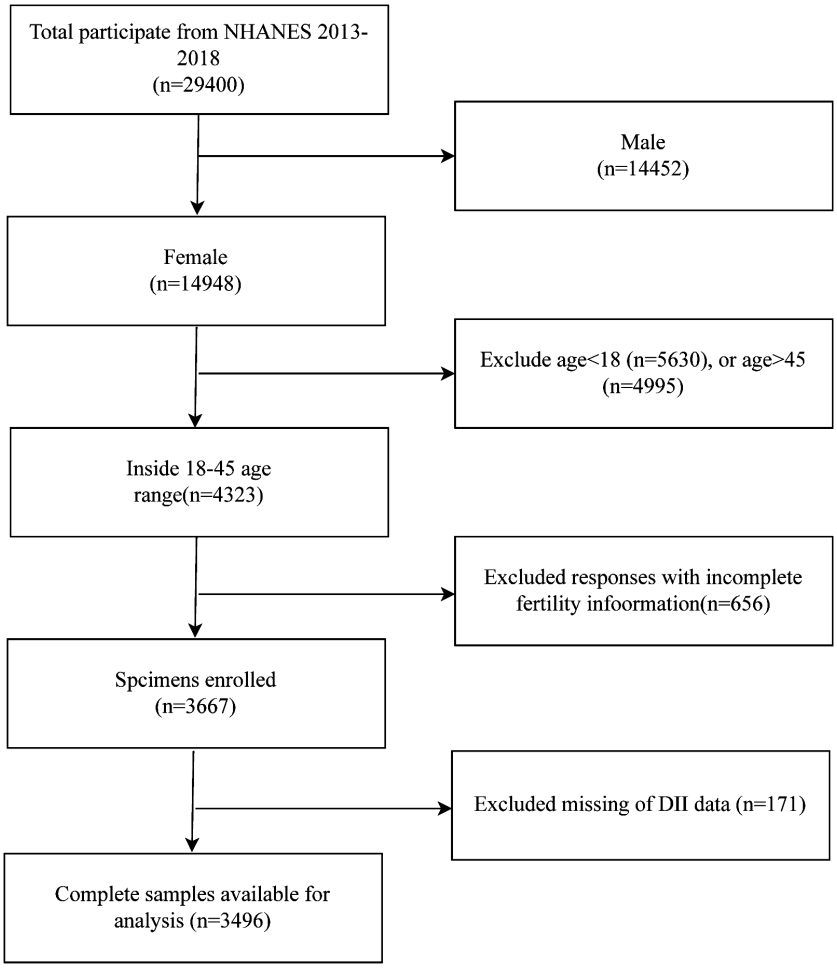

FIGURE 1
The flow chart of sample selection from NHANES 2013-2018.

Main outcomes

The outcome variable infertility was among the reproductive health questionnaires, RHQ074. The question for this variable was "Have you ever tried to get pregnant for at least one year without getting pregnant?" If the answer was "Yes", you were considered infertile.

Other variables

Demographic variables included age, race (Mexican American, Non-Hispanic Black, Non-Hispanic White, Other Hispanic, Other Race), marital status (non-single, single), education level (high school or below, high school, high school level or higher).

Comorbidities

Diagnostic criteria for diabetes were based on:(1) physician diagnosis of diabetes, (2) glycated hemoglobin HbA1c (%) >6.5, (3) fasting blood sugar (mmol/L) >7.0.4, (4) random blood sugar (mmol/L) ≥11.1 and (5) 2-hour OGTT blood sugar (mmol/L) ≥11.1,(6) Use of diabetes medications or insulin. Hypertension diagnosis was based on: (1) doctors' diagnosis of high blood pressure, (2) use of antihypertensive medications, and (3) abnormal blood pressure readings (>=3 times).

Some other variables

Diagnostic criteria for alcohol use were as follows: (1) Never: <12 drinks in a lifetime. (2) Former: ≥12 drinks in 1 year and no drinking last year, or no drinking last year but ≥12 drinks in a lifetime. (3) Yes: those except the above two. There were three categories of smoking: never, former, and now: (1) Never: smoked <100 cigarettes in a lifetime; (2) former: smoked >100 cigarettes in a lifetime and not currently smoking; (3) Now: smoked >100 cigarettes in a lifetime and smoking some days or every day. There was also physical activity, insurance status, and pregnancy status.

Statistical analysis

For the statistical analysis of this study, NHANES took survey weights into account. Continuous variables are presented as mean ± SD, and categorical variables are presented as percentages. Specifically,

multivariate logistic regression was used to assess the association between DII, E-DII and infertility while adjusting for covariates. To explore the relationship between DII, E-DII and infertility, DII and E-DII were divided by continuous variables into 4 subgroups respectively — categorical variables were used to calculate the differences between different DII and E-DII. In Model 1, adjustments were made for age, marital status and BMI. In Model 2, adjustments were made for age, household income ratio, BMI, sedentary time, race, divorce status, education, smoking, alcohol use, diabetes, hypertension, previous pregnancy, outdoor exercise intensity, and insurance. To further explore the relationship between DII and infertility, subgroup analyses were conducted. Additionally, we utilized a restricted cubic spline (RCS) to account for potential non-linear relationships between DII and infertility, which places knots at the 5th, 35th, 65th, and 95th percentiles of the predictor distribution. RCS provides flexibility by allowing the function to change at specific values of the predictor, known as knots. The statistical software packages R (http://www.R-project.org) and Empower Stats (http://www.empowerstats.com) were used for analysis. $P < 0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics

The study comprised 3496 participants with an infertile cohort (n=367) showing a significantly higher mean age (35.45 vs. 30.91 years, p<0.0001) and BMI. Presence of comorbidities (diabetes and hypertension) and lifestyle habits (drinking and smoking) were more prevalent in the infertile group (p<0.05). The analysis also involved diverse ethnic backgrounds i.e., Mexican Americans, other Hispanic, non-Hispanic white, non-Hispanic black, and Non-Hispanic Asian (Table 1).

Dietary inflammatory indexand infertility

A significantly higher DII was observed in the infertile group compared to controls (2.10 vs 1.75). Furthermore, each unit increase in DII was associated with a 10% increased odds of infertility. When DII was categorized into quartiles, the highest quartile (Q4) was associated with a 59% higher risk of infertility compared to the lowest quartile (Q1) (Table 2).

Empirical dietary inflammatory index and infertility

EDII was significantly higher in the infertile group compared to the control group (1.73 vs 1.41). An increasing trend in the risk of infertility was observed with increasing levels of EDII (Table 2).

Stratified analysis

Subgroup analysis revealed significant interaction effects of age and race on the relationship between DII and infertility. No significant interaction was observed across other strata (Figure 2).

TABLE 1 Baseline characteristics of participant.

Characteristic	infertility	
	negative	positive
Age, mean(sd),years	30.905 (30.484,31.327)	35.451 (34.550,36.352)
BMI,mean(sd),kg/m2	28.964 (28.487,29.440)	32.288 (31.001,33.575)
Poverty-to-income ratio, mean(sd)	2.633 (2.512,2.755)	2.832 (2.612,3.052)
Sedentary Time,mean (sd) (min)	383.282 (373.250,393.314)	400.270 (375.493,425.047)
DII	1.752 (1.626,1.877)	2.100 (1.891,2.310)
EDII	1.414 (1.298,1.530)	1.730 (1.438,2.022)
Race/Ethnicity n(%)		
Mexican American	12.059 (9.538,15.136)	10.132 (6.626,15.191)
Other Hispanic	13.679 (11.053,16.811)	12.112 (9.178,15.820)
Non-Hispanic White	55.789 (50.938,60.533)	63.170 (55.382,70.327)
Non-Hispanic Black	8.052 (6.577,9.824)	5.811 (3.563,9.339)
Non-Hispanic Asian	10.420 (8.912,12.149)	8.776 (6.346,12.015)
Education level n(%)		
Less than high school	3.134 (2.331,4.201)	2.190 (1.136,4.178)
High school	30.451 (27.513,33.557)	27.962 (22.940,33.605)
More than high school	66.416 (62.929,69.732)	69.848 (63.963,75.146)
Marital status n(%)		
Married and living with partner	57.423 (54.671,60.131)	78.186 (72.788,82.766)
Living alone	42.577 (39.869,45.329)	21.814 (17.234,27.212)
Smoking n(%)		
never	70.506 (68.086,72.816)	61.724 (55.602,67.496)
former	11.634 (10.149,13.305)	14.324 (10.318,19.545)
now	17.860 (16.101,19.766)	23.952 (18.162,30.891)
drinking n(%)		
never	15.705 (13.438,18.273)	10.954 (6.826,17.119)
former	4.686 (3.856,5.685)	8.728 (5.646,13.256)
now	79.609 (76.406,82.476)	80.318 (73.579,85.673)
Diabetes n(%)		
Yes	5.616 (4.784,6.584)	11.267 (8.679,14.505)

(Continued)

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TABLE 1 Continued

Characteristic	infertility	
	negative	positive
Diabetes n(%)		
No	94.384 (93.416,95.216)	88.733 (85.495,91.321)
Hypertension,n(%)		
yes	13.569 (12.094,15.192)	23.763 (18.808,29.548)
no	86.431 (84.808,87.906)	76.237 (70.452,81.192)
Vigorous recreational activities, n (%)		
yes	17.748 (15.761,19.927)	17.125 (12.668,22.742)
no	82.252 (80.073,84.239)	82.875 (77.258,87.332)
Moderate recreational activities, n (%)		
yes	43.369 (41.006,45.763)	41.970 (35.254,48.998)
no	56.631 (54.237,58.994)	58.030 (51.002,64.746)
Health insurance, n (%)		
none	17.933 (16.225,19.778)	21.240 (16.084,27.507)
prirate	58.111 (55.121,61.041)	59.032 (52.626,65.145)
public	23.957 (21.439,26.669)	19.729 (15.637,24.579)
Ever been pregant		
yes	66.841 (63.710,69.830)	86.153 (81.916,89.525)
no	33.159 (30.170,36.290)	13.847 (10.475,18.084)

For continuous variables: P-value was by survey-weighted linear regression. For categorical variables: P-value was by survey-weighted Chi-square test. BMI, body mass index; NHANES, National Health, and Nutrition Examination Survey; SD, standard deviation.

TABLE 2 The association between infertility and DII 、 E-DII.

Exposure	Non-adjusted model OR,95%CI	Minimally-adjusted model OR,95%CI	Fully-adjusted model OR,95%CI
DII	1.11 (1.03,1.12)0.007	1.12(1.04, 1.20)0.007	1.10 (1.01,1.19)0.034
DII			
Q1	Ref	Ref	Ref
Q2	1.02 (0.66,1.56)0.945	1.05(0.65,1.69)0.840	1.05 (0.65,1.70)0.829
Q3	1.20 (0.84,1.72)0.313	1.23(0.868,1.75)0.250	1.02 (0.70,1.49)0.900
Q4	1.54(1.053, 2.24)0.031	1.71 (1.14 ,2.58)0.014	1.59 (1.03,2.45)0.045
P for trend	0.023	0.011	0.068
Exposure	Non-adjusted model OR,95%CI	Minimally-adjusted model OR,95%CI	Fully-adjusted model OR,95%CI
E-DII	1.05 (1.00,1.09)0.034	1.06 (1.01,1.11)0.019	1.04 (0.99,1.08)0.109
E-DII			
Q1	Ref	Ref	Ref
Q2	1.06 (0.65,1.72)0.823	1.14 (0.69,1.88)0.620	1.07 (0.630, 1.82)0.804
Q3	1.35 (0.88,2.07)0.184	1.47(0.95,2.29)0.093	1.40 (0.86, 2.27)0.188
Q4	1.42 (0.99,2.04)0.066	1.46 (0.99,2.16)0.060	1.31 (0.89, 1.92)0.177
P for trend	0.025	0.021	0.086

Non-adjusted model: no covariates were adjusted for.
Minimally-adjusted model: we only adjusted for age 、 Marital status and BMI.
Fully-adjusted model: we adjusted for all covariates presented in Table 1.

Nonlinear relationship

Analysis using restricted cubic splines showed a linear relationship between DII and infertility risk, with no evidence of nonlinearity (Figure 3).

Discussion

This was a cross-sectional study of 3496 women aged 18-45 years, from which it was observed that there was a positive correlation between DII and infertility, suggesting that consumption of a pro-inflammatory diet increased the risk of infertility. After adjusting for covariates, the positive association between DII and infertility remained. However, after stratification, the positive association was affected by age and race. In the final curvilinear relationship, there was no nonlinear association between DII and infertility.

To our knowledge, there are few studies to explore the relationship between DII and infertility. A RaNCD cohort study verified the association between infertility and the quality of diet in women, the results showed the odds ratio of infertility in the pro-inflammatory diet was 1.76 times higher than in the anti-inflammatory diet of DII (95% CI: 1.57-2.02) (27). Its results are consistent with ours. There have been previous studies on the relationship between diet and infertility. One study has confirmed that a Mediterranean nutritional pattern reduces the risk of weight gain and insulin resistance, which may be responsible for increased pregnancy (28, 29). In 2007, a prospective cohort study created a "fertility diet" pattern that included a lower intake of animal proteins and higher availability of plant proteins. The results suggested that increased adherence to a "fertility diet" could improve infertility caused by ovulation disorders (30, 31). It is well known that inflammation occurs throughout almost the entire

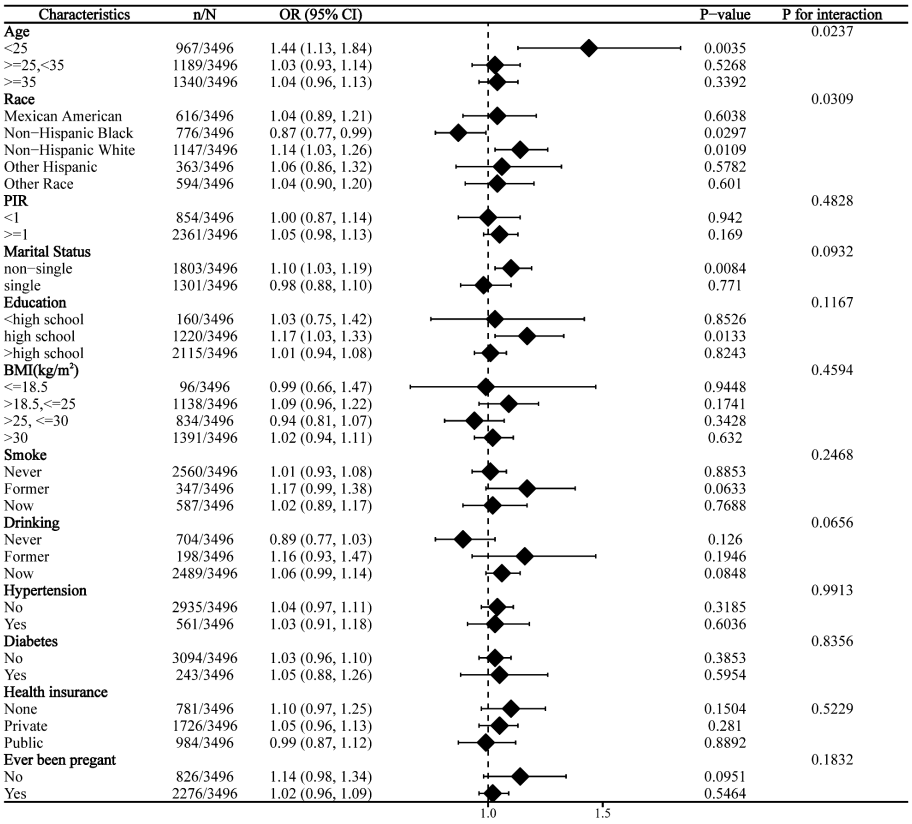

FIGURE 2
Subgroup analysis for the association between DII and infertility.

reproductive process, from ovulation, implantation, and fertilization of the egg to pregnancy. Inflammation is a normal process of injury and infection, but prolonged inflammation can impair fertility. Inflammation can damage the endometrium (1, 32), trigger oxidative stress that impairs folliculogenesis (33), and alter blood coagulation leading to thrombosis (13). A prospective study showed that chronic endometritis affected homeostatic imbalance in patients with endometrial fibrosis and was associated with a higher incidence of adhesions, thus leading to reproductive failure.

The odds of infertility are increased with higher DII scores, and the exact mechanism of this positive association remains unclear. However, high DII has a modulating effect on the inflammatory process, which can lead to an increase in inflammatory markers including CRP (34, 35), TNF-α (Kwak-Kim, Yang and Gilman-Sachs, 2009) (36), IL-6 (37), and other markers of inflammation, thus adversely affecting reproduction. TNF-α mediates immune and inflammatory responses; in addition, elevated concentrations of TNF-α in peritoneal fluid can directly reduce sperm viability, thereby affecting the entire fertilization and implantation process and exhibiting embryotoxicity (38). In one study in transgenic mice, the number of implantation sites or larval size was reduced in the absence of cytokines, such as CSF-1, GM-CSF, IL-1 and IL-6 (39). There was also a basic study from Michigan, USA, in which mouse oocytes were exposed to IL-6 (50, 100, and 200 ng/mL) for 30 min, as compared with untreated controls. It was found that IL-6 resulted in dose-dependent deterioration of microtubule and chromosome arrangement in the treated oocytes, compared with the untreated group, suggesting that elevated levels of IL-6 might be mediated through a mechanism involving impaired microtubule and chromosome architecture to reduce the fertilizing ability of human oocytes (40). There have also been several studies suggesting that anti-inflammatory diets may improve fertility outcomes. In a prospective study of 18,555 premenopausal women, this anti-inflammatory diet prevented ovulatory infertility by reducing carbohydrate intake and overall dietary glucose load (41). A recent randomized controlled trial investigating a subgroup of 150 overweight adult women with polycystic ovary syndrome found that the anti-inflammatory diet group and the physical activity group had improved menstrual cycles and spontaneous pregnancies, as well as a 7% weight loss, and these effects were not inferior to those observed in the metformin group.

One of the strengths of this study is that it is based on a weighted and representative population with a large base size. It is worth noting that we also performed a curve analysis. However, this study has several limitations. First, it is a cross-sectional analysis, therefore, we cannot determine causality. Second, for the DII the calculations were based on 24 h dietary recalls from the population, which may introduce bias in the data. Finally, for confounders, we merely included those shown in Table 1, which is obviously insufficient for the outcome variable. In our paper, we focused on

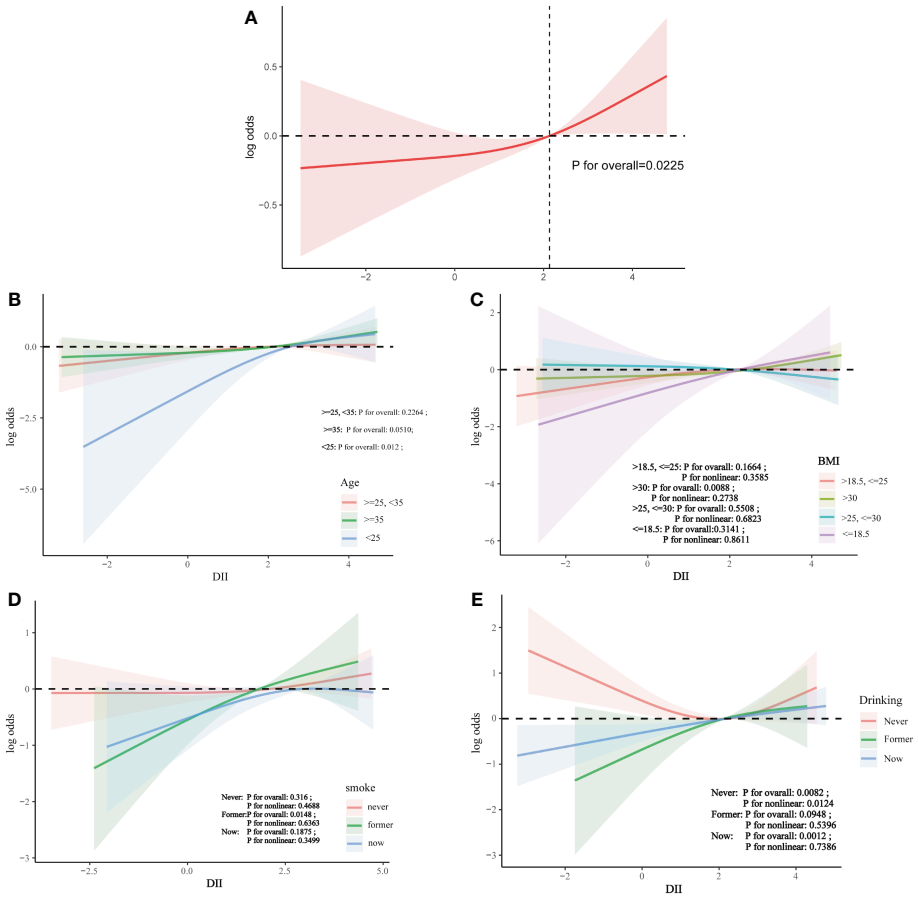

FIGURE 3
The association between DII and infertility. **(A)** Solid line plot of curve fitting with DII and infertility as variables.The red line indicates the smooth curve fit between the variables. The 95% confidence interval of the fit is shown by the red bar. **(B)** The association between DII and infertilit stratified by age. **(C)** The association between DII and infertilit stratified by BMI. **(D)** The association between DII and infertilit stratified by smoke. **(E)** The association between DII and infertilit stratified by drinking.

specific infertility risk factors. However, given the complexity of infertility, there are other potential risk factors that may have an impact on the observed indicators, such as environmental factors, genetic factors, as well as endometriosis, and polycystic ovary syndrome.These risk factors may influence the indicators we observe. For example, the basis of infertility caused by polycystic ovary syndrome is chronic inflammation caused by immune metabolism (42).Whilst our study has focused primarily on the DII, we recognise that a comprehensive understanding of the complex mechanisms of infertility requires consideration of a wider range of possible risk factors. Therefore, future research should further explore these additional risk factors and their specific impact on infertility. This will help us to gain a deeper understanding of the causes of infertility and may provide new ideas for treatment.

Conclusion

This study showed a significant positive correlation between DII scores and infertility, which suggests that there is a positive correlation between a pro-inflammatory diet and the incidence of infertility, and that management with an anti-inflammatory diet decreases the chances of infertility. However, further fundamental research is still needed to explore the potential association between them.

Data availability statement

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

Ethics statement

The studies involving humans were approved by National Center for Health Statistics. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

JQ: Writing – original draft. YS: Writing – review & editing. HZ: Data curation, Writing – review & editing. YR: Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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The role of epigenetics in women's reproductive health: the impact of environmental factors

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This paper explores the significant role of epigenetics in women's reproductive health, focusing on the impact of environmental factors. It highlights the crucial link between epigenetic modifications—such as DNA methylation and histones post-translational modifications—and reproductive health issues, including infertility and pregnancy complications. The paper reviews the influence of pollutants like PM2.5, heavy metals, and endocrine disruptors on gene expression through epigenetic mechanisms, emphasizing the need for understanding how dietary, lifestyle choices, and exposure to chemicals affect gene expression and reproductive health. Future research directions include deeper investigation into epigenetics in female reproductive health and leveraging gene editing to mitigate epigenetic changes for improving IVF success rates and managing reproductive disorders.

KEYWORDS

epigenetics, women, reproductive, environmental factors, reproductive health

1 Introduction

The World Health Organization (WHO) identifies Sexual and Reproductive Health (SRH) as integral to achieving the highest standard of health. Women's reproductive health remains a significant global concern, impacting not only physical well-being but also societal development, economic growth, and public health. Women's unique physiological structures and responses, including the glandular folds of their internal and external genitalia in a moist environment, create conditions conducive to pathogen survival. The presence of androgens in the body may promote the growth and proliferation of pathogens, significantly increasing the risk of tumor development (1). During menstruation, the disruption of the endometrial lining and the increased susceptibility to various infections

during sexual intercourse further exacerbate women's reproductive health challenges (2). According to WHO data, 40% of Chinese women suffer from various degrees of reproductive tract infections, with the prevalence among married women reaching up to 70%. This indicates that approximately 300 million women in China face reproductive health issues, a figure significantly higher than the incidence rate of common colds. The repercussions of reproductive health issues on women's lives and careers are profound, causing immense distress and suffering. Common gynecological conditions include endometriosis, uterine fibroids, ovarian cysts, vaginitis, cervicitis, cervical erosion, pelvic inflammatory disease, adnexitis, functional uterine bleeding, breast diseases, infertility, and menstrual disorders. Notably, endometriosis affects 10% (190 million) of women of reproductive age worldwide (3). The majority of gynecological diseases, along with psychological factors such as work stress and environmental conditions, can adversely affect ovarian function. This leads to metabolic disorders, endocrine disruptions, and imbalances in estrogen and progesterone levels, triggering conditions such as melasma, wrinkles, constipation, acne, obesity, hyperlipidemia, and even carcinogenesis, accompanied by mental lethargy (4, 5). Hence, reproductive system issues directly impact human health.

Over the past decade, notable progress has been made in the prevention and treatment strategies for common diseases of the female reproductive system. However, current research on the impact of environmental factors through epigenetic mediation on women's reproductive health remains fragmented. In this review, we summarize the latest findings on the influence of environmental factors on epigenetics and, consequently, on women's reproductive health. This includes an overview of several common types of epigenetics and the potential cellular and molecular mechanisms involved. Furthermore, we discuss the impact of both internal and external environmental factors on the female reproductive system. Therefore, our aim is to gain a more comprehensive understanding of the pathophysiological processes and potential mechanisms related to female infertility diseases, with the goal of enhancing fertility and pregnancy outcomes in women of childbearing age.

2 An introduction to epigenetics

Epigenetics refers to the transmission of genetic information that does not involve changes to the DNA sequence. It operates through chemical modifications on the genome, such as histones post-translational modifications, DNA methylation, and hydroxy methylation, thereby altering the way genes are expressed. The mechanism by which DNA methylation leads to gene silencing is not yet fully understood; however, three main forms are commonly considered. First, DNA methylation affects the transcriptional activity of genes. It can directly hinder the recognition and binding of transcription factors to specific DNA sequences, thus inhibiting gene transcription (6). Second, the mechanism by which methylation leads to gene silencing involves the methylation of CpG islands located in promoters or other regulatory regions playing a role in gene repression. Methylation of CpG islands in non-coding promoter regions recruits sequence-specific methylated DNA binding proteins and histone deacetylases (HDACs), forming complexes that suppress transcription by obstructing the binding of transcription factors to their target sequences, thereby affecting transcription (7, 8). Finally, DNA methylation can alter chromatin structure to suppress gene expression, where highly methylated promoters cause chromatin to condense, further affecting transcription (9).

Epigenetic modifications can regulate the splicing and expression patterns of genes (10). For instance, numerous studies have shown that histones post-translational modifications can regulate the binding and activity of splicing factors, thus influencing gene splicing. Results obtained from real-time quantitative Polymerase Chain Reaction(PCR) analysis of 16 epigenetic remodeling markers in the epidermal cells of 14 patients after *in vitro* amplification compared to freshly isolated epidermal cells (ISO), indicated a significant reduction in the transcription levels of genes involved in DNA methylation and histones post-translational modifications in cells cultured to the second generation of keratinocyte formation.

Beyond the aforementioned effects, epigenetic modifications can also regulate genomic stability and genetic memory, enabling cells to stably express specific phenotypes (11). Further research and understanding of the mechanisms of epigenetics are crucial for unraveling important processes in organism development, environmental adaptation, and disease onset.

3 The potential impact of environmental factors on women's reproductive health

Infertility is commonly defined as "a disease of the male or female reproductive system characterized by the failure to achieve a successful pregnancy after more than one year of regular, unprotected sexual intercourse." External harmful environmental factors can impair women's fertility. Non-gaseous pollutants, such as PM2.5, have a certain impact on female reproduction(12, 13). Gaseous pollutants (such as ozone (O_3), sulfur dioxide (SO_2), and nitrogen oxides (NOx)) can affect the endocrine system of women of childbearing age, leading to infertility and pregnancy complications, including reduced ovarian reserve (14, 15), uterine fibroids (16), and preeclampsia (17, 18).

Harmful chemicals also impact women's reproductive health (19). Polycyclic aromatic hydrocarbons have been shown to interact with estrogen receptors, activating the aryl hydrocarbon receptor leading to changes in steroid functionality and anti-estrogenic activity, resulting in adverse pregnancy outcomes such as preterm birth, miscarriage, and embryonic developmental arrest (20).Studies indicate that the widespread use of the heavy metal cadmium can cause endocrine disruption in women, potentially directly affecting the development of oocytes, the development of the uterus, and ovarian function, leading to decreased fertility, spontaneous miscarriage, and other reproductive issues (21).

4 Defining the purpose and scope of a literature review

In recent years, a growing body of evidence suggests that environmental exposures can leave epigenetic marks on genes, with various environmental factors proven to induce global or specific epigenetic changes (266). In this review, we discuss the interactions between environmental factors related to women's reproductive health risks and epigenetics. This includes more common epigenetic processes such as DNA methylation, histones post-translational modifications, and non-coding RNA. Key roles in genetic regulation are played by external environmental factors like heavy metals (cadmium), polycyclic aromatic hydrocarbons, air pollutants, and internal factors such as dietary and nutritional elements, as well as the influence of parental care and climate factors on epigenetics. Moreover, we comment on the regulation of female reproductive functions through epigenetics, such as the pathogenesis and regulatory processes related to Polycystic Ovary Syndrome (PCOS), Premature Ovarian Insufficiency (POI), and endometriosis mediated by epigenetics, elucidating the function of epigenetics as a mediator, bridging environmental factors and female reproduction. Besides the adverse effects on female reproductive functions, epigenetic processes and abnormal epigenetic marks can also impact offspring health. Therefore, understanding how maternal environmental factors can affect offspring health through epigenetic mechanisms is crucial for preventing and managing environmentally related women's reproductive health issues and accelerating the application of epigenetics in reproductive medicine.

5 The fundamentals of epigenetics

Epigenetics, also referred to as character genetics, exogenetics, paragenetics, postgenetics, or topogenetics, such as DNA methylation and histone modifications. DNA methylation is termed a carrier of epigenetic information, whereas variations and modifications of histones can directly or indirectly impact the structure of local chromatin. These chemical modifications to chromatin are both inheritable and reversible.

5.1 Epigenetic mechanisms

5.1.1 DNA methylation

DNA methylation is the most prevalent epigenetic regulatory mechanism, where methyl groups are covalently bonded to cytosine residues in DNA sequences through enzyme-mediated reactions catalyzed by specific methyltransferases. In vertebrates, three methylation states of DNA are recognized: a persistent hypomethylation state (22), an induced demethylation state, and a hypermethylation state, which is notably observed in the methylation modifications of the inactivated X chromosome (23). Recent studies have demonstrated that genome-wide hypermethylation can impede the epithelial-to-mesenchymal transition, thereby further inhibiting the healing of chronic wounds (24).

In mammals, DNA methylation involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring in CpG dinucleotides. As a form of chemical modification, DNA methylation alters the structure of the cytosine residues, creating the "fifth base" - 5-methylcytosine (5mC), which is the most significant form of DNA methylation in mammals. DNA methylation is essential for the organism's normal growth and development, including the formation of genetic imprints(11

) and the promotion of dispersed chromatin to become condensed. DNA methylation also impacts the normal expression of genes (25), leading to a decrease in gene transcriptional activity. The mechanism by which DNA methylation impedes gene transcription is complex: it interferes with the binding of transcription factors to promoters, thus blocking transcription (1). Moreover, transcription factors can recognize methylated DNA and reactivate gene transcription (26). DNA methylation is catalyzed by DNA methyltransferases (DNMTs), with DNMT1 and DNMT3 being the active enzymes responsible for establishing and maintaining DNA methylation (27). DNA methyltransferases add methyl groups to CpG islands using S-adenosyl methionine (SAM) as a substrate. Zhu Bing was the first to use the methylation profile of mouse oocytes to confirm that DNMT1 indeed functions as an initiating DNA methyltransferase (28).

De novo methylation refers to the addition of methyl groups to previously unmethylated cytosines under the action of DNMT3 methyltransferases. DNMT3A and DNMT3B are the primary enzymes for mammalian DNA methylation, known as *de novo* methyltransferases (29). DNMT3A-mediated DNA methylation plays an indispensable role in the spermatogenesis of male germ cells (30),Mutations in DNMT3A render it insensitive to the inhibition of H3K4me3, resulting in the aberrant methylation of promoter subgroups marked by H3K4me3 in mouse embryonic stem cells (ESCs). This aberrant methylation leads to the downregulation of associated genes (31).While the ectopic expression of DNMT3B can enhance the genome-wide methylation level of haploid embryonic stem cells, shorten the transition from the G2 phase to the M phase of cell mitosis, alleviate spontaneous diploidization of haploid cells, and extend the survival time of semiclone mice (32).Weinberg et al. (33) demonstrated that H3K36me2 is essential for the recruitment and maintenance of DNA methylation in intergenic regions by DNMT3A. In contrast, Shirane et al. (34) showed that H3K36me2, deposited by NSD1, plays a crucial role in *de novo* methylation in germ cells. Typically, *de novo* methyltransferases preferentially bind to CpG-rich regions that are not protected by H3K4 methylation. These enzymes with methyltransferase activity prioritize the methylation of unmethylated CpG dinucleotides. For instance, the flexible N-terminal guides DNMT3AA1 to its bivalent target catalytic methyltransferase domain, thereby regulating DNA methylation and gene expression (35). DNMT3L, while having no catalytic activity, can interact with DNMT3A and DNMT3B to regulate their activity (36) and is expressed only in specific rodents like mice, rats, gerbils, and hamsters (37). In human oocytes, it is transcriptionally silent and involved in the regulation of repetitive

elements and imprinting in germ cells. Although DNMT3L cannot bind the methyl donor S-adenosyl-L-methionine (SAM), it facilitates the interaction of SAM with DNMT3A2, aiding the *de novo* methyltransferases (38).

DNMT1 is the key enzyme for maintaining DNA methylation (39), preserving the methylation pattern from the parental DNA strand to the daughter strand through cell division and DNA replication (40). The C-terminal domain of DNMT1 consists of two subdomains: the Target Recognition Domain (TRD), which identifies hemimethylated cytosines, and the methyltransferase domain.

DNMT1 preferentially methylates hemimethylated DNA, a process primarily facilitated by the TRD domain recognizing cytosines within hemimethylated DNA. Once the DNA methylation pattern is established, the DNA methyltransferase DNMT1 maintains this pattern during DNA replication. The faithful replication of the DNA methylation pattern during cell division makes it an ideal mechanism for preserving epigenetic memory.

A recent review on DNA methylation proposed a regulatory model for DNMTs, where *de novo* methyltransferases adopt an autoinhibitory conformation until they are locally activated upon binding to the N-terminal tail of histone H3. Additional research indicates that this regulatory principle is applicable not only to classical *de novo* methyltransferases but also to DNMT1 (41). In this context, the replication foci targeting sequence (RFT) domain interacts with conformational activators, such as UHRF1, thereby exposing the catalytic site (42, 43).

DNA methylation can suppress the activity of certain genes, while DNA demethylation induces the reactivation and expression of genes (267). TET enzymes play a key role in the DNA demethylation process (44), including TET1, TET2, and TET3. These enzymes convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (45), which can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Subsequently, the TET and base excision repair (BER) pathways intervene in the repair process, converting these modified bases back to unmethylated cytosine, thereby achieving DNA demethylation. Although the BER pathway is commonly considered the final step in DNA demethylation, there remains controversy regarding the specific enzymes and chemical intermediates formed during this process (46). The implications of DNA demethylation are increasingly recognized; for instance, aberrant DNA hypermethylation has been detected in the leptotene spermatocytes of some patients with non-obstructive azoospermia, suggesting that DNA demethylation can influence male meiotic recombination and fertility (47).

5.1.2 Histone post-translational modification

Histones are primarily composed of a globular domain (48) and tails that protrude outside the nucleosome, made up of basic amino acids forming a fundamental structural protein. Two H2A-H2B dimers and one H3-H4 tetramer assemble into a histone octamer. Histones and DNA are the basic components of the nucleosome, providing appropriate sites for DNA winding when nuclear DNA is in a highly condensed state. Timothy J. Richmond and others determined the crystal structure of the chromatin nucleosome core particle, with a 2.8A high-resolution X-ray structure detailing the internal binding mode of the histone octamer and the superhelical organization of its surrounding 146 base pairs of DNA (49). Unmethylated nucleosome DNA spontaneously extends, forming four superhelical turns (50), a phenomenon that strongly evidences the crucial role of histone tails in maintaining the overall nucleosome structure (51).As illustrated in Figure 1, the diagram represents the spectrum of histones post-translational modifications. histones post-translational modifications mainly include methylation, acetylation, ubiquitination, sumoylation, and citrullination.

Methylation of histones that wrap DNA in eukaryotic chromosomes can have significant implications for human health. It occurs on all basic residues, including arginine (52), lysine (53), and histidine (54). Protein methylation can occur at the N-, O-, and S-centers of amino acid residues, with arginine and lysine residues being the most common sites of histone methylation. The effects of methylation on gene activity vary (55), depending on the modified amino residue (56), the genetic context of methylation, its level and pattern. Amino acid methylation primarily changes chromatin structure by increasing or decreasing DNA-histone interactions, leading to the activation or suppression of gene transcription. The core histone H3 plays a key role in nucleosome structure and gene expression regulation, being a primary site of histone methylation, along with other core histones like H2A, H2B, and H4, which are central components of the nucleosome and participate in histone methylation (57). During histone methylation, S-adenosylmethionine (SAM) (58) serves as the substrate, and under the catalysis of histone methyltransferases (HMTs, also known as "writers"), it transfers its methyl group to the lysine residues of histones (59). On the ε-amino group of lysine, it can be monomethylated, dimethylated, or trimethylated (60–62), referred to as me1, me2, and me3 (63). Currently, extensive research has been conducted on methylated histones, including H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 (64). Studies show that H3K4, H3K36, and H3K79 are mainly found in transcriptionally active regions of chromatin, playing a role in activating gene transcription. Conversely, H3K9, H3K27, and H4K20 often act as markers for gene transcription repression, closely associated with gene silencing (65–68).

Histone acetylation is a reversible process where acetyl groups are transferred by histone acetyltransferases (HATs), with lysine acetylation being a common form of histone acetylation. Simply put, this process involves adding an acetyl group to the positively charged lysine residues. Histone acetylation decreases the electrostatic affinity between histones and negatively charged DNA, loosening chromatin structure and activating open chromatin regions (69), making it easier for transcription factors and RNA polymerase to access DNA, thereby promoting transcription (70). Histone 1 is the most commonly acetylated protein, and H2A, H2B, H3, and H4 form the core of the nucleosome, referred to as core histones. Acetylation modifications within these core histones affect the dynamics of the nucleosome core particle (71), with the acetylation of H2B, H3, and H4 having minimal impact on the dynamics of the nucleosome core particle. In contrast, H2A shows significantly increased dynamics after acetylation modification (72). Histone acetylation

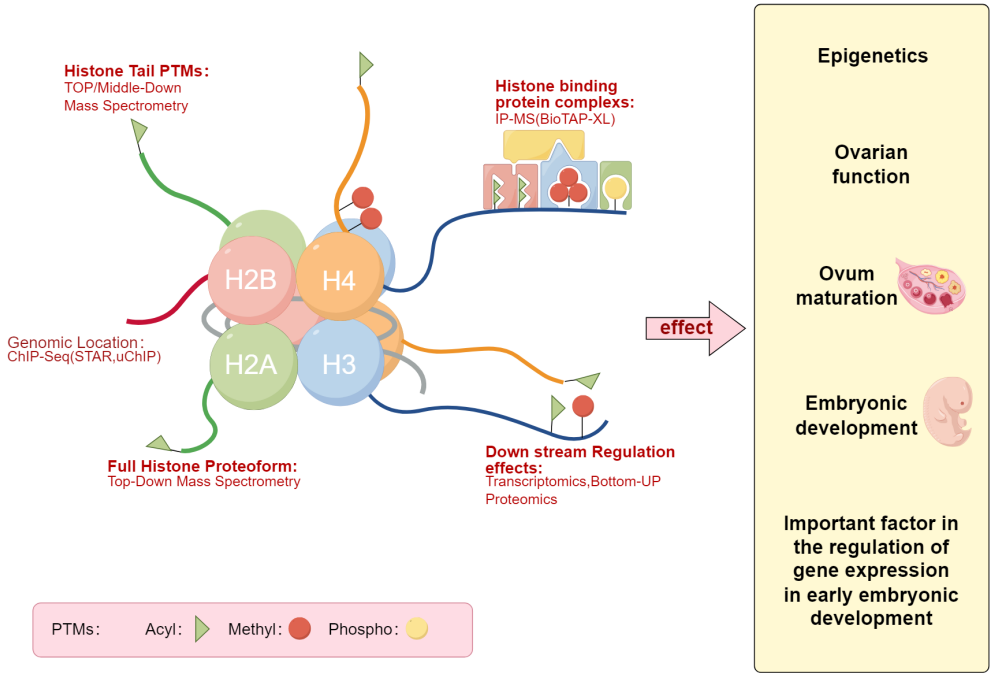

FIGURE 1
Histones post-translational modifications Map. The left panel describes the patterns of histones post-translational modifications, while the right panel lists the potential impacts of histones post-translational modifications on the pregnancy process, such as embryonic development (Draw by figdraw.).

primarily occurs at the amino-terminal lysine sites of H3 and H4, including H3K9ac, H3K14ac, H3K18ac, H3K23ac, H3K27ac, H3K56ac, H4K5ac, H4K8ac, H4K16ac, and H4K20ac (73), catalyzed by HATs such as CBP/p300, MOF, HBO1, or KAT6A (74). Previous extensive research has revealed a link between histone acetylation levels and the expression of pro-inflammatory cytokines and other antimicrobial products (75), with acetylases playing an indispensable role.

Acetyl-CoA is the donor of the acetyl group, produced by metabolic enzymes in the nucleus and having a direct effect on histone acetylation (76, 77). To date, studies have shown that three enzymes are crucial for maintaining acetyl-CoA levels: the Acyl-CoA Synthetase Short-Chain Family Member 2 (ACSS2) (78–80). Extensive research indicates that ACSS2 catalyzes the synthesis of acetyl-CoA from acetate, recruited to neuronal sites associated with organismal memory, crucial for memory consolidation (81). Furthermore, ACSS2 is an enzyme required for alcohol-induced neuron-specific gene expression and alcohol-associated associative learning (82). In summary, extensive research highlights the key role of ACSS2 in enhancing spatial memory and regulating histone acetylation.

Zhang et al. (83) were the first to discover histone lactylation, where lactate-derived histone lysine lactylation emerged as a novel epigenetic modification and demonstrated that histone lactylation directly stimulates chromatin gene transcription. Wan et al. reported the formation of cyclic immonium ions of lactylated lysine during tandem mass spectrometry analysis, enabling the identification of protein lactylation. Their findings highlighted that lactylation is common on glycolytic enzymes and is conserved on Aldolase A. Additionally, widespread lactylation was identified on Reductase SDR Family Member 7(DHRS7) in a draft of the human tissue proteome (84).

5.1.3 Non-coding RNA

In eukaryotes, approximately 90% of genes are transcribed, of which only 1%-2% are responsible for encoding proteins, with the majority being transcribed into non-coding RNAs (ncRNAs). ncRNAs refer to RNA molecules transcribed from genes that do not have the capacity to encode proteins.

ncRNAs can be primarily classified into two forms: small ncRNAs (SncRNAs, 18-200 bp) and long ncRNAs (lncRNAs, >200 bp), neither of which can be translated into proteins. SncRNAs include small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), microRNAs (miRNAs), circular RNAs (circRNAs), and extracellular RNAs (exRNAs) (85, 86). Extensive research on ncRNA biology has demonstrated that ncRNAs play a critical regulatory role in shaping cellular activity. ncRNAs include molecules that act as oncogenes or tumor suppressors, and their aberrant expression has been linked to carcinogenesis and metastasis regulated by epigenetic mechanisms (87–89). ncRNAs can regulate the transcription of individual genes or entire transcriptional programs, affecting the expression of hundreds to thousands of genes (90–92).

MicroRNA (miRNA) is one of the most extensively studied ncRNAs, consisting of single-stranded molecules approximately 20-24 bp in length. Thousands of miRNAs can function as tumor suppressors or oncogenes by base-pairing with complementary

sequences in the 3'UTR of target mRNAs, inhibiting gene translation and leading to gene silencing (93). Competitive endogenous RNAs (ceRNAs) are common post-transcriptional regulators. Notably, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) within the ceRNA category can induce gene silencing, affecting gene expression and thereby influencing tumor progression (94). lncRNAs regulate various physiological processes such as tumor cell invasion, migration, proliferation, and tumor microenvironment (TME) remodeling by modulating mRNA processes and gene transcription (95, 96). The unique circular structure of circRNAs, resulting from the back-splicing of pre-mRNA, confers them with exceptional stability. CircRNAs, as endogenous ncRNAs with their linear transcriptional 3' and 5' ends removed, are implicated in biological processes related to tumor suppression and carcinogenesis (97, 98).

5.2 Advances in research methods and techniques in epigenetics

Current research in epigenetics primarily focuses on three aspects: DNA methylation, histones post-translational modifications, and non-coding RNA. The methodologies for studying DNA methylation (99) are categorized into two main types. The first type examines the overall level of DNA methylation, including techniques such as whole-genome bisulfite sequencing (WGBs), methylation 450K array, and immunoprecipitation techniques (MeDIP). The second type investigates DNA methylation at specific sites; after bisulfite treatment, unmethylated cytosines are converted into uracil, followed by detection methods such as methylation-specific PCR (MSP), reduced representation bisulfite sequencing (RRBS), and methylation-sensitive high-resolution melting (MS-HRM).

Classic methods for detecting histones post-translational modifications (100) include chromatin immunoprecipitation followed by sequencing (ChIP-seq), Cut&Tag, and Edman degradation. Edman degradation, a low-throughput sequencing method, is generally limited to analyzing the N-terminal 50 amino acids of proteins and cannot detect multiple proteins simultaneously; it is used for analyzing chemically unmodified N-terminal α-amino acids. Immunosequencing methods cannot detect unknown modification sites. More widely used methods for histones post-translational modifications detection now include mass spectrometry (MS) and ELISA techniques.

Research methods for non-coding RNA mainly include transcriptome sequencing (RNA-seq), Northern blotting, fluorescence *in situ* hybridization (FISH), and RNA-binding protein immunoprecipitation (RIP-seq). Currently, Solexa high-throughput sequencing (101) is extensively used. This method can simultaneously analyze hundreds of millions of nucleotide fragments, offering low cost, high precision, and requires a small sample volume. Additionally, ACAT-seq, as a crucial tool for understanding chromatin states and epigenetic regulation, aids researchers in analyzing chromatin accessibility, chromatin structure and three-dimensional organization, and the association between epigenetic changes and diseases. An overview of these common epigenetic research methods, including their advantages, disadvantages, and applicability, is summarized in Table 1.

5.2.1 Environmental chemical substances

Heavy metal pollution has become a significant and widespread environmental issue that poses multiple hazards to human health. Cadmium, a typical heavy metal, exerts its reproductive toxicity, carcinogenicity, and other toxic effects by mediating changes in epigenetic modifications to regulate gene expression (268). Cadmium exposure can affect the overall level of genomic methylation. It leads to abnormal expression of DNMTs enzymes, with significant reductions observed in the mRNA and protein levels of DNMT1, DNMT3A, and DNMT3B, thereby inducing global DNA hypomethylation *in vitro* (124). Similarly, cadmium exposure can also cause DNA hypermethylation to regulate gene expression. Aigner GP et al. (125), using earthworms as a model organism, revealed cadmium-induced hypermethylation of adenine and cytosine in spot imprinting, thus elucidating the time- and dose-dependent effects of Cd on global and gene-specific DNA methylation and its potential mechanisms.

Research has shown that continuous exposure of Drosophila melanogaster to cadmium during growth results in a significant increase in H3K4me3 levels and a significant decrease in H3K9me3 and H3K27me3 levels in third-instar larvae of the offspring. The expression of histone methylation-related genes dSet-1, ash1, and Lsd1 is significantly increased. Cadmium-induced wing phenotypic defects can be inherited by offspring, suggesting a potential transgenerational effect related to histone methylation's epigenetic regulation (126).

Chen et al. (127)studied 31-week-old Hy-Line Brown hens fed with dietary cadmium chloride (150 mg/kg) and found a close association between miR-33 and cadmium toxicity. Cadmium toxicity significantly inhibited the expression of miR-33 and significantly increased the mRNA and protein levels of AMP-activated protein kinase(AMPK), altering the expression pattern of the miR-33-AMPK axis in the spleen and causing dysregulation of the miRNA-33-AMPK axis.

Mercury (Hg) is also a heavy metal that, due to its toxicity and widespread human exposure, has become a significant public health concern. Mercury is found in a variety of sources including seafood, household products, medical devices, and cosmetics, making it a common occupational hazard. Mercury pollution and volcanic eruptions are significant sources of its presence in soil, water, and the atmosphere. High doses of mercury are widely recognized as neurotoxic substances that negatively affect the female reproductive system (128).

Studies have shown that whole-body exposure to 2.5 mg/m^3 of mercury for 6 hours per day over 6-8 weeks leads to prolonged estrous cycles in treated female rats and increased mortality rates in offspring (129). Davis et al. (130) found immature corpora lutea in rats exposed to mercury vapor. Lamperti et al. (131) observed inhibition of follicle maturation after injecting HgCl2 into hamsters. Dansereau et al. directly demonstrated that female minks exposed to a dietary concentration of 1.0 microgram/gram

TABLE 1 Comparative summary of epigenetic research methods.

Serial Number	Method	Applicable Epigenetic Type	Advantages	Disadvantages	References
1	WGBs	DNA Methylation	Whole genome coverage; high resolution; quantitative and precise analysis of methylation levels and patterns at each CpG site	High cost; complex data processing; not suitable for time-sensitive studies	(102, 103)
2	MeDIP	DNA Methylation	High throughput; high specificity; low cost; suitable for various species and sample types, especially for CpG-rich (methylation) or specific methylation	Low resolution; presence of affinity bias; not suitable for other types of DNA modifications (e.g., hydroxymethylation) or specific methylation	(104, 105)
3	450k Chip	DNA Methylation	High throughput; high resolution; accuracy; covers most functional regions and key genes of the genome	Lacks coverage of unknown or specific regulatory regions; high cost; strict sample processing requirements	(106)
4	MSP	DNA Methylation	High specificity; high sensitivity; simple and fast	PCR bias; not suitable for unknown methylation sites or large-scale methylation analysis	(107)
5	RRBS	DNA Methylation	High resolution; low initial DNA amount requirement; low cost	PCR bias; complex data processing	(108, 109)
6	MS-HRM	DNA Methylation	High resolution; simple and fast; no primer design required	PCR bias; limited by methylation range; semi-quantitative	(110)
7	ChIP-seq	Protein Modification	Whole genome coverage; high resolution; discovery of new gene regulatory elements	High sample requirements; presence of enrichment bias	(111, 112)
8	Cut&Tag	Protein Modification	High resolution; simple, low cost; initial cell number can be as low as 50	Antibody specificity requirements; potential for background noise and non-specific binding	(113)
9	MS	Protein Modification	Comprehensive; high resolution	Limited by enzyme cleavage; expensive	(114)
10	ELISA	Protein Modification	High sensitivity; high throughput; simple, low cost	Risk of cross-reactivity; limited dynamic range; not suitable for small molecule analysis; requires high-quality antibodies	(115, 116)
11	Edman Degradation	Protein Modification	Efficiently determines the N-terminal sequence of proteins, suitable for small samples or high precision sequence information	Low throughput; time-consuming	(117)
12	RNA-seq	Non-coding RNA	Whole genome coverage; high throughput; detection of new genes and variants	Complex data analysis; high cost; high requirements for RNA integrity	(118, 119)
13	Northern Blot	Non-coding RNA	Can measure RNA size and relative abundance; shows RNA expression patterns and differential expression; quantitative	Low throughput; time-consuming; does not provide comprehensive transcriptome information	(120)
14	RIP-seq	Non-coding RNA	High throughput; high sensitivity; full transcriptome coverage	Relies on antibody specificity; not suitable for all RNA	(121)
15	FISH	Non-coding RNA	High resolution; can detect multiple targets simultaneously; maintains sample structure	Limited to static analysis; limited to known sequences	(122)
16	Solexa Sequencing	Non-coding RNA	High throughput; high sensitivity and accuracy; short time requirement	Complex data processing; relies on reference genome	(123)

of mercury had fewer births compared to those exposed to 0.5 micrograms/gram and 0.1 micrograms/gram (132).

The theory of endocrine disruptors was initially proposed in the 1990s, identifying certain exogenous chemicals that interfere with the endogenous hormonal axis (133). These chemicals can interact in various ways within the body and affect different physiological areas. They include a variety of substances found in the environment, such as various pesticides, industrial chemicals (like Bisphenol A and phthalates), and dioxins. The presence and persistence of endocrine-disrupting chemicals (EDCs) in the environment impact organisms, and increasing evidence suggests that EDCs may be etiologically linked to the development and severity of diseases. The reproductive system is a primary target for most endocrine disruptors (134). Universal exposure during early development has been linked to the incidence of female cancers, especially reproductive organ cancers such as breast and ovarian cancer (135). In particular, *in utero* exposure might affect processes that initiate tumor growth years later. Additionally, some gynecological diseases are associated with exposure to various environmental toxins, particularly during critical developmental

stages. Bisphenol A (BPA) was the first synthetic chemical found to cause selective estrogen receptor modulation, particularly as a hormone-like pollutant. Recent studies have shown that prenatal exposure to BPA might cause a phenotype similar to endometriosis in mice (136). Phthalates and BPA are chemicals widely present in many products, such as food packaging and household items. They are typical endocrine disruptors present in various substances and are frequently exposed to the public. Inhalation, ingestion, and skin contact are all possible exposure pathways (137). Urine analysis is a feasible method to confirm human exposure to these chemicals. Studies have shown that endometriosis is associated with elevated levels of phthalate metabolites and BPA metabolites in bodily fluids (138).

In the environment, pesticides are also endocrine disruptors. Current data indicate that approximately 2.5 million tons of pesticides enter the environment annually (139). Pesticides tend to accumulate in the environment due to their lipophilicity, long half-lives, and long mobility, causing significant environmental pollution (140). Pesticides can enter the human body through inhalation or skin penetration, but the highest toxicity is from ingesting contaminated food or water, including fish, meat and dairy products (especially the high-fat parts), drinking water, indoor and environmental air, and dust and soil (141, 142). Dichloro-Diphenyl-Trichloroethane(DDT), one of the most widely used pesticides, can bind with lipids and accumulate in adipose tissue (143). Long-term presence of pesticides in the human body can affect fertility and alter the levels of male and female reproductive hormones. These chemicals have anti-androgenic and estrogen-like properties (144), which may lead to stillbirth, birth defects, spontaneous abortions, and infertility. Animal studies suggest that daily exposure to DDT during prenatal and postnatal development could cause gender differences in steroid levels, possibly through direct interference by DDT and its impact on the hypothalamic-pituitary system (144). Research has also found that the disruptive effects of DDT involve competition with testosterone and damage to androgen receptor binding and signal transduction, as well as an association with increased estrogen synthesis (145). These changes in hormone secretion may be related to reproductive problems and physical diseases in later life, as excessive estrogen secretion and an imbalanced testosterone/estradiol ratio are associated with increased risks of feminization, metabolic disorders, estrogen-related cancers, and cardiovascular diseases (146).

Among the environmental pollutants that have been confirmed to promote transgenerational inheritance of epigenetic phenotypes, endocrine disruptors constitute a heterogeneous group of substances capable of interfering with hormone signaling pathways, directly altering germ cell epigenetic modifications, and changing metabolism and reproductive function (147). For instance, exposure to BPA increases DNA methylation and histone acetylation in zebrafish testicular cells (148). Male zebrafish exposed to BPA early in spermatogenesis and analyzed for F1 embryos showed increased histone acetylation induced by BPA, resulting in cardiac toxicity (149). Interestingly, research has identified three pairs of miRNA-mRNA involved in hypoxia-induced reproductive disorders, including novel miRNA-525-DIAPH2, novel miRNA-525-myocardium, and novel miRNA-525-RAI14, indicating for the first time that miRNAs may participate in hypoxia-induced reproductive disorders through transgenerational inheritance (147).

Furthermore, exposure to other endocrine disruptors such as organic compounds like benzo[a]pyrene (150) and air pollution particulate matter like CO2 (151) is associated with epigenetic changes, mediating DNA methylation, histones post-translational modifications, RNA expression alterations, and inducing human cancers and other diseases. These changes can be transgenerationally inherited and manifest as alterations in fertility, metabolic function, or behavioral traits.

Environmental pollution's impact on human health is becoming increasingly apparent, with particulate matter (PM) pollution now recognized as one of the most critical public health risks. Particulate matter pollution encompasses various airborne particles, ranging in size from a few micrometers to visible particles up to 100 micrometers. Long-term exposure to environmental particulate matter can lead to cardiac and pulmonary diseases. Most studies focus on particulate matter with an aerodynamic diameter less than 10 micrometers (PM10) or less than 2.5 micrometers (PM2.5), which may adversely affect fetal development, the normal course of pregnancy, and lead to premature birth (152).

Research indicates that high concentrations of PM10 are closely associated with an increased risk of pregnancy complications throughout the gestation period and its various stages. Particularly during the late stages of pregnancy, exposure to high levels of PM2.5 significantly increases the risk of pregnancy complications. In the middle stage of pregnancy and throughout the entire gestational period, every 10 $\mu g/m^3$ increase in PM10 concentration increases the risk of preterm birth (PTB) by 24% and 27%, respectively. Additionally, exposure to high concentrations of PM10 during the mid-pregnancy stage increases the risk of gestational diabetes mellitus (GDM) by 30%. For PM2.5, every 5 $\mu g/m^3$ increase in concentration raises the risk of GDM by 15% in the mid-pregnancy stage and 25% throughout the entire pregnancy. In the first three months of pregnancy, exposure to high concentrations of PM10 and PM2.5 increases the risk of having a small for gestational age (SGA) infant by 96% and 26%, respectively (153).

5.2.2 Other factors

There are reports indicating a positive correlation between the highly prevalent sexually transmitted protozoan parasite *richomonas vaginalis* and vaginal and cervical neoplasms in women, as well as prostate cancer in men (154). Infection with *T. vaginalis* significantly alters the structure of the vaginal microbiome, shifting from a lactobacilli-dominated community to one that favors the widespread transmission of bacterial vaginosis (155). This parasite releases metabolites, such as indoles, which aid in the survival of intracellular spreading bacteria like *Chlamydia trachomatis*, which has been independently associated with cancer (156). Given the positive correlation between bacterial vaginosis and precancerous lesions of the cervix (157), it is necessary to conduct research to clarify the role of the microbiome in *T. vaginalis*

-associated vaginal carcinogenesis, whether as a cofactor or a necessary factor.

Climate factors such as global warming (158) can also lead to transgenerational transmission of specific histones post-translational modifications, DNA methylation modifications, and other epigenetic marks. This article provides a concise summary of common environmental factors along with their epigenetic impacts and mechanisms of action, as presented in Table 2 and Figure 2.

5.3 The regulation of female reproductive function by epigenetics

5.3.1 Ovarian function and epigenetic regulation

PCOS, as a heterogeneous disease leading to reproductive and metabolic disorders in women, is the most common cause of infertility in women of childbearing age (171). A study (172) analyzed the DNA methylation in ovarian tissues of PCOS-like mice, and these findings suggest that PCOS can be transmitted to offspring through changes in DNA methylation in epigenetics, proposing that methylation biomarkers may serve as potential diagnostic indicators for this disease. Another study indicated that the histone deacetylase inhibitor valproic acid can reduce metabolic dysfunction in the skeletal muscles of PCOS rats by inhibiting PDK4/NLRP3 inflammasome (173). Therefore, inhibiting histone acetylation may aid in the diagnosis and treatment of PCOS. Additionally, alterations in non-coding RNAs (ncRNAs) are one of the mechanisms of PCOS (174). In recent years, numerous studies have shown significant abnormalities in the expression of ncRNAs in follicular fluid, serum, ovarian granulosa cells (175), and other tissues of women with PCOS. Therefore, analyzing the abnormal expression of ncRNAs in PCOS patients can serve as diagnostic biomarkers and play crucial roles as therapeutic targets in the treatment of PCOS.

As shown in Figure 3, premature ovarian insufficiency depicts common ovarian dysfunctions and their epigenetic mechanisms. POI refers to the ovarian function decline characterized by elevated gonadotropins and estrogen deficiency in women before the age of 40, mainly manifested as menstrual abnormalities, ultimately progressing to premature ovarian failure (POF) with varying degrees of perimenopausal symptoms (176), affecting the fertility and quality of life of women of reproductive age. N6-methyladenosine (m6A) modification can effectively regulate the epigenetics of mammalian transcriptome. A case-control study (177) measured the m6A content in the RNA of POI patients and controls. Compared with the control group, the m6A content in the granulosa cells of POI patients was significantly increased, accompanied by a significant decrease in FTO mRNA and protein expression levels. The results indicate a strong association between m6A content and the risk of POI, which may impair ovarian function and further lead to complications of POI.

5.3.2 Fertility capability

In non-disease conditions, epigenetics can influence the female reproductive environment, thus affecting fertility. The proper development and maturation of oocytes not only directly impact fertility but also relate to embryo health and the likelihood of successful pregnancy. Appropriate methylation of oocytes is crucial for coordinating gene expression patterns, driving oocyte developmental programs, and ensuring oocyte quality. N6-methyladenosine (m^6A) is the most common internal modification of mRNA (178), playing a role in oocyte maturation. Specific knockout of METTL3 in Gdf9-Cre mouse oocytes disrupts normal mRNA methylation, leading to DNA damage, follicular

TABLE 2 Environmental factors that can induce epigenetic changes affecting female reproduction.

Environmental Factors			Reproductive Impact	Involved Epigenetic Mechanism	References
Heavy Metals	Cadmium		Endometriosis, uterine fibroids, miscarriage	Activates certain cellular signals, suppresses DNA methylation, increases miR-146a expression	(159)
	Lead		Breast cancer, heart disease	Gene-specific hypomethylation	(160, 161)
Endocrine Disruptors	Bisphenol A		Breast cancer, oocyte development defects	Induces DNA hypomethylation, increases miR-146a overexpression	(162, 163)
	Phthalates		Uterine fibroids, endometrial hyperplasia	Induces DNA hypermethylation	(164, 165)
	Pesticides	DDT	Ovarian tumors, polycystic ovary syndrome, endometriosis, infertility	Affects DNA methylation	(166)
		Atrazine	Pregnancy complications, anemia, breast cancer	Gene-specific CpG methylation changes, affects gene expression, chromatin remodeling, and DNA methylation	(167)
Environmental Pollutants	PM10, PM containing heavy metals		Gestational diabetes, preterm birth, low birth weight, stillbirth, birth defects	Induces gene expression changes, rapid changes in miR-21 and miR-222 expression	(168, 169)
	Chemicals		Infertility, decreased ovarian reserve, uterine fibroids, ovarian cancer	Affects microRNA expression and regulation	(170)

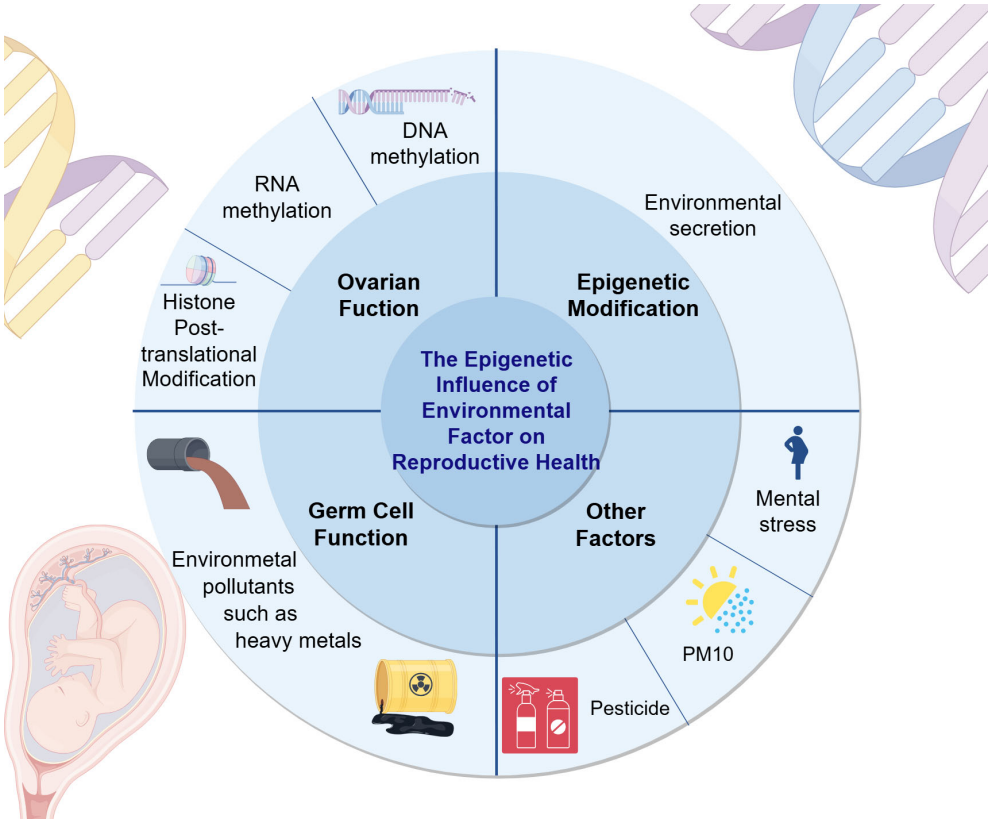

FIGURE 2
The epigenetic impact of environmental factors on reproductive health. This figure elucidates the influence of environmental factors on reproductive health from three aspects: ovarian function, epigenetic modifications, germ cell function, and other examples are given to illustrate the impact of environmental factors on reproductive health. For example, the negative effects of PM10 and pesticides on women's reproductive health. (Draw by figdraw.).

development defects, and ovulation abnormalities. It has been shown that the methyltransferase METTL3 may enhance the stability of m6A modifications on Itsn2, affecting oocyte meiosis (179, 180). Similarly, studies have found that the negative mutant H3.3-K4M specifically expressed in mouse oocytes reduces H3K4 methylation levels, leading to decreased transcriptional activity and increased DNA methylation in oocytes, disrupting oocyte development and female mouse fertility. Early embryos from H3.3-K4M oocytes exhibit developmental arrest and reduced activation of the zygotic genome (181).

Epigenetics also influences the role of hormones in the reproductive system by regulating gene expression, including

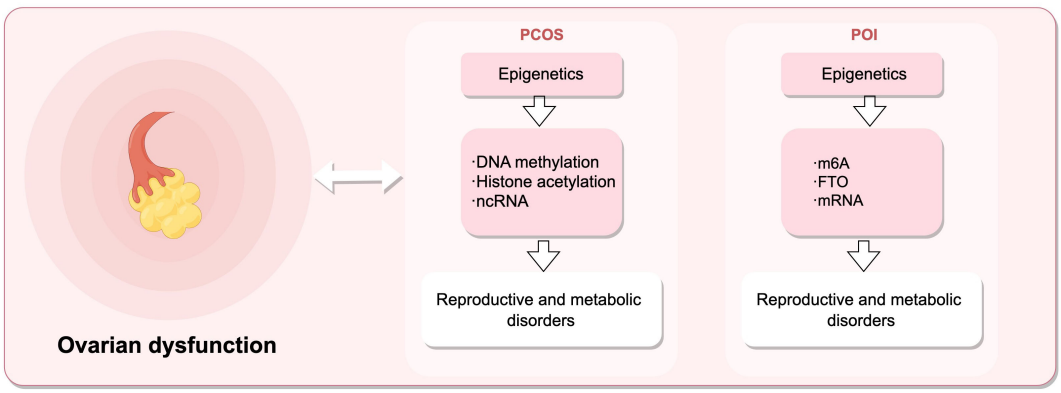

FIGURE 3
Epigenetic mechanisms regulate female reproductive capacity, leading to ovarian dysfunction. Dysregulation of DNA methylation, histones post-translational modifications, and ncRNAs can result in polycystic ovary syndrome (PCOS), while alterations in m6A, FTO, and mRNA levels contribute to premature ovarian insufficiency (POI). (Draw by figdraw.).

hormone synthesis, secretion, and receptor sensitivity, thus affecting ovarian function, the uterine environment, and cyclic changes. Research has shown that the estrogen receptor α (ERα) can recruit various coregulators (such as histone modifiers, transcription factors, or other auxiliary proteins), where histone modifiers alter the chromatin structure and organization, regulate gene accessibility and transcriptional activity, and promote chromatin opening, facilitating the binding of ERα and transcription mechanisms to estrogen response elements (ERE) (182). SMYD2 is a proven negative regulator of ERα, primarily inhibiting the activation of estrogen receptor target genes by aiding methylation of ERα protein at the K266 site (183). An epigenetic axis exists between TET2 and ERα, with endogenous TET2 occupying active enhancers and promoting proper recruitment of ERα. DNA demethylation activates enhancers to coordinate transcription programs, enhancing estrogen response (184). Besides influencing reproductive hormone receptors, epigenetics can cause changes in reproductive hormone synthesis. Endometriosis or adenomyosis cells often exhibit aberrant epigenetic programming mechanisms. Binding of NR5A1 to the proximal promoter of the CYP19A1 gene may promote demethylation of the NR5A1 gene promoter region, leading to overexpression of estrogen receptor-b (ESR2), excessive estrogen formation, abnormal interaction of estrogen with ESR2, and progesterone resistance (185). In regulating the internal hormonal environment in women, the hypothalamus serves as a key hub of the endocrine system, regulating the secretion of multiple hormones. By secreting gonadotropin-releasing hormone (GnRH), it controls the anterior pituitary secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (186). Epigenetic mechanisms regulate the expression of related hypothalamic genes, affecting the synthesis and secretion of GnRH, thereby controlling the release of FSH and LH, stimulating follicular development and ovulation, thus maintaining a normal reproductive cycle (187). A study showed that perinatal exposure to EDCs can reprogram DNA methylation and steroid hormone receptor expression through epigenetic mechanisms to regulate female fertility (188).

When embryos develop to the blastocyst stage, they can enter the uterine cavity and interact with the endometrium, a process regulated by epigenetics. During this process, the miRNA processing enzyme Dicer is upregulated, and microRNA Let-7a is downregulated, enabling the blastocyst to acquire the ability to implant in the uterus (189). During embryo implantation, DNA methylation can regulate the expression of HOXA10. Abnormal DNA methylation can downregulate HOXA10 expression (190), affecting endometrial receptivity. Targeted destruction of HOXA10 in female mice can lead to embryo death and implantation failure (191). Endometrial angiogenesis is a necessary condition for good endometrial receptivity, and a study showed that the KLF4 (Krüppel-like factor 4)-VEGFA (vascular endothelial growth factor A) positive feedback loop is regulated by epigenetics, promoting proliferation and migration of human endometrial microvascular endothelial cells (HEMECs), inhibiting apoptosis, thus enhancing endometrial receptivity (192). Abnormal expression of non-coding RNA miRNAs can lead to defects in human endometrial receptivity. Patients with recurrent implantation failure (RIF) in IVF exhibit lower mRNA levels of cell adhesion molecules, Wnt signaling components, and cell cycle pathways, including N-cadherin, H2AFX, netrin-4, and secreted frizzled-related protein-4 (193).

Endometriosis is a common benign inflammatory gynecological disease characterized by the presence and growth of endometrial-like glands and stroma outside the uterus, leading to pelvic pain and reduced fertility in reproductive-aged women, significantly impacting the quality of life of affected women 194. Epigenetic processes can regulate gene expression during endometrial development throughout the menstrual cycle through various mechanisms, altering the function and morphology of the endometrium. Epigenetic dysregulation plays an important role in the pathogenesis and pathophysiology of endometriosis, with aberrant expression of epigenetic processes found in the endometrium of affected women, holding great potential as therapeutic targets, diagnostic, and prognostic markers (195). DNA methylation is one of the most common epigenetic modifications in endometrial biology, with changes in DNA methylation occurring during different stages of the menstrual cycle (196). As a representative example, high methylation of the HOXA10 gene promoter in the endometrium of women with endometriosis leads to gene silencing, resulting in decreased levels of HOXA10 in the ectopic endometrium, potentially impairing female fertility (197). Similarly, histones post-translational modifications are associated with endometrial function. Reduced protein levels of histone deacetylase 3 (HDAC3) in the ectopic endometrium of infertile women with endometriosis may impair fertility, as HDAC3 is crucial for endometrial receptivity and decidualization. As a typical example, studies by Samartzis, E.P. et al. on the impact of Hdac3 deletion in mouse uteri on fertility demonstrated that Hdac3 deficiency can lead to aberrant transcriptional activation of two direct targets of mouse and human HDAC3, COL1A1 and COL1A2(COL1A1 and COL1A2, genes that encodes the human collagen Iαchain), resulting in implantation and decidualization abnormalities and consequent loss of fertility (198). Thus, aberrant expression of HDACs can better explain the causes and mechanisms of endometriosis. A study evaluating differential expression of microRNAs in serum cultures of severe endometriosis patients and controls found significant dysregulation of six microRNAs. This study confirmed the role of miRNAs in the pathogenesis of endometriosis, demonstrating that serum-derived eMSCs from severe endometriosis patients can induce abnormal expression of miRNAs and their target genes, leading to the development of endometriosis (199). Figure 4 illustrates the common epigenetic mechanisms and associated genes involved in endometriosis.

Pre-eclampsia is a multisystem disease broadly affecting pregnancy, annually responsible for over 60,000 maternal deaths globally and causing more than 500,000 cases of preterm birth (200). Studies have established an association between the ACVR2A gene and pre-eclampsia. Although single nucleotide polymorphisms (SNPs) related to the ACVR2A gene do not reside within its coding region, and thus do not directly alter the coding sequence of the ACVR2A protein, they may still influence

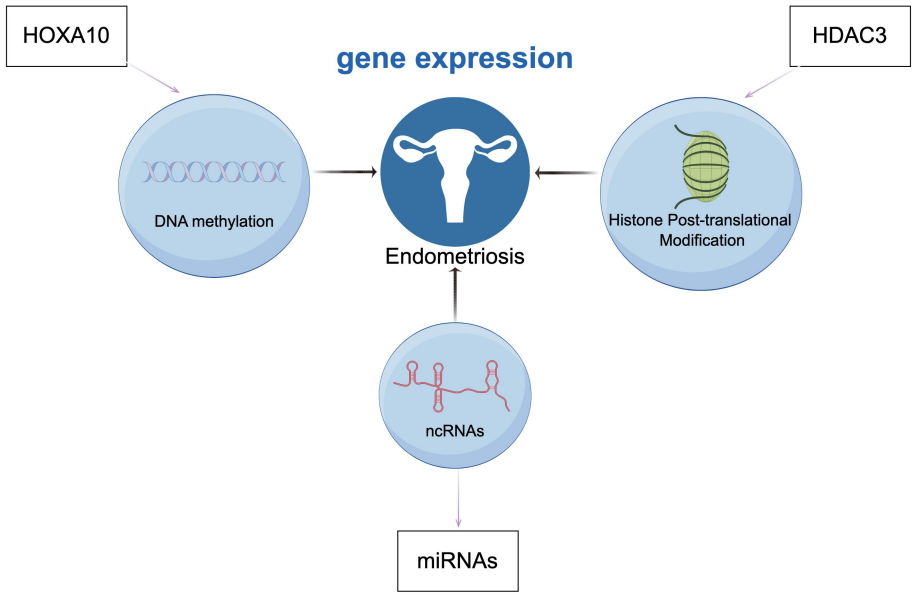

FIGURE 4
Endometriosis is associated with epigenetic mechanisms related to gene regulation, DNA methylation (HOXA10), histones post-translational modifications (HDAC3), and non-coding RNAs (ncRNAs). (Draw by figdraw.).

the transcription of the ACVR2A gene by affecting the binding of transcription factors or the regulation by microRNAs. ACVR2A is located on chromosome 2q22, and its encoded type II activin receptor binds the activin A ligand (201). In maternal tissues, activin A promotes decidualization of the endometrial stroma cells and regulates the invasion process of the trophoblast (202); in fetal tissues, it is essential for trophoblast differentiation, placental development, and the functional regulation of the trophoblast (203).

Adenomyosis, a common gynecological disorder in women of reproductive age, is characterized by the aberrant invasion of endometrial tissue into the myometrium and is closely associated with infertility (204). Studies on the expression and localization of DNMT in healthy females and those with adenomyosis revealed heightened immunoreactivity to DNMT1 and DNMT3B in ectopic endometrium (265), whereas DNMT3A staining levels significantly decreased in both eutopic and ectopic endometrium. Beyond DNA methylation, abnormal expression and localization of class I histone deacetylases (HDACs) in the endometrium have also been confirmed (205). Additionally, lower total m6A levels in the myometrium of patients with adenomyosis have been linked to differential expression of METTL3 and ZC3H13 (206), suggesting that m6A RNA methylation regulatory factors may participate in the pathogenesis of adenomyosis through aberrant expression in the endometrium.

Furthermore, improper maintenance of heritable epigenetic markers can result in aberrantly activated or suppressed signaling pathways, leading to malignant tumors in the reproductive system (207). Research has shown that epigenetic modifications like DNA methylation can influence TMB and play an indispensable role in tumor onset, as proposed by Liu B et al., where DNA methylation in the tumor microenvironment (TME) affects the expression levels of certain cervical cancer genes, further influencing the immune response in the TME, thereby inducing cervical cancer (208). Moreover, other reproductive system cancers such as endometrial cancer, ovarian cancer, and uterine fibroids can also arise due to epigenetic induction, leading to infertility and life-threatening conditions for the patient.

Uterine fibroids (also known as leiomyomas), the most common benign gynecological tumors among women of reproductive age worldwide, can cause severe anemia, pelvic pain, and infertility by affecting the overall function of the endometrium (209). Compared to normal myometrial layers, uterine fibroids exhibit altered levels of DNA methylation, increased estrogen receptor mRNA, and DNA methyltransferase levels. The activation of the PANKL gene plays a crucial role in the development of uterine fibroids by activating stem cells in the myometrial layer (210), while DNA methylation and MED12 gene mutations form a complex regulatory network affecting the expression of the PANKL gene mediated by progesterone and its receptors. Furthermore, post-translational modifications of histones in uterine fibroid tissues have changed, particularly through genomic activation-related histone acetylation; acetylated histone H3K27 is involved in regulating genes related to cell signaling, transport, angiogenesis, and extracellular matrix formation, promoting the development of uterine fibroids (211). The miR-29 family is associated with the deposition of the extracellular matrix, and the miR-3 family can regulate cyclins, also interacting with long non-coding RNAs (lncRNAs) to promote the production and deposition of the extracellular matrix, providing a physical platform for tumor cell growth, migration, and spread. Overproduction of extracellular matrix components not only increases the hardness and volume of the fibroids but also regulates the TGF-β signaling pathway through miRNA

expression, thereby affecting the normal biological functions of the uterine wall, such as menstrual bleeding, fertility, and embryo implantation. In this context, research by Włodarczyk et al. (212) further indicates that abnormal changes in TET protein and 5hmC levels may be key factors leading to the formation of uterine fibroids. Additionally, lncRNAs can directly activate the Wnt/β-catenin pathway through the estrogen receptor, promoting the proliferation of uterine fibroid cells (213).

Ovarian cancer poses a serious threat to female reproductive health, with miRNAs playing a critical role in the pathogenesis and progression of the disease (214). Studies have found that certain miRNAs are aberrantly expressed in ovarian cancer tissues, showing upregulation or downregulation, directly affecting multiple key biological processes including cell proliferation, apoptosis, metastasis, and invasion capabilities (215). For instance, the significant downregulation of miR-126-3p in ovarian cancer cells is closely related to tumor cell proliferation and invasion (216). In the treatment process, aberrant miRNA expression is also associated with resistance of ovarian cancer cells to chemotherapy drugs, potentially leading to disease relapse and progression (217). Additionally, single-gene methylation biomarkers such as RASSF1A and BRCA1 are significant factors in the onset and progression of ovarian cancer. Studies have shown that high methylation levels of BRCA1 and RASSF1A are prevalent in ovarian cancer patients, with 82% of patients exhibiting the same high methylation pattern in serum or plasma BRCA1 and RASSF1A (218). Methylation of these genes is highly correlated with clinical features of ovarian cancer such as FIGO stage, plasma CA-125 levels, and histological type (219).

In the development of cervical cancer (CC), persistent HR-HPV infection, aberrant methylation of the host cell genome and HPV genome DNA in cervical squamous epithelial cells can lead to dysfunction of various tumor suppressor genes, promoting the development of CC (220). The cervix, a critical gateway for nurturing life, is highly sensitive to estrogens, and research in an HPV transgenic mouse model has shown that estrogens and their nuclear receptors, in conjunction with HPV oncogenes, promote the onset of cervical cancer (221). Highly methylated and lowly expressed genes (Hyper-LGs) are significantly enriched in estrogen receptor pathways and the Wnt/β-catenin signaling pathway, affecting estrogen expression (222). Current research on post-translational modifications of CC proteins has focused on histone acetylation. One study showed that histone H3 and H4 acetylation is associated with the activation of HPV16 gene expression levels in CC cells, with histone acetylation levels increasing as HPV16 gene expression increases, thereby advancing the development of CC (223). Moreover, extensive research has demonstrated that lncRNAs are involved in the malignant transformation of cervical epithelial cells. For example, lncRNAMIR210HG is overexpressed in CC tissues and promotes cell proliferation and invasion through hypoxia-inducible factor-1α (HIF-1α) (224).

The endometrium, a hormone-sensitive tissue, undergoes numerous biochemical and morphological changes during the normal menstrual cycle under the control of steroid hormone levels, while abnormal exposure to estrogens can significantly increase the risk of endometrial cancer (225). DNA methylation can affect the functional changes of endometrial tissue, for instance, overall DNA methylation status and progesterone receptor levels are significantly increased during the proliferative phase and decrease at the end of the secretory phase (226). Apoptotic cell death mediated elimination of senescent cells in the functional layer of the endometrium helps maintain cellular homeostasis, thus avoiding apoptosis remains a major issue in the successful treatment of late-stage endometrial cancer. Abnormal changes in DNA methylation can lead to the deregulation of key apoptotic proteins during the development of endometrial cancer, resulting in the development of apoptosis resistance (227).

5.3.3 Specific studies

Women exposed to various chemical, biological, physical, and sociopsychological factors may experience impacts on their reproductive systems. These effects can manifest as changes in sex hormone levels, sexual dysfunction, menstrual disorders, early menopause, delayed menarche, impaired ovarian function, reduced fertility, and adverse pregnancy outcomes. During pregnancy, maternal exposure can disrupt normal fetal development, such as intrauterine growth retardation, preterm birth, birth defects, and impacts on cognitive development and immune function (228). Thakur et al.'s study showed that in areas affected by heavy metals and pesticides, the rate of spontaneous abortions is 20.6 per 1000 live births, and the rate of preterm births is 6.7 per 1000 live births, significantly higher than in non-polluted areas. Petrelli et al. (229) found that the abortion/pregnancy ratio for pesticide applicators was 0.27, compared to 0.07 for food retailers. In a multivariable logistic regression model, after adjusting for the wife's age and parents' smoking habits, the odds ratio for spontaneous abortion was 3.8 times higher compared to the control group; considering interaction effects, this ratio increased to 7.6 times. Both men and women exposed to certain pesticides face increased risks of abnormal sperm, reduced fertility, increased spontaneous abortions, male birth defects, birth defects, or fetal growth retardation (230). Logan and Chen (231) noted that exposure to bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) might reduce the rate of preterm births, thereby lowering infant mortality in malaria control. Additionally, Salazar-García et al. (232) reported that occupational exposure to DDT is associated with an increased risk of birth defects. It has been reported that levels of DDT metabolites (p, p'-DDE) are higher in 100% of infertile women. Furthermore, studies (233) have also found that pesticide exposure can lead to reduced fertility. Moreover, parents working in agriculture may increase the risk of congenital anomalies in their children, such as hemangiomas, orofacial clefts, neurological damage, and musculoskeletal defects.

In conclusion, environmental factors can induce epigenetic changes that affect female reproductive function. Epigenetics serves as a bridge and mediator, establishing a connection between environmental factors and female reproductive function. Below are specific research examples. A cross-sectional study based on 1647 American women aged 20-54 with endometriosis from 1999 to 2006 showed a positive correlation between urinary cadmium levels and the prevalence of endometriosis (234). Additionally, research exposed fruit flies to cadmium during egg

development to adulthood, then cultured the offspring in a cadmium-free environment. Under cadmium exposure, the expression of histone methylation-related genes significantly increased in the ovaries of third instar larvae and adult flies, with a marked increase in histone H3K4me3 post-translational modification and a decrease in H3K9me3 and H3K27me3 levels. These changes could be transmitted to the offspring's ovaries, leading to changes in reproductive ability (126). Female reproductive capacity begins in fetal ovaries, and early steps in folliculogenesis are sensitive to environmental factors. The quality of oocytes is closely linked to the process of folliculogenesis, with a long window of susceptibility to environmental damage. After fertilization, fertilized eggs and pre-implantation embryos undergo extensive epigenetic reprogramming. The FEDEXPO project studied potential transgenerational inheritance based on epigenetic markers in F1 offspring gametes, demonstrating that early and perinatal environments can have adverse effects on female reproductive capacity. Abnormalities in epigenetic processes and imprints may affect the health of future generations (235). Therefore, it is crucial to closely monitor and assess the toxicity and risks of environmental factors, avoid inducing epigenetic changes that may harm female reproductive capacity, and consider the adaptability of future generations.

5.4 The role of epigenetics in female reproductive health

In the field of female reproductive health, epigenetic processes such as DNA methylation and histones post-translational modifications play a pivotal role. These changes are crucial in the transition from the maternal environment of the oocyte to the embryo-driven developmental expression program (236), thereby significantly influencing the regulation of ovarian function, oocyte maturation, and embryo development.

DNA methylation, particularly in the regulation of ovarian function, plays a key role. Alterations in DNA methylation can affect the activity of specific genes, thereby regulating the levels of hormones in the ovaries, which are vital for fertility. For instance, methylation changes in genes that affect oocyte maturation and the ovulatory cycle may lead to fertility-related issues.

Taking m6A as an example, N6-methyladenosine, also known as m6A, is a widely occurring base modification on mRNA and represents the most prevalent form of RNA modification in the human body. Due to the dynamic and reversible regulation of various biological processes by m6A (237), abnormal increases or decreases in its levels can lead to the occurrence of different diseases, including many female reproductive endocrine disorders such as endometriosis, polycystic ovary syndrome, and malignancies of the female reproductive system like endometrial cancer and cervical cancer (172, 198, 238).

In the development of these diseases, DNA methylation and m6A modifications may exert their effects through pathways such as influencing the expression of specific genes or regulating hormone levels in the ovaries. These findings underscore the importance of in-depth research into the role of epigenetics in female reproductive health to better understand, prevent, and treat related diseases.

5.4.1 The epigenetic impact of environmental factors on reproductive health

Environmental factors such as diet, lifestyle, psychological stress, and exposure to chemicals play a significant role in female reproductive health. These factors can alter patterns of DNA methylation and histones post-translational modifications, affecting gene expression and thereby significantly impacting reproductive health.

For instance, long-term exposure to certain environmental pollutants, such as heavy metals, organic pollutants, and endocrine-disrupting chemicals, may lead to changes in the epigenetic markers of eggs and sperm (239, 240). These changes can affect the functionality of reproductive cells, such as the maturation of oocytes and the vitality of sperm, thereby impacting the healthy development of embryos and fertility.

Studies have shown that environmental endocrine disruptors may affect epigenetic modifications, interfering with the development of reproductive cells (241).

Increasing evidence suggests that widely prevalent environmental pollutants known as endocrine-disrupting chemicals (EDCs), such as BPA, polychlorinated biphenyls (PCBs), and phthalates, negatively impact reproductive health in animals and humans and are associated with various diseases, including infertility. EDCs can exhibit estrogen-like activity, mimicking or blocking the actions of endogenous hormones and affecting related genes, thus altering phenotypes. Hormones cause developmental changes in offspring through embryonic methylation and maintain these changes in germ cells. Evidence indicates that exposure to EDCs impacts female reproductive potential, as measured by ovarian reserve and outcomes of assisted reproductive technologies (ART) (242).

In addition to natural pregnancies, women undergoing ART are also susceptible to environmental influences. Studies have examined women who underwent ART and were closely monitored during early pregnancy to explore the association between exposure to types of environmental air pollution and the timing of miscarriages. It was found that higher NO2 exposure was associated with an increased risk of miscarriage within 30 days after a positive Human Chorionic Gonadotropin(HCG) test (243). Beyond miscarriages, immediate maternal complications related to ART are among the more apparent and recognizable issues, such as ovarian hyperstimulation syndrome (OHSS), and risks associated with ART procedures including egg retrieval, embryo transfer, and fetal reduction surgeries (244). Additionally, a range of derived issues such as risks associated with operative anesthesia, endometrial biopsy, and hysteroscopy can impact the individual and potentially affect the offspring.

Despite ART being a core technology for treating infertility in contemporary settings, a woman's reproductive potential still largely depends on the quality of her oocytes and the maternal environment that supports embryo implantation and development. The influence of the perinatal environment on the epigenetics of developing embryos has become a focal point in research into the

effects of the environment, nutrition, and assisted reproductive technologies on human development and health (245).

These findings indicate that environmental factors and lifestyle choices are crucial for maintaining reproductive health, especially in preventing and managing reproductive issues related to the environment. Therefore, understanding how environmental factors influence reproductive health through epigenetic mechanisms is essential for developing effective prevention and intervention strategies.

5.4.2 Epigenetics and female fertility

Moreover, the impact of epigenetic changes on female fertility is a significant area of research. As age increases, the quantity and quality of eggs in the ovaries gradually decline, a phenomenon that may be related to changes in the methylation patterns of specific genes. Age-related changes in the methylation patterns of genes in eggs may affect their maturation process and fertilization capability. The decline in egg quality is not only related to epigenetic changes but also involves abnormalities in mitochondrial function, mutations in nuclear DNA, shortening of telomeres, misalignment of chromosomes, and inactivation of the spindle checkpoint. These changes may lead to early pregnancy loss, neonatal death, or chromosomal aneuploidy genetic diseases such as Down syndrome (246). In terms of epigenetic regulation, METTL3 is a key factor. Knocking out METTL3 severely inhibits the maturation of oocytes, affecting the transition from oocyte to zygote (247). Although the pathogenesis of most patients with ovarian dysfunction is not entirely clear, related experiments indicate (248) that the development of oocytes in Zmettl3m/m zebrafish (a zygotic defect mutant line targeting METTL3 exons) is delayed, with most remaining at an early stage. The m6A modification evidently affects the maturation rate of follicles; the experiment also pointed out that due to the significant reduction in m6A content in the oocytes of Zmettl3m/m zebrafish, the key factors related to *in vivo* sex hormone synthesis and gonadotropin signaling cannot be normally expressed. Consequently, this leads to a decrease in the secretion of 11-ketotestosterone and 17β-estradiol in the offspring embryos, ultimately causing gamete maturation disorders and reduced fertility. Additionally, the methylation status of some genes may affect the cell cycle regulation and aging of the oocyte (249), thereby impacting its maturation and quality.

5.4.3 The role of epigenetics in pregnancy and embryonic development

During pregnancy and embryonic development, epigenetics plays a crucial role. The nutritional status of pregnant women, environmental exposure, and psychological state can influence the development of the embryo by altering its epigenetic status (250). These factors not only have a significant impact on embryonic development during pregnancy but may also have a profound effect on the long-term health of the offspring, including their metabolic status and disease susceptibility in adulthood.

Epigenetics plays a vital role at every stage of embryonic development. For example, during the early stages of embryonic development (251), the nutrition and environmental factors provided by the mother can regulate the gene expression and development of the embryo by affecting gene methylation and histones post-translational modifications. This epigenetic regulation is not only crucial for the normal development of the embryo but may also affect the long-term health and disease susceptibility of the embryo.

Moreover, the regulation of gene expression during the early stages of embryonic development is highly complex and dynamically ordered, supported by various factors, including transcription factors and epigenetic information such as chromatin accessibility, DNA methylation, and histones post-translational modifications. Transcription factors and epigenetic information (such as chromatin accessibility, DNA methylation, histones post-translational modifications, etc.) are important factors in the regulation of gene expression during early embryonic development (252, 253). These factors work together to ensure the smooth progression and transition of important biological processes at various stages of early embryonic development.

Therefore, in-depth research into the role of epigenetics in pregnancy and embryonic development is of great significance for understanding the mechanisms of normal embryonic development, predicting and preventing developmental abnormalities, and improving the health of offspring.

5.4.4 The epigenetic effects of environmental factors on offspring health

Embryonic development and infancy are two critical periods that are particularly sensitive to environmental factors. During these stages, epigenetic programming is highly susceptible to various environmental factors such as diet, temperature, environmental toxins, maternal behavioral habits, and even childhood experiences of abuse. The epigenetic modifications of imprinted genes induced by these factors may lead to poor development of multiple organs in the fetus and may increase the risk of various diseases in adulthood (254). For instance, Professor Li Jingwen from Fujian Medical University has pointed out that exposure to cadmium during pregnancy in rats has shown effects on the regulation of miRNA and DNA methylation patterns in the offspring's ovarian granulosa cells, revealing the potential for epigenetic changes to be inherited across generations. This cross-generational impact highlights the significance of environmental factors in affecting the health of offspring, especially how exposure to harmful substances during early pregnancy can have long-lasting effects on the health of the offspring.

The intrauterine environment and the early environment of newborns can provoke permanent responses in fetuses and newborns, thereby increasing their susceptibility to diseases later in life (255). Today, the mode of conception (such as *in vitro* fertilization), maternal metabolic conditions (such as malnutrition, overnutrition, diabetes), and pregnancy complications (such as preeclampsia and intrauterine growth restriction) are suspected to be negative predictors of long-term health in offspring.

Moreover, as ART become more widespread, while they compensate for familial deficiencies, they also bring subsequent health risks to offspring. During ART, significant epigenetic

reprogramming occurs, which is crucial for the normal destiny of the embryo. This epigenetic reprogramming is highly susceptible to changes in environmental conditions, such as those inherent in *in vitro* fertilization, including *in vitro* culture, nutrients, lighting, temperature, oxygen tension, embryo-maternal signaling, and the general lack of protection against exogenous elements that could destabilize this process (256). Professor Zhuan Ning Xia of Shanghai Jiao Tong University, using the Shanghai area as an example, found that exposure to pesticides affects not only the reproductive health of women undergoing ART but also adversely affects their offspring. As more infants are born, some involving embryo culture and exposure to potentially inappropriate environmental factors, this could alter the phenotype of the offspring, such as Large Offspring Syndrome in cattle (257).

Like in cattle, ART can facilitate the development of human congenital overgrowth conditions, known as Beckwith-Wiedemann syndrome (BWS), which may later lead to molar pregnancies and embryonal tumor formation. BWS is an overgrowth and embryonal tumor susceptibility disorder linked to genetic or epigenetic abnormalities in the chromosome 11p15.5 region, causing abnormal expression of parental alleles. ART disrupts the DNA methylation of imprinting sites, supporting the notion that ART could lead to imprinting disorders, including BWS. Children conceived through ART are at a 4 to 10 times higher risk of developing BWS compared to those conceived naturally (258). Furthermore, long-term exposure to inappropriate environmental factors aligns with the "DOHaD theory" – that in addition to adult lifestyle and genetic inheritance, early life environmental factors, including nutrition, influence the risk of some non-communicable diseases in adulthood, such as obesity, diabetes, and cardiovascular diseases.

These studies indicate that the environmental and behavioral choices of mothers during pregnancy not only affect their own health but can also have profound effects on the health of their offspring. Therefore, understanding how maternal environmental factors influence the health of offspring through epigenetic mechanisms is of significant importance for the prevention and management of environment-related health issues.

5.4.5 The application of epigenetics in reproductive medicine

The application of epigenetics in the field of reproductive medicine has significantly enhanced our understanding of the fertility process and provided new therapeutic strategies for treating infertility. According to a 2021 study published in "The Lancet," the current global live birth rate for ART, such as *in vitro* fertilization (IVF), is below 30%. Epigenetics plays a crucial role in the development of embryos and the trophoblast layer. The use of ART during this critical period introduces a potential window of vulnerability where epigenetic changes can occur. This susceptibility is due to the significant epigenetic reprogramming that embryos undergo during early development, which can be influenced by the ART procedures. These procedures may include culture conditions, media composition, and handling techniques, all of which could potentially impact the epigenetic landscape of developing embryos, leading to lasting effects on gene expression and function. By regulating the methylation status of specific genes in ovarian cells, the success rate of IVF can be effectively improved (259), and the yield of high-quality embryos can be increased. This method increases the chances of fertilization and healthy pregnancy by optimizing the quality of oocytes and the early development of embryos.

Furthermore, research in epigenetics provides a solid scientific foundation for future reproductive health intervention strategies. These interventions could address the issue of declining fertility due to ovarian aging as age increases (260) and may play a role in treating reproductive endocrine diseases. For example, treatments for reproductive endocrine disorders like PCOS might find new directions from an epigenetic perspective.

In the realm of female reproductive health, studies in epigenetics have not only revealed the crucial roles of DNA methylation and histones post-translational modifications in ovarian function, oocyte maturation, and embryonic development but also emphasized the significant impact of environmental factors such as diet, lifestyle, and chemical exposures on gene expression. These impacts may extend to the pregnancy process and the long-term health of offspring.

On the other hand, epigenetic changes are reversible, and understanding the pathogenesis of epigenetic diseases could allow for therapeutic interventions by targeting these modifications. In-depth studies on epigenetics help elucidate the mechanisms of human reproduction and related diseases and offer potential therapeutic approaches. For example, abnormal DNA methylation plays a crucial role in the initiation and progression of endometrial cancer, leading to the silencing of Estrogen Receptor(ER) and Progesterone Receptor(PR) expression, increased genomic DNA instability, activation of oncogenes, and inactivation of tumor suppressor genes. Yanokura et al. (261) found that the abnormal hypermethylation of the CHFR mitotic checkpoint gene in endometrial cancer tumor cells is closely related to the sensitivity to taxane-based drugs, providing new intervention targets and guidance for tumor treatment.

As basic and clinical research advances, epigenetic regulation has been found to be significant in the pathogenesis, diagnosis, treatment, and prognosis assessment of various malignancies. Recent studies have shown that DNMT inhibitors, which competitively inhibit DNMT activity and block methylation reactions, have been effective in treating endometrial cancer in clinical settings (262). Clinical trials indicate that DNA demethylating agents can reverse platinum resistance in ovarian cancer patients, suggesting that epigenetic drug therapy has clinical benefits in treating chemotherapy-resistant or recurrent advanced ovarian cancer (263).

Exploring the epigenetic molecular regulatory mechanisms of human germ cell development not only provides a theoretical basis for inquiries into issues like epigenetic reprogramming of human germ cells, the establishment of pluripotency in early embryos, directed differentiation of stem cells into gametes, and transgenerational inheritance of DNA methylation but also holds significant implications for assessing the safety of assisted reproductive technologies, determining whether reproductive disorders will be inherited by offspring or across generations, researching recurrent miscarriages and embryonic arrest, and studying diseases related to abnormal development of reproductive cells in clinical settings.

In summary, research and applications of epigenetics in the field of reproductive medicine have provided new insights and therapeutic strategies, especially showing great potential in improving IVF and embryo quality.

6 Conclusion and future perspectives

Epigenetics plays a crucial role in women's reproductive health, and environmental factors also have a potential impact on it by inducing epigenetic changes that affect female reproductive functions. External harmful environmental factors, such as PM2.5 and gaseous pollutants, can impact the female reproductive system, leading to infertility, pregnancy complications, and other issues. Additionally, harmful chemicals such as polycyclic aromatic hydrocarbons and the heavy metal cadmium can affect women's reproductive health, potentially leading to preterm birth, miscarriage, and halted embryonic development among other adverse pregnancy outcomes. Long-term exposure to these environmental pollutants may lead to changes in the epigenetic markers of eggs and sperm, affecting the function of reproductive cells and thereby impacting the development of the embryo and fertility. Furthermore, environmental endocrine disruptors may affect epigenetic modifications, thereby interfering with the development of reproductive cells. Therefore, environmental factors and lifestyle choices are crucial for maintaining reproductive health.

The revelation of the impact of epigenetics and environmental factors on women's reproductive health provides new approaches for treating infertility, pregnancy complications, and other diseases. By regulating the methylation status of specific genes in ovarian cells, the success rate of *in vitro* fertilization can be effectively increased and embryo quality improved. Meanwhile, studying how environmental factors influence reproductive health through epigenetic mechanisms is important for predicting and preventing developmental anomalies and improving offspring health.

Future research directions may include more in-depth studies on the role of epigenetics in women's reproductive health and intervening in epigenetic changes through gene editing. Technologically, researchers might utilize epigenetics to address issues such as infertility and might also reduce the risk of embryos developing other diseases in adulthood by altering methylation in ovarian cells. For instance, the preconception period, pregnancy, and prebirth are becoming recognized as sensitive periods to the epigenetic impacts of environmental factors, which may increase the risk of chronic diseases in adulthood (including neurodegenerative diseases) (264). Additionally, future research will delve deeper into how environmental factors such as diet, lifestyle, and chemical exposure affect gene expression, and how these impacts, through epigenetic mechanisms, influence the health of offspring, thereby preventing and intervening in women's reproductive health issues.

Author contributions

XY: Writing – original draft. JX: Writing – original draft. BS: Writing – original draft. RZ: Writing – review & editing. JL: Writing – original draft. YL: Writing – original draft. YM: Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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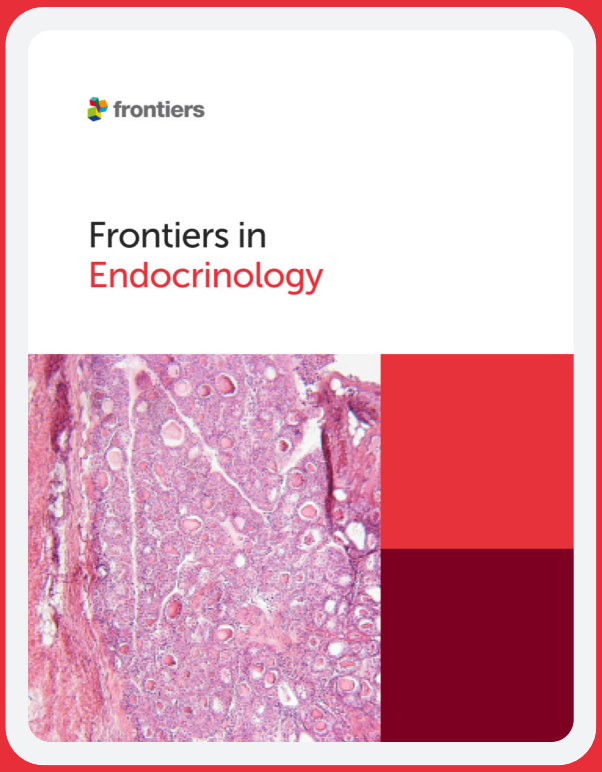

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