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Original Article

Protective effect of ellagic acid on epididymal sperm profile changes and testicular tissue apoptosis in male rats receiving nicotine

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Abstract

Nicotine (NC) is the major alkaloid in tobacco and has been strongly associated with male reproductive toxicity, mainly through mechanisms related to oxidative stress and apoptosis. Ellagic acid (EA), a naturally occurring polyphenol with well-documented antioxidant and anti-inflammatory properties, has been reported to counteract oxidative tissue damage in various experimental models. The present study investigated the protective effects of EA against NC-induced reproductive impairment in adult male Wistar rats. To do this, a total of twenty-four adult male Wistar rats were randomly divided into four groups (n = 6), including control group, EA group (60 mg/kg, orally), NC group (1 mg/kg, intraperitoneally), and NC combined with EA group. Following 30 days of treatment, the animals were euthanized, and testicular tissues were examined for gonadosomatic index (GSI), sperm quality parameters (count, motility, viability, and morphology), and expression levels of key apoptotic genes (Bcl-2, caspase-3, p53, and BAX). The administration of NC significantly reduced GSI, sperm count, motility, viability, and normal sperm morphology compared to the control group. Also, NC significantly increased the expression of p-53, caspase-3, and BAX genes and decreased Bcl-2 gene expression. Co-treatment with EA and NC significantly attenuated these adverse effects and improved reproductive parameters and apoptotic gene expression. In conclusion, these findings suggest that EA mitigates NC-induced reproductive toxicity in rats by reducing apoptosis, indicating its potential as a therapeutic agent for NC-related reproductive disorders.

Key words: Nicotine, Ellagic acid, Reproductive toxicity, Apoptosis, Testis

Introduction

Nicotine (NC) is the major alkaloid found predominantly in tobacco plants (Nicotiana tabacum), and is widely recognized as a key mediator of the adverse health effects associated with tobacco use (Sansone et al, 2023). As a psychoactive compound, nicotine exerts systemic effects by binding to nicotinic acetylcholine receptors and triggering a cascade of physiological responses (Benowitz et al, 2020). In the

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context of reproductive health, nicotine is a well-documented toxicant that impairs male fertility (Omolaoye et al, 2022). Studies have shown that nicotine exposure, whether through smoking or other forms of tobacco consumption, disrupts spermatogenesis, reduces sperm quality, and compromises testicular function (Harlev et al, 2015; Cui et al, 2025). These effects manifest as decreased sperm count, motility, and viability, as well as increased morphological abnormalities, which collectively contribute to male infertility (Aitken, 2020). Furthermore, nicotine induces oxidative stress in testicular tissue by increasing the production of reactive oxygen species (ROS), which overwhelms endogenous antioxidant defenses and leads to lipid peroxidation and cellular damage (Oztekin et al, 2020). This oxidative imbalance disrupts the integrity of the blood-testis barrier and impairs Leydig cell function, reducing testosterone levels and exacerbating reproductive dysfunction (Ukwenya et al, 2020).

The mechanisms underlying nicotine-induced reproductive toxicity are closely tied to oxidative stress and apoptosis. Nicotine promotes excessive ROS generation, which depletes antioxidant enzymes (Das et al, 2012; Mahmoud et al, 2021). This oxidative stress damages testicular germ cells and Sertoli cells, impairing spermatogenesis and sperm maturation (Budin et al, 2017). The previous studies have demonstrated that nicotine-induced apoptosis disrupts the balance between cell survival and death in the testes, leading to germ cell loss and reduced fertility (Paccola and Miraglia, 2016; Mosadegh et al, 2017). For instance, Madi et al. reported that nicotine administration in rats significantly increased caspase-3 activity, correlating with reduced sperm quality (Madi et al, 2021). Similarly, human studies have linked smoking-related nicotine exposure to elevated oxidative stress markers and sperm DNA fragmentation (Ni et al, 2016; Ribas-Maynou et al, 2021).

It is essential to identify natural compounds capable of counteracting nicotine-induced oxidative and apoptotic damage in testicular tissue. Ellagic acid (EA) is a naturally occurring polyphenolic compound widely distributed in various plant sources, including fruits such as pomegranates, strawberries, raspberries, blackberries, and grapes, as well as in nuts, seeds, and certain medicinal plants and oak species (Gupta et al, 2020; Li et al, 2025). Chemically, EA is a dimeric derivative of gallic acid with a well-documented ability to scavenge free radicals and enhance the activity of antioxidant enzymes (Evtyugin et al, 2020). In reproductive health, EA has demonstrated protective effects against chemically induced testicular damage. Experimental studies have shown improvements in sperm quality, testicular histoarchitecture, and steroidogenesis following EA administration (Rostami et al, 2022). Antioxidant effects of EA are mediated by increasing SOD, CAT, and GPx levels while reducing MDA, thereby mitigating lipid peroxidation (Bhattacharjee et al, 2021). Additionally, EA exerts anti-apoptotic effects by modulating the expression of Bcl-2, caspase-3, and BAX, promoting cell survival in various tissues (Aslan et al, 2020; Liu et al, 2024). Although the therapeutic potential of EA has been widely studied in conditions such as diabetes, oxidative organ injury, and chemotherapy-induced toxicity (ALTamimi et al, 2021), its role in mitigating nicotine-induced reproductive dysfunction remains largely unexplored. Therefore, this study aimed to evaluate the protective effects of ellagic acid against nicotine-induced reproductive failure in male rats.

Materials and Methods

Animals

Twenty-four adult male Wistar rats (8–10 weeks old, weighing 200–250 g) were selected from the Animal Research Center of Shahid Chamran University of Ahvaz. The rats were housed in polycarbonate cages under controlled conditions (22 ± 2°C, 50–60% humidity, 12:12 h light/dark cycle) with ad libitum access to standard rodent chow and filtered water. Animals were acclimatized for one week before the experiment to minimize stress. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Shahid Chamran University of Ahvaz (Approval No. IR.SCU.REC.1404.113).

Experimental Design

Twenty-four adult male Wistar rats were randomly divided into four groups (n=6 per group), including: (1) vehicle control, receiving 0.9% normal saline (1 ml/kg, intraperitoneally [IP]); (2) ellagic acid (EA) group, receiving EA (60 mg/kg/day, orally); (3) nicotine (NC) group, receiving nicotine (1 mg/kg/day, IP); and (4) nicotine + EA group, receiving nicotine (1 mg/kg/day, IP) and EA (60 mg/kg/day, orally). Treatments were administered daily between 8:00 and 9:00 AM for 30 days. The 30-day treatment duration was selected to cover at least one complete spermatogenic cycle in rats, which lasts approximately 48–52 days, allowing sufficient time to evaluate the sub-chronic effects of nicotine and the potential protective role of ellagic acid on reproductive parameters. This duration has also been widely used in the previous studies assessing nicotine-induced reproductive toxicity and antioxidant interventions (Aprioku and Ugwu, 2015). The doses were selected based on the previous studies demonstrating reproductive toxicity for nicotine (Zhang et al, 2022) and antioxidant efficacy for EA (Alkully et al, 2025).

Sampling and gonadosomatic index (GSI) measurement

On day 31, body weights were recorded using a digital scale (CAS CA, South Korea). Then, the rats were anesthetized with a combination of ketamine (10%, 80 mg/kg, intraperitoneally) and xylazine (2%, 10 mg/kg, intraperitoneally) before sample collection (Sadeghi et al, 2023). Both testes were excised, weighed using a precision balance, and the gonadosomatic index (GSI) was calculated as: GSI = (testicular weight/body weight) × 100. The left epididymis was minced in 5 ml of Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 15 min to release sperm for analysis (Khazaeel et al, 2022). The left testis was snap-frozen in liquid nitrogen for molecular assays. All procedures were performed under sterile conditions to prevent contamination.

Assessment of sperm parameters

The epididymal sperms were analyzed for count, motility, viability, and morphology. Sperm suspensions from the left epididymis were diluted 1:20 in phosphate-buffered saline (PBS). The sperm count was determined using a Neubauer hemocytometer under a light microscope (400× magnification, Nikon, Tokyo, Japan), with counts expressed as sperm per milliliter (Sadeghi et al, 2020). Motility was assessed by classifying 200 sperm per sample as progressive, non-progressive, or immotile, expressed as a percentage of total motile sperm. Viability was evaluated using eosin-nigrosin staining, where 200 sperm were scored as live (unstained) or dead (stained). Morphology was examined by staining smears with Papanicolaou stain, assessing 200 sperm for normal versus abnormal head and tail structures under 1000× magnification. All assessments were

performed by a single trained technician to ensure consistency (Kalantari et al, 2014).

Assessment of apoptosis-related genes

Apoptosis in testicular tissue was evaluated by analyzing the expression of Bcl-2, caspase-3, p53, and BAX genes. Testicular tissues were homogenized, and total RNA was extracted for gene expression analysis. Gene expression levels were quantified using real-time quantitative polymerase chain reaction (qPCR). Primers were designed using Primer-BLAST and validated for specificity (Table 1). GAPDH was used as the housekeeping gene for normalization.

Table 1: List of primers employed for quantitative real-time RT-PCR in rat target genes

Gene name	Sequences	Product (bp)	GenBank
Caspase-3	F: CTATCCATGGAAGCAAGTCGATG R: TTGCGAGCTGACATTCCAGT	136	NM_014215
Bcl-2	F: ATCGCTCTGTGGATGACTGAGTAC R: AGAGACAGCCAGGAGAAATCAAAC	135	NM_017531
BAX	F: TGCTACAGGGTTTCATCCAG R: AAATGCAGACAGGCTTTGCAG	144	NM_017059
P-53	F: ATGCTGGTGCTGAGTATGTC R: AAATGCAGACAGGCTTTGCAG	122	NM_030989
GAPDH	F: ATGCTGGTGCTGAGTATGTC R: AGTTGTCATATTTCTCGTGG	162	NM_017008

RNA isolation and cDNA synthesis

Approximately 50 mg of snap-frozen testis was homogenized in 1 ml TRIzol (Yekta Tajhiz Azma, Tehran, Iran), followed by phase separation with chloroform and RNA precipitation with isopropanol. RNA pellets were washed with 75% ethanol, air-dried, and resuspended in RNase-free water. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), ensuring that A260/A280 ratios of 1.8–2.0. cDNA was synthesized from 1 µg of RNA using a reverse transcription kit (Takara Bio, Kusatsu, Japan) with oligo-dT primers in a 20 µl reaction volume. The reaction was incubated at 42°C for 60 min, followed by 70°C for 5 min to inactivate the enzyme. cDNA was stored at -20°C for qPCR analysis.

Real-time quantitative real-time polymerase chain reaction

Real-time qPCR was performed using a SYBR Green-based system on a StepOnePlus Real-Time PCR System. Reactions (20 µl) contained 10 µl SYBR Green Master Mix, 1 µl cDNA, 0.5 µM forward and reverse primers for Bcl-2, caspase-3, p53, BAX, and GAPDH, and nuclease-free water. Cycling conditions were initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis confirmed the amplicon specificity. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH.

Statistical Analysis

Data were analyzed using GraphPad Prism (Version 9, GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± standard deviation (SD). Differences between the groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Normality was confirmed using the Shapiro-Wilk test, and homogeneity of variances was verified with Levene's test. Statistical significance was set at $P<0.05$.

Results

Body and testicular weight

Nicotine administration significantly affected both body and testicular weights. The nicotine group exhibited a significant reduction in body weight, testicular weight, and gonadosomatic index (GSI) when compared with the control group ($P<0.01$). Notably, co-administration of EA with nicotine significantly increased these values compared to the nicotine-only group ($P<0.01$). Body and testis weights and GSI in the nicotine + EA group did not show significant differences compared to the control group ($P>0.05$; Figure 1).

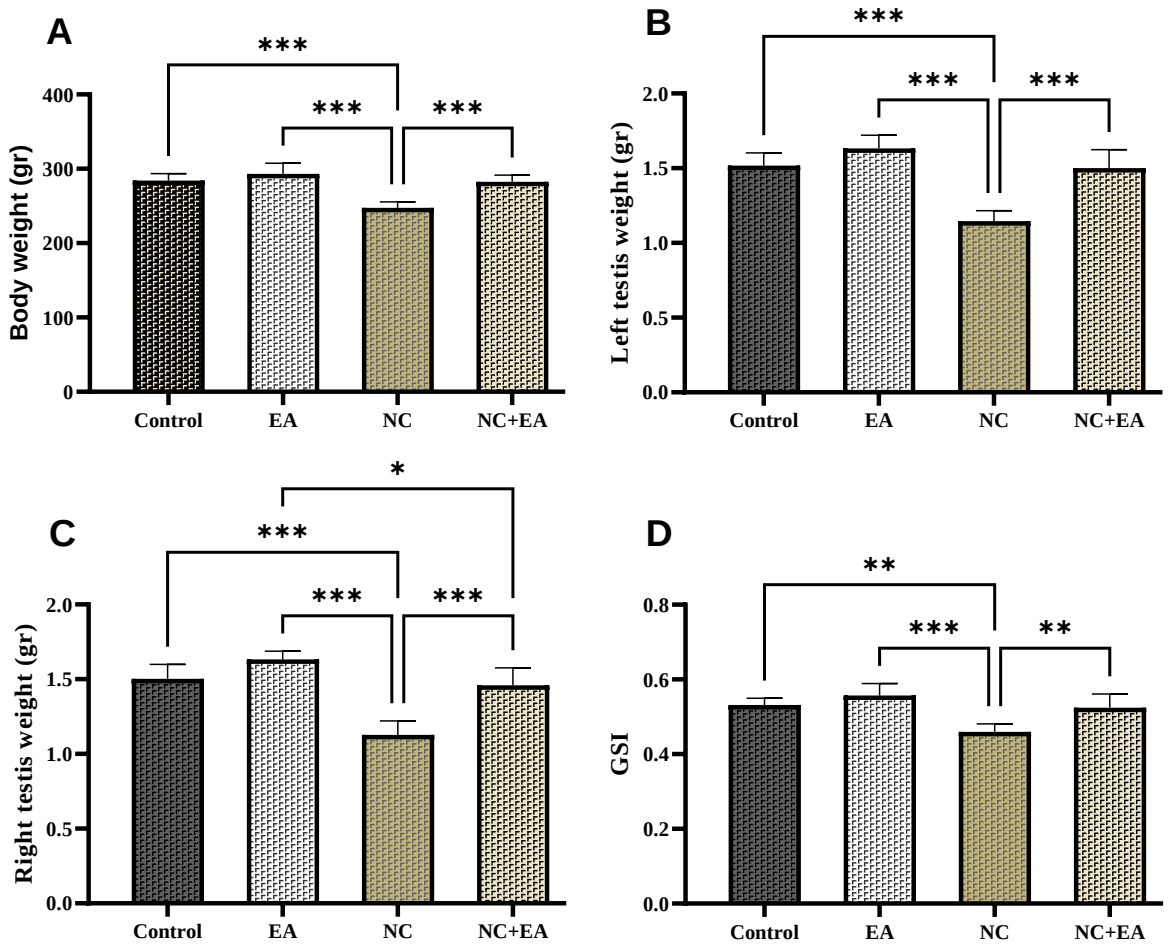

Figure 1. Effect of nicotine (NC) and ellagic acid (EA) on body weight (A), left (B) and right (C) testicular weight, and GSI (D) in rats. * P < 0.05, ** P < 0.01, *** P < 0.001 indicates statistically significant differences between groups.

Sperm parameters

The administration of nicotine significantly decreased the sperm count, motility, viability, and normal morphology compared to the control group ($P < 0.001$). The nicotine + EA group displayed significantly higher sperm count, motility, viability, and normal morphology compared to the nicotine group ($P<0.001$; Table 2; Figure 2).

Table 2: Effect of nicotine (NC) and ellagic acid (EA) on sperm parameters (count, motility, viability, and normal morphology) in rats

	Control	EA	NC	NC + EA
Mean of sperm count (10^6)	34.43±1.71	35.39±1.41	25.68±1.36***	31.14±1.51**###
Sperm motility (%)	88.50±3.27	90.33±3.44	65.67±3.77***	83.17±2.31*###
Sperm viability (%)	86.83±3.06	88.17±3.48	66.67±3.26***	79.17±2.63**###
Sperm normal morphology (%)	89.00±3.34	90.67±3.67	76.83±2.85***	83.17±2.31*###

* P<0.05, ** P<0.01, *** P<0.001 indicates statistically significant differences compared with the control group and # P<0.05, ## P<0.01, ### P<0.001 indicates statistically significant differences compared with NC group.

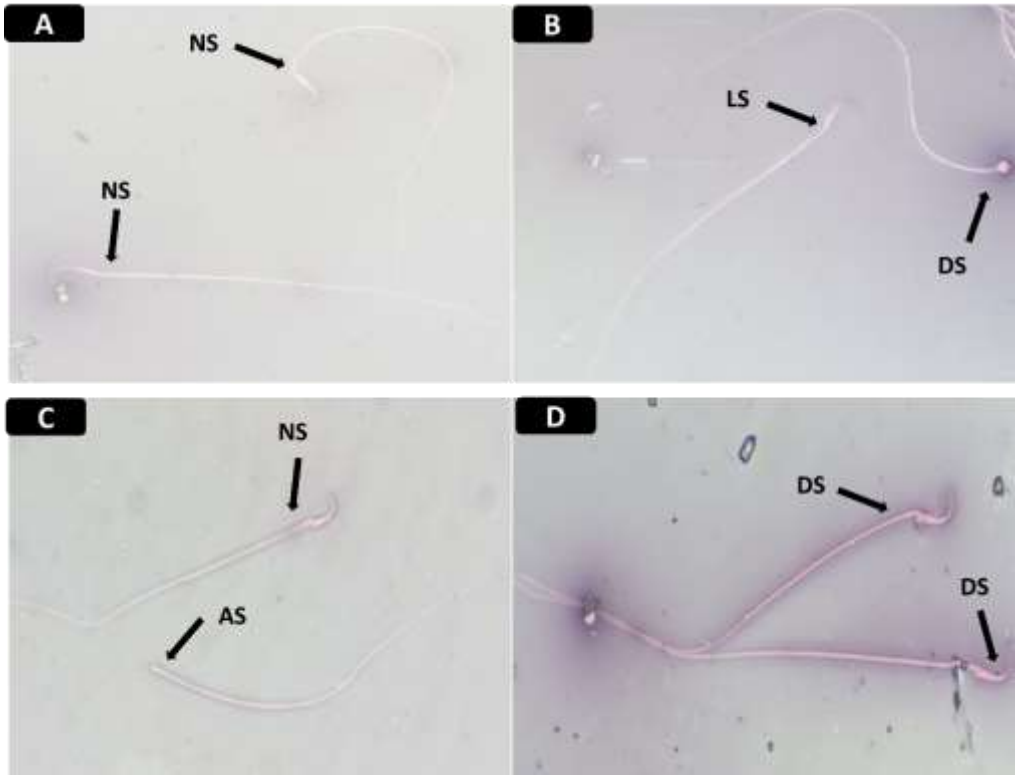

Figure 2: Photomicrograph of rat epididymal spermatozoa (A-D). NS: Normal sperm, AS: acephalic sperm (Headless sperm); DS: Dead sperm, LS: Live sperm (Eosin-Nigrosin staining; 400×).

Expression of apoptosis-related genes

Nicotine significantly altered testicular apoptosis-related gene expression. The nicotine group showed a significant increase in caspase-3, p53, and BAX expression, and a significant decrease in Bcl-2 expression compared to the control group (P<0.001). The EA group exhibited no significant changes in these genes compared to controls (P>0.05). The expression of caspase-3, p53, and BAX significantly (P<0.001) reduced in the nicotine + EA group, while Bcl-2 expression in the nicotine + EA group significantly (P<0.001) increased compared to the nicotine group (Figure 3).

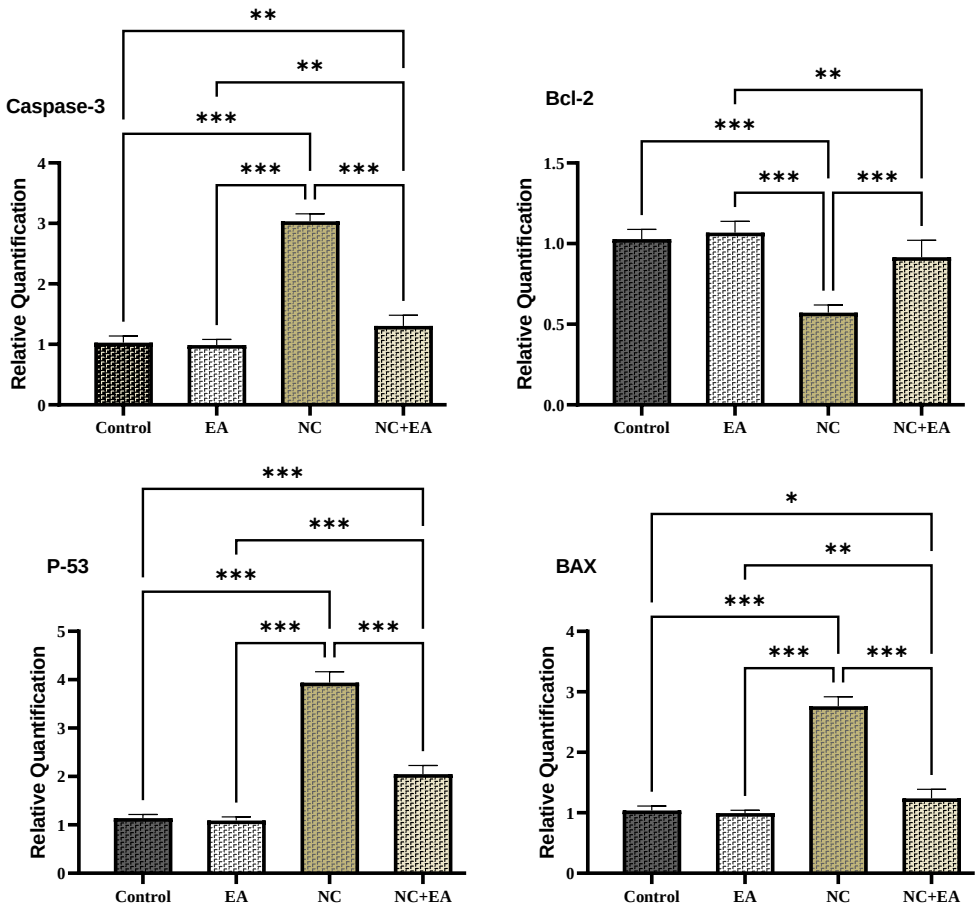

Figure 3. Effect of nicotine (NC) and ellagic acid (EA) on expression of Bcl-2, caspase-3, P-53, BAX genes. * P<0.05, ** P<0.01, *** P<0.001 indicates statistically significant differences between groups.

Discussion

The present study investigated the protective effects of ellagic acid (EA) on nicotine-induced reproductive failure in male Wistar rats, with a specific focus on its ability to alleviate apoptosis within testicular tissue. Findings revealed that nicotine administration of 1 mg/kg intraperitoneally for 30 days significantly impaired reproductive function, as evidenced by reduced gonadosomatic index (GSI), sperm count, motility, viability, and normal morphology. Moreover, molecular analyses indicated a downregulation of the anti-apoptotic protein Bcl-2, coupled with an upregulation of pro-apoptotic markers including caspase-3, p53, and BAX. Co-administration of EA at 60 mg/kg orally effectively reversed these alterations, restoring reproductive parameters to levels comparable to the control group. These findings suggest that EA confers reproductive protection, likely through mechanisms involving the suppression of apoptotic pathways within testicular tissue. Overall, EA demonstrates strong potential as a natural therapeutic candidate for alleviating nicotine-associated reproductive toxicity.

Our findings on the adverse effects of nicotine on male reproductive health are in agreement with a growing body of evidence demonstrating that tobacco-derived alkaloids exert testicular toxicity primarily through oxidative stress and apoptotic mechanisms (Cui et al, 2025). The previous studies have shown that nicotine disrupts spermatogenesis by increasing ROS production, which in turn triggers lipid

peroxidation and compromises sperm quality in animal models (Erfani et al, 2013; Oyeyipo et al, 2014). Consistent with these studies, Mosbah et al. (2015) reported that nicotine administration in male rats caused significant oxidative damage, histopathological changes in testicular tissue, and reductions in fertility indices (Mosbah et al., 2015). Similarly, our study revealed decreases in gonadosomatic index (GSI) and sperm parameters, suggesting a convergent mechanism in which nicotine impairs Leydig cell function and disrupts seminiferous tubule architecture, thereby interfering with testosterone synthesis and gametogenesis (Guo et al., 2017). However, variations across studies highlight that the effects of nicotine are not entirely uniform. Investigations utilizing lower doses have reported milder impairments, such as reduced sperm motility without significant changes in GSI. These discrepancies may be attributable to differences in dosage, duration of exposure, or strain-dependent susceptibility (Rahimi-Madiseh et al., 2020). Additionally, methodological factors such as the route of administration, intraperitoneal in our study compared to oral or inhalation routes in others, could account for discrepancies. Moreover, the absence of comorbid conditions such as diabetes, which are known to intensify nicotine's toxic effects, may further influence the observed outcomes (Kushwaha and Jena, 2014; Ansari et al, 2022).

At the molecular level, the present study demonstrates that nicotine induces significant apoptotic signaling in testicular tissue, as evidenced by the upregulation of pro-apoptotic genes (caspase-3, p53, BAX) and the downregulation of the anti-apoptotic marker Bcl-2. These findings are consistent with earlier reports linking nicotine exposure to enhanced apoptotic activity in male reproductive organs. For instance, Mosadegh et al. reported elevated expression of p-53 and caspase-3 mRNA in nicotine-treated rat testes, attributing these changes to oxidative stress-induced DNA damage that triggers mitochondrial apoptotic pathways (Mosadegh et al, 2017). Similarly, Jalili et al. reported altered apoptotic gene expression in several organs following nicotine administration, with a pronounced BAX/Bcl-2 imbalance that favored cell death (Jalili et al, 2017). Our study extends these observations by demonstrating that nicotine's pro-apoptotic effects are not only confined to germ cells but also disrupt Sertoli cell function, as evidenced by the significant reduction in Bcl-2 expression. Sertoli cells are essential for maintaining testicular microenvironmental homeostasis and supporting spermatogenesis (Zangene et al, 2025). Therefore, their impairment may accelerate spermatogenic arrest and contribute to infertility (Marinucci et al, 2020). The marked increase in p-53 expression observed in our nicotine-treated group further supports its role as a central regulator of DNA damage responses, potentially activating BAX translocation to mitochondria, thereby initiating cytochrome c release and caspase-3 activation (Azad et al, 2019). This mechanistic cascade aligns with the findings of Mohammadghasemi et al, 2021 who demonstrated that nicotine-induced oxidative stress amplifies p53-dependent apoptosis, thereby disrupting seminiferous tubule architecture and impairing sperm development.

The protective role of ellagic acid (EA) against nicotine-induced reproductive toxicity highlights a novel contribution, as limited research has investigated this polyphenol in the context of tobacco-related testicular damage. The present findings show that EA not only improves sperm parameters but also restores apoptotic gene expression, effects likely mediated by its strong free radical scavenging ability and anti-inflammatory action. By correcting the BAX/Bcl-2 imbalance and suppressing caspase-3 and p53 expression, EA appears to counteract the pro-apoptotic signaling

activated by nicotine exposure. This multifaceted protective action aligns with evidence from other toxicant models. For instance, EA has been reported to alleviate arsenic-induced testicular oxidative stress by activating the Nrf2 pathway and modulating key steroidogenic regulators such as Nfe2l2, Ppargc1a, and StAR, thereby preserving testosterone synthesis (Guvvala et al, 2019). Similarly, EA reduced testicular inflammation and apoptosis in phthalate-exposed mice by downregulating BAX and caspase-3 (Hosseinzadeh et al, 2021). Mechanistically, the efficacy of EA may involve activation of the Nrf2/HO-1 signaling axis, which enhances endogenous antioxidant defenses and attenuates nicotine-induced oxidative stress. This mechanism has also been demonstrated in neuronal cells, where EA enhanced Nrf2 nuclear translocation and increased expression of antioxidant enzymes such as superoxide dismutase (Liu et al, 2024). In the testicular context, this pathway likely preserves germ cell viability by stabilizing mitochondrial membrane potential, reducing lipid peroxidation, and preventing DNA strand breaks that would otherwise activate p53-dependent apoptosis (Naraki et al, 2022; Mehrzadi et al, 2018). Beyond oxidative stress regulation, EA may also exert epigenetic effects, such as inhibiting histone deacetylases (HDACs), thereby suppressing transcription of pro-apoptotic genes (Golmei et al, 2024).

In conclusion, the present study demonstrates that nicotine exposure impairs male reproductive function through oxidative stress and apoptosis, leading to significant declines in sperm quality, gonadosomatic index, and testicular integrity. Importantly, ellagic acid (EA) supplementation effectively counteracted these adverse effects by restoring antioxidant balance, modulating apoptotic gene expression, and preserving testicular architecture. The dual mechanisms of attenuating oxidative damage and suppressing apoptosis signaling by ellagic acid highlight its potential as a natural therapeutic agent against nicotine-induced reproductive toxicity. While these findings provide compelling experimental evidence, further investigations, including clinical trials, are warranted to validate the protective efficacy of EA and establish its translational relevance for managing tobacco-related male infertility.

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Conflict of interest

The authors declare no competing interests.

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اثر حفاظتی الاژیک اسید بر تغییرات پروفایل اسپرم اپیدیدیمی و آپوپتوز بافت بیضه در موش‌های بزرگ آزمایشگاهی نر دریافت کننده نیکوتین

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چکیده

نیکوتین اصلی‌ترین آلکالوئید موجود در تنباکواست و به طور قوی با سمیت تولید مثلی در مردان مرتبط می‌باشد که این ارتباط عمدتاً از طریق مکانیسم‌های مرتبط با استرس اکسیداتیو و آپوپتوز است. الاژیک اسید، یک پلی‌فنول طبیعی با خواص آنتی‌اکسیدانی و ضدالتهابی است که در مدل‌های مختلف تجربی برای مقابله با آسیب اکسیداتیو بافتی گزارش شده است. مطالعه حاضر، اثرات محافظتی الاژیک اسید را در برابر اختلال تولیدمثلی ناشی از نیکوتین در موش‌های بزرگ آزمایشگاهی نر بررسی کرد. بیست و چهار موش بزرگ آزمایشگاهی نر بالغ ویستار به طور تصادفی به چهار گروه (n=۶) تقسیم شدند که شامل گروه کنترل، الاژیک اسید (۶۰ میلی‌گرم/کیلوگرم، خوراکی)، نیکوتین (۱ میلی‌گرم/ کیلوگرم، داخل صفاقی) و نیکوتین همراه با الاژیک اسید بود. پس از ۳۰ روز تیمار، بافت‌های بیضه از نظر شاخص گنادوسوماتیک (GSI)، پارامترهای کیفیت اسپرم (تعداد، تحرک، زنده‌مانی و مورفولوژی) و سطوح بیان ژن‌های کلیدی آپوپتوز (Bcl-2، کاسپاز-۳، p53 و BAX) مورد بررسی قرار گرفت. تجویز نیکوتین به طور معنی‌داری شاخص گنادوسوماتیک، تعداد اسپرم، تحرک، زنده‌مانی و مورفولوژی طبیعی اسپرم را در مقایسه با گروه کنترل کاهش داد. همچنین نیکوتین به طور معنی‌داری بیان ژن‌های p53، کاسپاز-۳ و BAX را افزایش و بیان ژن Bcl-2 را کاهش داد. تیمار همراه با الاژیک اسید و نیکوتین به طور معنی‌داری این اثرات نامطلوب را کاهش داد و پارامترهای تولیدمثلی و بیان ژن‌های آپوپتوزی را بهبود داد. در نتیجه، این یافته‌ها نشان می‌دهند که الاژیک اسید با کاهش آپوپتوز، سمیت تولیدمثلی ناشی از نیکوتین را در موش‌ها کاهش می‌دهد که نشان‌دهنده پتانسیل آن به عنوان یک عامل درمانی برای اختلالات تولیدمثلی مرتبط با نیکوتین است.

کلمات کلیدی: نیکوتین، الاژیک اسید، سمیت تولیدمثلی، آپوپتوز، بیضه

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