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Prenatal Neem Exposure Induces Lasting Testicular and Reproductive Deficits in Male Rats

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Abstract | This research was designed to illustrate the consequences of prenatal dietary administration of the neem leaves extract, *Azadirachta indica*, on the pattern of reproduction in adult male Wistar albino rats. Sixteen female and four male Wistar rats were used in the study. The experimental rats were allowed to mate. The pregnant females were divided equally into two groups: the control group (n = 8) received no treatment, and the treated group (n = 8) received 10 mL of neem leaf extract/kg diet, which was administered from day 1 of gestation (GD1) until full term. The birth weight of male offspring was recorded in the control (n=22) and the neem-treated group (n=24). After sexual maturity in males, body weight was measured, and serum testosterone, luteinizing hormone (LH), testicular malondialdehyde (MDA), and catalase (CAT) levels were estimated. Additionally, testicular histopathology and immunohistochemistry of Caspase-3 as an apoptotic marker were performed. Results showed a statistically significant decline in serum levels of testosterone and LH in the neem-treated group compared to the control rats. The testicular histoarchitecture of the neem group was markedly deteriorated. Moreover, the intensity of the immune-stained Caspase-3 area was increased in neem-administered rats as compared with the control group. It was concluded that the dietary administration of neem leaves extract hindered male fertility via alteration of male reproductive hormones manifested by lowered serum testosterone, LH, alterations in testicular architecture, as well as an increase in Caspase-3 expression.

Keywords | Neem, Testes, Testosterone, MDA, Catalase, Caspase-3

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INTRODUCTION

An endocrine disruptor (ED) is an exogenous substance or mixture that interferes with the normal functioning of the endocrine system either in the organism or its progeny (Amir *et al.*, 2021; Cargnelutti *et al.*, 2021).

Moreover, EDs alter hormonal activity via mimicking the effect of steroid hormones, followed by disrupting the homeostatic mechanisms that adjust tissue growth and development (Guarnotta *et al.*, 2022).

Exposure to EDs is through contact with these

MATERIALS AND METHODS

PLANT EXTRACT

In electric blender, the dried leaves of Neem plant were ground and homogenized with distilled water. For filtration of the homogenate, triple-folded gauze was used. The ethanolic solvent was evaporated using a rotatory vacuum evaporator for preparation of 70% dilution of the extract as described by (Mamoon-ur-Rashid *et al.*, 2011).

ANIMALS AND GROUPING

Sixteen female (6 months old, 300-320 g) and 4 male (7 months old, 300-350 g) Wistar rats were used in this research. The experimental rats were allowed to mate. The pregnant females were divided equally into two groups: the control group (n= 8) received no treatment, and the treated group (n= 8) received 10 mL of neem leaf extract/kg diet (Al-Awadhi *et al.*, 2024), administered on day 1 of gestation (GD1) until full term. The male offspring were split into a control group (n= 22) and a prenatal neem-administered group (n= 24). The birth weight (BW) of male offspring was recorded in both groups. The study was approved by the ethical committee in the Faculty of Veterinary Medicine, Suez Canal University, with Registration No. SCU-VET-AREC-R-2025032.

BLOOD SAMPLES

Under the influence of light diethyl ether anesthesia, blood samples were poised from the retro-orbital orifice on plain tubes. Collected blood was subjected to centrifugation at 3000 rpm for 15 min. The harvested sera were kept at -20° C for the estimation of testosterone and LH. Afterwards, animals were euthanized using an overdose of chloroform.

ORGAN RELATIVE WEIGHTS AND BODY WEIGHT

The final body weight for each animal in each group was obtained prior to sacrifice. The testes and epididymis were then weighed. For each experimental rat, the relative testicular and epididymal weights were estimated conferring to the subsequent formula: organ weight/body weight X 100.

SAMPLE COLLECTION

Testes and epididymis were dissected and weighed from euthanized rats. By dissecting the epididymal tail, epididymal sperms were evaluated for motility, vitality, abnormalities, and count (Aldaddou *et al.*, 2022). The left testis from each rat in each group was preserved at -80°C for evaluation of catalase activity. While the right testis was used for immunohistochemistry and histopathology.

SERUM ANALYSIS

Serum LH and testosterone were determined using commercial ELISA kits (San Diego, USA and Sunlong

compounds. EDs are liberated into the environment, leading to exposure via food and water consumption, inhalation, or dermal absorption (Zhang *et al.*, 2021). During fetal and neonatal life, transmission can also occur through the placenta and breastfeeding (Puche-Juarez *et al.*, 2023).

In males, the adverse effects of EDs on the genital system are caused by deterioration of the synthesis and/or function of steroid hormones, which are essential for the masculinization of the Wolffian ducts (Schiesaro *et al.*, 2022). Disruptions during fetal testis development can manifest as a variety of reproductive disorders (Schiesaro *et al.*, 2022). These conditions are often grouped under the “testicular dysgenesis syndrome” (TDS) hypothesis, which encompasses a spectrum of male reproductive disorders (Schiesaro *et al.*, 2022).

Castration is a procedure that alters the normal testicular function, resulting in testicular degeneration and atrophy. This can be achieved either through surgical excision or by administering chemical agents (Hess *et al.*, 2024). Castration can be achieved through various approaches, such as hormonal, mechanical, physical and chemical methods (Hess *et al.*, 2024). For this reason, the herbal sterilization of animals should be achieved to overcome the deleterious effects of surgical procedures, including tenderness, distension and inflammatory condition (Seriana *et al.*, 2019).

Neem (*Azadirachta indica*) is a natural contraceptive for both males and females (Saleem *et al.*, 2018). The antifertility criteria of neem are associated with its alteration to the spermatogenic cycle and steroidogenesis in males (Umar *et al.*, 2022). Additionally, in females, neem exerts its action by downregulating sex steroids and inhibiting ovarian caspase-3 expression, as well as deteriorating ovarian and uterine histology (Al-Awadhi *et al.*, 2024). Also, it led to the interruption of the estrous cycle and anti-implantation influence, as well as abortion (Sharma *et al.*, 2013). Furthermore, neem leaves possess numerous pharmacological properties, including antibacterial, antifertility, antihyperglycemic, antiulcer, antifungal, immunomodulatory, antimalarial, antimutagenic, anti-inflammatory, anticancer, antiviral, antioxidant, and contraceptive (Gbotolorun *et al.*, 2008; Gupta *et al.*, 2017, 2019).

Therefore, the experiment demonstrates the potential effects of neem leaf extract on male fertility, as indicated by serum testosterone and LH levels, antioxidant markers, testicular caspase-3 expression, and testicular histology in prenatally exposed male Wistar Albino rats.

Biotech, China), respectively.

TESTICULAR CATALASE ASSAY

Homogenization of frozen testes was performed in phosphate buffer (pH 7.4). The homogenates of the testes were exposed to cold centrifugation (4 °C) at 3000 rpm for 20 min. The supernatant fluids were stored at -80 °C till their analysis. according to Aebi (1984) catalase (CAT) activity and lipid peroxidation expressed as malondialdehyde (MDA) were determined using Biodiagnostic, Egypt, Kits.

HISTOPATHOLOGY

From all experimental rats, testicular specimens were collected and subjected to fixation in 10% buffered formalin, dehydrated in an ethyl alcohol gradient (70–100%). According to Bancroft (2013) tissue samples were equipped by means of standard dealings for Hematoxylin and Eosin stain.

CASPASE 3 IMMUNOHISTOCHEMISTRY

Paraffin-embedded testicular tissues were sectioned into 5 µm slices and put on positively charged slides for immunohistochemistry (IHC) of caspase-3 using specific antibodies (Cat. No. PAI-29157; Thermo Fisher Scientific, USA). The IHC procedure and quantification of reaction were conducted following the method defined by Abdelrazek *et al.* (2016).

STATISTICAL ANALYSIS

All data were exposed to statistical assessment using GraphPad Prism software (Version 8.4, San Diego, USA). A Student's t-test was used to identify the differences between the experimental groups. The obtained data were offered as means with standard error.

RESULTS

RELATIVE ORGANS AND BODY WEIGHTS

The prenatal neem-treated males demonstrated a statistical ($P \leq 0.05$) decline in birth and final body weight. Furthermore, the relative testicular and epididymal weights demonstrated a statistical ($P \leq 0.05$) lessening in exposed males to neem extract prenatally equated to the control rats (Table 1).

SEMEN CRITERIA

Semen analysis revealed significant ($P \leq 0.05$) deteriorations in progressive forward motility, vitality, morphology, as well as sperm count of prenatal neem-treated males when equated to the control-untreated group, as declared in Table 2.

SERUM HORMONES AND TESTICULAR OXIDANT MARKERS

Males subjected to neem extract prenatally demonstrated a

statistically significant ($P \leq 0.05$) decrease in serum levels of LH and testosterone compared to the control group. Additionally, testicular oxidation in prenatal neem extract-treated males was pronounced by significant elevations in testicular oxidant markers, including catalase activity and MDA, compared to the control rats (Table 3).

Table 1: Body weights and relative testicular and epididymal weights of prenatal neem-treated males.

Parameter	Control group	Prenatal neem treated group
Birth weight (g)	6.69±0.14 ^a	4.99±0.13 ^b
Final body weight (g)	379.60±8.84 ^a	298.40±20.15 ^b
Relative testicular weight (g)	3.46±0.23 ^a	1.37±0.17 ^b
Relative epididymal weight (g)	0.66 ±0.06 ^a	0.20±0.03 ^b

Values are expressed as mean ± SE. Different superscript letters (a, b) within the same row indicate significant differences between groups at $P \leq 0.05$.

Table 2: Semen criteria of prenatal neem-treated males.

Parameter	Control group	Prenatal neem treated group
Progressive motility	56.84±3.21 ^a	9.14±11 ^b
Sperm vitality (%)	66.23±12.25 ^a	20.58±10.25 ^b
Sperm abnormalities(%)	10.25±9.98 ^b	60.25±5.21 ^a
Sperm count (x10 ⁶)	29.58±7.25 ^a	10.25±5.78 ^b

Values are expressed as mean ± SE. Different superscript letters (a, b) within the same row indicate significant differences between groups at $P \leq 0.05$.

Table 3: Serum levels of LH and testosterone of prenatal neem-treated males.

Parameter	Control group	Prenatal neem treated group
LH (ng/mL)	44.04±3.66 ^a	22.22±2.59 ^b
Testosterone (ng/mL)	5.84±0.49 ^a	2.96±0.48 ^b
Catalase activity (U/g)	0.50±0.07 ^b	1.18±0.10 ^a
MDA (U/g)	0.98±0.07 ^b	2.02±0.24 ^a

Values are expressed as mean ± SE. Different superscript letters (a, b) within the same row indicate significant differences between groups at $P \leq 0.05$.

HISTOPATHOLOGY

Histological examination revealed that the control group (Figure 1A) had well-organized seminiferous tubules with well-arranged spermatogenic layers and mature spermatozoa. In contrast, the neem-treated group displayed testicular degeneration, characterized by atrophied seminiferous tubules with peritubular fibrosis, necrotic changes in spermatogenic cells and spermatozoa, depletion of mature sperm, edema in interstitial tissues, and congested blood vessels (Figure 2B).

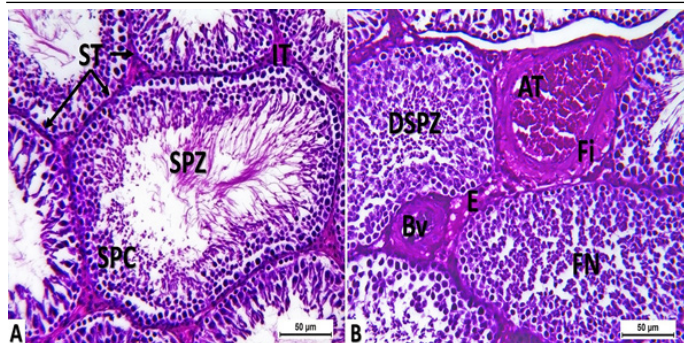


Figure 1: Representative testicular tissue in control and neem-treated *Wistar albino* rats (H&E stain, Mag = 400X, Scale bar = 50 µm). (A) The control testis section displayed normal seminiferous tubules (ST) with spermatogenic cells (SPC) and mature spermatozoa (SPZ). (B) The neem-treated rat revealed marked degenerative alterations, including atrophied seminiferous tubules (AT) with peritubular fibrosis (Fi), focal necrotic changes (FN) in spermatogenic cells and spermatozoa, depletion of spermatozoa (DSPZ), edema (E) in interstitial tissues (IT), and congested blood vessels (BV).

CASPASE-3 EXPRESSION

Control albino rat testes exhibited weak Caspase-3 immunoreactivity in some spermatogenic and interstitial cells, while neem-treated testes exhibited intense brown staining in these cells (Figure 2A, B). Quantitative analysis showed a mean integrated optical density (IOD) of 17.4 ± 0.99 in the control group and 62.6 ± 3.55 in the neem group, indicating a significant upregulation ($P < 0.0001$) of Caspase-3 expression in the neem group (Figure 2C).

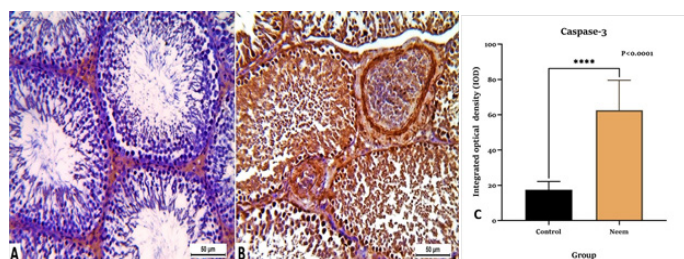


Figure 2: Immunohistochemical expression of Caspase-3 in testicular tissue of control and neem-treated *Wistar albino* rats. (A) The control group exhibited weak Caspase-3 immunoreactivity (Mag = 400X, Scale bar = 50 µm). (B) The neem-treated group showed strong positive cytoplasmic staining for Caspase-3 in all spermatogenic cells and interstitial tissue (Mag = 400X, Scale bar = 50 µm). (C) The integrated optical density (IOD) of Caspase-3 showed a significant upregulation in the neem-treated group compared to the control ($P < 0.0001$). Data are expressed as mean $\pm$ SE using the unpaired t-test.

DISCUSSION

The prenatal period is crucial for the development

of the male reproductive system. Exposure to neem (*Azadirachta indica*) extract during gestation may disrupt the hypothalamic-pituitary-gonadal (HPG) axis and affect fetal testicular development, potentially leading to long-term reproductive issues. The testicular dysgenesis syndrome hypothesis proposes that maternal exposure to ED compounds can impair masculinization and result in reproductive abnormalities, such as hypospadias, cryptorchidism, and reduced anogenital distance at the time of birth, or an underprivileged quality of semen and an amplified risk of testicular tumors later in life (Skakkebaek *et al.*, 2001). This aligns with the “fetal origins of adult disease” theory, which posits that early environmental insults can cause lasting physiological changes evident in adulthood (Barker, 2004).

The study found that male reproductive health is negatively impacted by prenatal exposure to neem leaf extract, which is consistent with these frameworks. Exposed offspring had lower birth and body weights, reduced testicular and epididymal weights, and impaired semen quality, along with decreased serum LH and testosterone levels. Increased testicular MDA and catalase activities indicate oxidative stress and dysfunction. These results align with previous findings on the long-term reproductive effects of endocrine disruption during the development of fetus (Cargnelutti *et al.*, 2021; Guarnotta *et al.*, 2022; Schiesaro *et al.*, 2022).

In this study, prenatally male neem-treated animals exhibited considerable reductions in both birth and final body weight, accompanied by significant decreases in relative testicular and epididymal weights. These findings are consistent with earlier research highlighting growth-retarding effects. Mahajan *et al.* (2015) reported diminished testicular parameters in males subjected to prenatal neem exposure, while the study of El-Dakdoky (2014) reported reduced fetal body weights in rats treated with the extract of neem bark at a dosage of 300 mg/kg. Furthermore, maternal consumption of neem seed oil or azadirachtin during pregnancy has also been associated with significant decreases in fetal weight, as indicated by Dallaqua *et al.* (2012). The more pronounced reductions observed at higher exposure levels suggest a dose-dependent effect on fetal growth restriction (El-Dakdoky, 2014).

Semen analysis in this study displayed significant reductions in progressive motility, sperm vitality, normal morphology, and overall sperm count in males prenatally exposed to neem extract. This suggests that early exposure has a lasting adverse impact on sperm quality and reproductive organs. A previous study by Mahajan *et al.* (2015) confirmed similar reproductive performance issues following prenatal neem exposure. Another study found

that high doses of neem leaf extract in adult male mice led to degeneration of seminiferous tubules and disrupted spermatogenesis, resulting in lower sperm count and motility (Mishra and Singh, 2005). Additionally, *in vitro* studies showed that the neem oil volatile fraction inhibits sperm activities in both rat and human spermatozoa (Riar *et al.*, 1990). Overall, these findings highlight neem's potent anti-fertility properties and the postnatal influences of prenatal exposure on sperm production.

Testosterone is the major hormone governing spermatogenesis, manufactured by Leydig cells under stimulation by LH. Following synthesis, testosterone functions in a paracrine manner, spreading into the seminiferous tubules to promote the maturation of spermatids, maintain the elongated spermatid adhesion, facilitate the progress of germ cells, and complete meiosis (Smith and Walker, 2014; Wistuba *et al.*, 2023). Testicular testosterone levels below the physiological threshold cause spermatogenesis to stop before meiosis is finished, which lowers the amount of sperm produced.

In the hereby study, serum testosterone and LH levels were significantly reduced in male offspring prenatally administered neem extract in their prenatal life, indicating a clear disruption HPG axis. This hormonal suppression is consistent with earlier studies demonstrating neem's anti-androgenic properties. Ekaluo *et al.* (2011) declared that intraperitoneal (IP) injection of neem leaf extract at doses between 50 to 150 mg/kg for 15 days significantly lowered testosterone concentrations in male Wistar rats. Similarly, neem leaf extracts have been demonstrated to lessen serum testosterone, FSH, and LH in a dose-dependent manner in Wistar rats (Akpantah *et al.*, 2011). Additional evidence confirms neem's ability to interfere with endocrine regulation: aqueous neem leaf extract alters hormonal balance and exerts anti-androgenic effects in various experimental models (Mugisha Byaruhanga; Puri, 2002). Kasturi *et al.* (1995) also demonstrated that high doses of *A. indica* leaf powder markedly decrease serum testosterone levels of male rats.

The histopathological findings in the hereby study, including atrophied seminiferous tubules, depleted spermatozoa, and interstitial edema, indicated significant abnormalities of the testicular tissues. These lesions are strongly associated with reduced testosterone and altered LH secretion (Abdullah and Bondagji, 2011). Testosterone is essential not only for intratesticular spermatogenesis but also for normal epididymal epithelial function, which supports sperm membrane remodeling, ion transport, and antioxidant protection (Cornwall, 2009). Therefore, Low testosterone levels observed here likely contributed to defective epididymal maturation, resulting in impaired sperm motility and reduced membrane integrity, as

reflected in the current reductions in vitality and motility (Bhattacharya *et al.*, 2023).

Previous study indicated that neem exposure reduces LH levels and causes histological changes in the anterior pituitary, impairing endocrine regulation (Akpantah *et al.*, 2011). As LH is crucial for Leydig cell activation and testosterone production, its reduction leads to decreased testosterone synthesis and impaired spermatogenesis (Aigbiremolen and Odigie, 2018). These findings support the conclusion that prenatal neem exposure has lasting endocrine-disruptive effects.

Reactive oxygen species (ROS) are produced during normal cellular metabolism and are typically neutralized by antioxidant systems. When ROS production exceeds this capacity, oxidative stress occurs, leading to lipid peroxidation and cellular injury (Walsh *et al.*, 1998). CAT helps protect against oxidative damage, while MDA is a marker of tissue injury from ROS (Koracevic *et al.*, 2001). Reduced antioxidant capacity is linked to male reproductive dysfunction and poor semen quality (Potts *et al.*, 1999).

In this study, males prenatally exposed to neem exhibited an increase in testicular MDA level and CAT activity, thus indicating elevated oxidative stress. Previous studies have linked neem preparations to ROS-induced apoptosis and damage to reproductive tissues (Girish and Shankara, 2008). High levels of ROS or low antioxidant defenses can compromise reproductive cell viability (Agarwal *et al.*, 2005). The elevated levels of MDA reflected lipid peroxidation, while elevated CAT activity suggests a compensatory antioxidant response. These findings confirm that prenatal exposure to neem extract induces significant oxidative stress, resulting in testicular damage and reduced reproductive performance in male offspring.

The histological study revealed significant degeneration in the testes of males prenatally exposed to neem. In contrast to the control group, which had normal seminiferous tubules, the neem-treated group exhibited atrophied tubules with fibrosis, focal necrosis of spermatogenic cells, and a reduction in mature spermatozoa, along with edema within the interstitial tissue. These changes reflected a severe disruption of spermatogenesis, correlating with hormonal suppression and oxidative imbalance, as detected in the same rats here.

This study suggested that oxidative stress, as indicated by elevated MDA levels, can damage membrane lipids, germ cell DNA, and tubular structure. Previous research has documented the ROS-induced degeneration of the seminiferous epithelium and disruption of germinal layers following neem exposure, thus supporting the herein findings (Seriana *et al.*, 2019). As a consequence for the

marked oxidative stress happened in testes, the levels of testosterone and LH levels were reduced in the present study likely contributed to these structural abnormalities. Testosterone plays a decisive part in preserving the integrity of the seminiferous tubules, supporting Sertoli cell function, and facilitating the progression of germ cells through meiosis (Awasthy, 2001; Srivastava and Raizada, 2007). Decreased androgen support weakens Sertoli cell cytoskeletal stability, leading to sloughing of germ cells, impaired spermiation, and tubule atrophy (Johnson, 2014). The findings support the observed reduction in spermatids and spermatozoa. Additionally, previous studies have shown that neem extracts can lead to Leydig cell degeneration, decrease tubular diameter, and significantly impair spermatogenic elements (Khillare and Shrivastav, 2003; Aigbiremolen and Odigie, 2018).

The increase in Caspase-3 immunoexpression in neem-treated testes indicates heightened apoptosis. Offspring exposed to neem showed a threefold increase in IOD values. Caspase-3, an executioner protease linked to DNA fragmentation and germ cell death, shows that intrinsic apoptotic pathways were activated. This apoptotic response was inconsistent with the elevated oxidative stress observed here in the same rats. Increased ROS and lipid peroxidation are known triggers of mitochondrial damage, leading to the cytochrome-c release and caspase activation (Schuler *et al.*, 2000). Neem extracts have also been publicized to encourage ROS-mediated apoptosis in reproductive cells through H₂O₂ accumulation, mitochondrial disruption, and activation of Caspase-9 and Caspase-3 pathways (Tripathi *et al.*, 2012; Chaube *et al.*, 2014). Thus, the herein IHC findings complement the biochemical evidence of oxidative stress, providing mechanistic confirmation that prenatal neem exposure enhances germ cell apoptosis.

Increased germ cell death also helps explain the marked decline in sperm count, motility, and morphology recorded in this study. Apoptosis of developing germ cells leads to incomplete spermatogenesis, reduced sperm output, and structural defects in surviving spermatozoa, thereby linking the Caspase-3 overexpression to the functional reproductive impairments documented (Tamura *et al.*, 2008; Alexander *et al.*, 2014).

A limitation of the study is to investigate the lifelong lasting adverse effect of neem leaves extract on spermatogenic cells and fertility that should be addressed on future study. Another limitation to the current study is that the use of a whole-leaf extract which has not analyzed for active ingredients therefore the obtained effect was not optimized due to major active ingredient or a synergistic combination. Therefore, further studies to analyze ingredients of the extract should be done in the future.

DECLARATIONS

The study protocol was approved by the ethical and research committee at the Faculty of Veterinary medicine Suez Canal University SCU-VET-AREC-R-2025032.

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NOVELTY STATEMENT

The study provides insights on the life long lasting effect of neem exposure during pregnancy on male fertility parameters.

AUTHOR'S CONTRIBUTION

HMAA: Conceptualization. HMAA, HMA, NAA, AMZ, SJM, DHE, NAE: Metshodology. DHE, SJM, HMAA: Formal analysis. HMA, NAA, AMZ, NAE: Investigation. HMAA, SJM, DHE: Resources. HMA, NAA, AMZ, SJM, DHE, NAE: Writing- original draft preparation. HMAA, DHE, SJM, NAE: Writing-review and editing. All authors have read and agreed to the published version of the manuscript.

GENERATIVE AI AND AI-ASSISTED TECHNOLOGY STATEMENT

The authors of this study affirm that no generative AI tools, such as text-to-image generators and large language models (such ChatGPT and Copilot), were used in any way during the preparation, writing, or editing of this publication.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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