



## Protective effect of gallic acid on nicotine-induced testicular toxicity in mice

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### Abstract

**Background and purpose:** Nicotine is an alkaloid found in many nutrients and tobacco that can cause infertility in men. Gallic acid is a powerful antioxidant that possesses antimutagenic and anticancer activities. This study aimed to determine the potential protective effect of gallic acid against nicotine-induced testicular toxicity in male mice.

**Experimental approach:** In this *in vivo* study, forty-eight mice were equally divided into eight groups intraperitoneally receiving normal saline (control), nicotine (0.6 mg/kg), gallic acid (5, 10, and 15 mg/kg), and gallic acid (5, 10, and 15 mg/kg) plus nicotine. Nicotine was injected intraperitoneally for 14 days and gallic acid was administered concomitantly with nicotine and continued for 7 days later. Then, body and testicular weights, the sperm parameters (viability, number, motility, and morphology of sperm), and testicular histology were evaluated. Also, serum levels of nitric oxide, total antioxidant, superoxide dismutase, malondialdehyde, and testosterone were measured.

**Findings/Results:** The results showed that the administration of nicotine significantly reduced testis and body weight, sperm count, viability, normal morphology and motility, seminiferous tubules diameter, testosterone levels, serum levels of total antioxidants, and superoxide dismutase compared to the control group ( $P < 0.05$ ). It also significantly increased the level of nitric oxide and malondialdehyde ( $P < 0.05$ ). Increasing the dose of gallic acid along with nicotine significantly increased body weight, sperm count, viability, normal morphology and motility, the diameter of seminiferous, testosterone concentration, total antioxidant levels ( $P < 0.05$ ). This combination also significantly decreased malondialdehyde and nitric oxide levels compared to the nicotine-receiving group ( $P < 0.05$ ).

**Conclusion and implications:** Gallic acid had a protective effect on nicotine-induced testicular toxicity in mice. It can neutralize the harmful effect of nicotine on male fertility in smokers.

**Keywords:** Gallic acid; Infertility; Nicotine; Testis.

### INTRODUCTION

Nicotine is a toxic alkaloid extracted from the tobacco plant. It is quickly absorbed by the body (1) and easily crosses the cell membrane and reacts with some intracellular components such as tubulin protein in the cytoplasm of dividing cells such as germ cells and finally disrupts cell division (2). This toxic alkaloid can cause several functional disorders in the reproductive system (3). There are clear

evidences that nicotine given in laboratory animals causes testicular degeneration and sperm abnormalities (4). Also, nicotine causes disruption in the spermatogenesis process and testicular atrophy in laboratory mice (5). Nicotine consumption in the form of cigarettes or other products, such as hookah and pipe, is very widespread in human societies (6).

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Gallic acid (also known as 3,4,5-trihydroxy benzoic acid) is a type of phenolic acid. The chemical formula of gallic acid is  $C_6H_2(OH)_3COOH$ . It is found both free and as part of hydrolyzable tannins. Several beneficial effects are reported for gallic acid, including antioxidant, anti-inflammatory, and antineoplastic properties. This compound has been reported to have therapeutic activities in gastrointestinal, neuropsychological, metabolic, and cardiovascular disorders. This compound is in the form of red crystals and it is found in various plants such as oak, tea, sumac, grape seed, and apple (7). Gallic acid is an important part of traditional medicine in some countries. It prevents cell damage induced by oxidative stress (8).

Given the importance of nicotine effects on the body and also due to the antioxidant and anti-inflammatory effects of gallic acid, this study aimed to investigate the possible protective effect of gallic acid on nicotine-induced testicular toxicity in mice.

## MATERIALS AND METHODS

### *Chemicals*

In this *in vivo* experimental study, eosin Y stain, disodium hydrogen phosphate, glacial acetic acid, absolute ethanol, hematoxyline, mercuric oxide, potassium alum, and xylene were purchased from Merck (Germany). Nicotine and gallic acid were purchased from Sigma Corporation (Germany). All experiments were performed in triplicates and repeated independently at least three times. The study was approved by the Ethical Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Ethic Code: IR.KUMS.REC.1398.165).

### *Animals and experimental protocol*

Forty-eight adult male BALB/c mice (initial weight: 27-30 g) were purchased from Pasteur Institute of Iran (Tehran, I.R. Iran), and maintained at a constant temperature of  $24 \pm 1$  °C with a relative humidity of 55% and standard 12/12-h light/dark cycles. They had free access to standard food and tap water for a week before the experiment (9). Then, the animals were randomly divided into eight groups (n = 8) and

received normal saline, nicotine, and gallic acid as follows: group 1, normal saline (control); group 2, nicotine (0.6 mg/kg); group 3, gallic acid (5 mg/kg); group 4, gallic acid (10 mg/kg); group 5, gallic acid (20 mg/kg); group 6, nicotine (0.6 mg/kg) + gallic acid (5 mg/kg); group 7, nicotine (0.6 mg/kg) + gallic acid (10 mg/kg); group 8, nicotine (0.6 mg/kg) + gallic acid (20 mg/kg).

Normal saline solution and nicotine were injected intraperitoneally once a day for 14 consecutive days. Gallic acid was injected intraperitoneally for 21 consecutive days. At the end of the experiment, the body weight of each mouse was measured, the animals were anesthetized, and a blood sample was collected.

### *Biochemical analysis*

The blood sample was collected from the left ventricle of the heart using a syringe, 24 h after the last injection. The blood samples were incubated for 30 min, and then centrifuged at 2500 rpm for 30 min. Finally, the serum was removed and kept at -70 °C. The serum levels of testosterone and nitric oxide (NO) were determined by ELISA and Griess tests using nitric oxide assay kit (Abcam, Cambridge, USA). The Zell Bio kit (Germany) was used to measure the superoxide dismutase (SOD) and malondialdehyde (MDA). Randox kit (UK) was used to determine the total antioxidant (TAC) levels.

### *Spermatological studies*

The mice were sacrificed by cervical dislocation following treatments. The cauda epididymis was excised, minced, and incubated in 1.5 mL pre-warmed phosphate-buffered saline (PBS) pH = 7.4 at 37 °C. The spermatozoa were allowed to disperse into the buffer (20 min). Then the suspension was gently shaken to homogenize, and sperm parameters were analyzed according to criteria of the World Health Organization (fifth edition) (10) with some modifications under a light microscope at a magnification of  $\times 400$ .

To assess the percentage of motile sperm, the suspension was prepared by pipetting. Then well-mixed semen (40  $\mu$ L) was placed on a clean glass slide for film recording with a video microscope. Randomly ten fields from each

slide were recorded with a camera for sperm motility assessment *via* analyzing the recorded films. Sperm motility was characterized as (i) zero (rapid progressive motility), grade 1 (progressive motility), grade 2 (non-progressive motility), and grade 3 (immotile) (10).

Viability was assessed by eosin Y staining (5% in saline). Forty  $\mu$ L sperm suspension was transferred on a glass slide, mixed with 10  $\mu$ L eosin and observed under a light microscope ( $\times$  400 magnification). After treatment, live sperms remained unstained; whereas, those that showed any pink or red coloration were classified as dead. At least 200 sperm were counted from each sample in ten fields of vision randomly, and the percentage of live sperms was recorded (10).

For sperm counting, 500  $\mu$ L of the sperm suspension was diluted with 500  $\mu$ L of formaldehyde fixative (10% formalin in PBS). Ten  $\mu$ L of the sample was transferred into a hemocytometer and the settled sperms were counted and evaluated per 250 small squares of a hemocytometer (10). For the evaluation of sperm morphology, 20  $\mu$ L of sperm suspension was placed on the microscope slides and swiped. Slides were dried and stained with Papanicolaou. After drying, they were observed in the light microscopy at 400  $\times$ . A differential count of 200 spermatozoa per slide was performed and observed changes for the head, middle piece, and tail. The results were expressed as a percentage of normal cells (10).

### Histopathological studies

Testes were removed, weighed, fixed, and processed for histological observations. Briefly, after the fixation of the testis at room temperature for 24 h, the alcohol dehydration

process was performed automatically in the tissue processing machine. Subsequently, samples were embedded in paraffin using routine procedures. Paraffin-embedded tissues were cut with microtome at a thickness of 3  $\mu$ m and then deparaffinized in xylene and rehydrated through graded ethanol. They were stained with the hematoxylin-eosin protocol. The stained slides were observed in a research microscope and images were captured.

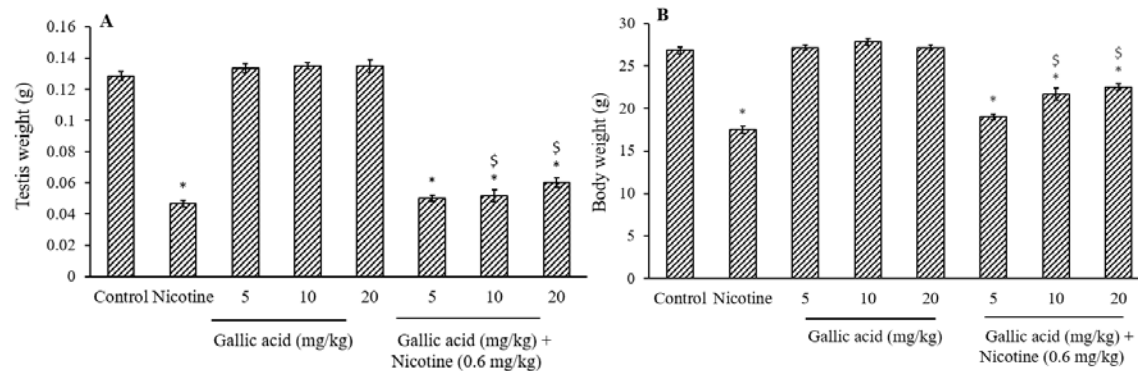
### Statistical analyses

Data were analyzed using SPSS 16.0 software and shown as mean  $\pm$  SD. Analysis of variance, the Duncan test for multiple comparisons, and the Mann-Whitney analysis were performed. Differences were considered not significant when  $P > 0.05$ .

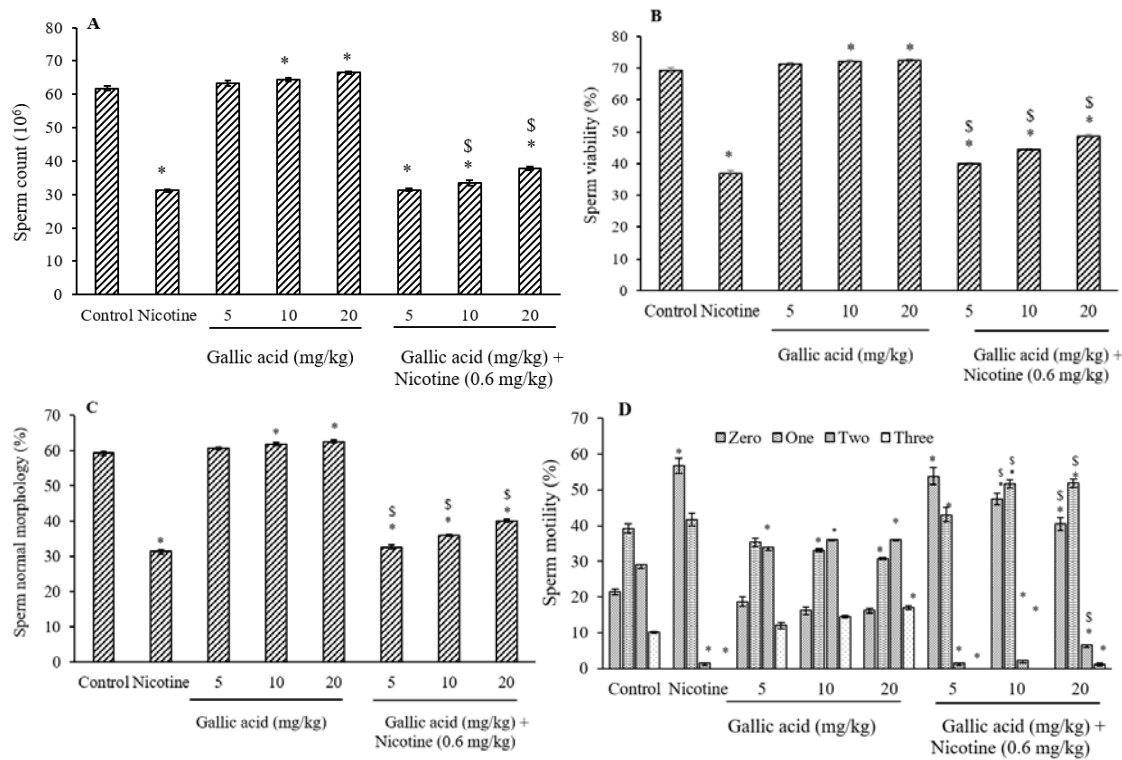
## RESULTS

### Weight of testis and body

There was a significant reduction in testicular weight in the nicotine receiving group compared to the control group ( $P < 0.05$ ). In nicotine plus gallic acid receiving groups (10 and 20 mg/kg), a significant increase in testicular weight was observed compared to the nicotine receiving group ( $P < 0.05$ ). Also, there was a significant reduction in mouse body weight in the nicotine receiving group compared to the control group. In nicotine plus gallic acid receiving groups (10 and 20 mg/kg), a significant increase in body weight was observed compared to the nicotine receiving group ( $P < 0.05$ ). There was no significant difference in the body and testis weight in gallic acid receiving groups compared to the control ( $P < 0.05$ ) (Fig. 1).



**Fig. 1.** The effect of treatment with nicotine and/or gallic acid on the weight of (A) testis and (B) body of mice. \* $P < 0.05$  indicate significant differences compared to the control group and  $^{\S}P < 0.05$  versus nicotine group.



**Fig. 2.** The effect of treatment with nicotine and/or gallic acid on sperm (A) counts, (B) viability, (C) normal morphology, and (D) motility. \* $P < 0.05$  indicate significant differences compared to the control group and <sup>s</sup> $P < 0.05$  versus nicotine group.

### Sperm parameters

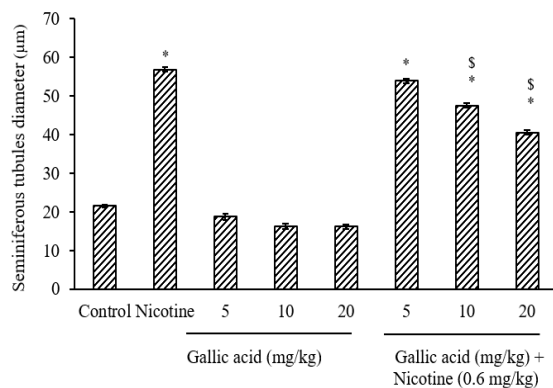
The results of the sperm count test showed that there was a significant reduction in the nicotine receiving group compared to the control group ( $P < 0.05$ ). Also, there is a significant increase in sperm count in the gallic acid (20 mg/kg) receiving group compared to the control group ( $P < 0.05$ ). In gallic acid (10 and 20 mg/kg) plus nicotine receiving groups, there was a significant increase in sperm count compared to the nicotine receiving group ( $P < 0.05$ ) (Fig. 2A).

The results of the sperm viability test showed that there was a significant reduction in the nicotine receiving group compared to the control group ( $P < 0.05$ ). Also, a significant increase was observed in the groups receiving gallic acid (10 and 20 mg/kg) in sperm viability compared to the control group ( $P < 0.05$ ). In groups receiving nicotine plus gallic acid, there was a significant improvement in sperm viability compared to the nicotine receiving group ( $P < 0.05$ ) (Fig. 2B).

The results of sperm morphology evaluation showed that there was a significant reduction in normal sperm in the nicotine receiving group

compared to the control group ( $P < 0.05$ ). Also, a significant improvement in sperm normality was observed in gallic acid (10 and 20 mg/kg) receiving groups compared to the control group ( $P < 0.05$ ). In gallic acid (10 and 20 mg/kg) plus nicotine receiving groups, a significant increase in sperm normality was observed compared to the nicotine group ( $P < 0.05$ ) (Fig. 2C).

The results of the sperm-motility study showed that there was a significant increase in immobility in the nicotine receiving group compared to the control group ( $P < 0.05$ ). A significant reduction in sperm motility was seen in nicotine plus gallic acid (5, 10, and 20 mg/kg) receiving groups ( $P < 0.05$ ). There was also a significant reduction in local, fast, and progressive mobility in the nicotine receiving group compared to the control group ( $P < 0.05$ ). Conversely, a significant increase in rapid and progressive mobility was observed in nicotine plus gallic acid (5, 10, and 20 mg/kg) receiving groups compared to nicotine receiving groups ( $P < 0.05$ ). Also, in gallic acid (5, 10, and 20 mg/kg) receiving groups, there was a significant increase in local, rapid, and progressive forward mobility compared to the control group ( $P < 0.05$ ) (Fig. 2D).



**Fig. 3.** The effect of treatment with nicotine and/or gallic acid on seminiferous diameter. \* $P < 0.05$  indicate significant differences compared to the control group and  $^{\S}P < 0.05$  versus nicotine group.

### Seminiferous tubules diameter

There was a significant decrease in the diameter of the seminiferous tubules in the nicotine receiving group compared to the control group ( $P < 0.05$ ) and a significant increase was observed in nicotine plus gallic acid receiving groups (10 and 20 mg/kg) compared to the nicotine receiving group ( $P < 0.05$ ). In groups receiving gallic acid, no significant changes were observed in the diameter of the seminiferous tubes compared to the control group ( $P < 0.05$ ) (Fig. 3).

### Serum testosterone, NO, SOD, TAC, and MDA levels

There was a significant reduction in serum testosterone levels in the nicotine receiving group compared to the control group ( $P < 0.05$ ). There was also a significant increase in testosterone levels in nicotine plus gallic acid (10 and 20 mg/kg) receiving groups compared to the nicotine receiving group ( $P < 0.05$ ). In gallic acid (5, 10, and 20 mg/kg) receiving groups, a significant change in testosterone levels was not observed compared to the control group ( $P > 0.05$ ) (Fig. 4A).

The results of the NO assay showed that there was a significant increase in NO levels in the nicotine receiving group compared to the control group ( $P < 0.05$ ). Gallic acid did not significantly reduce NO levels ( $P < 0.05$ ). Also, in nicotine plus gallic acid (5, 10, and 20 mg/kg) receiving groups, a significant

decrease in serum NO levels was observed compared to the nicotine receiving group ( $P < 0.05$ ) (Fig. 4B).

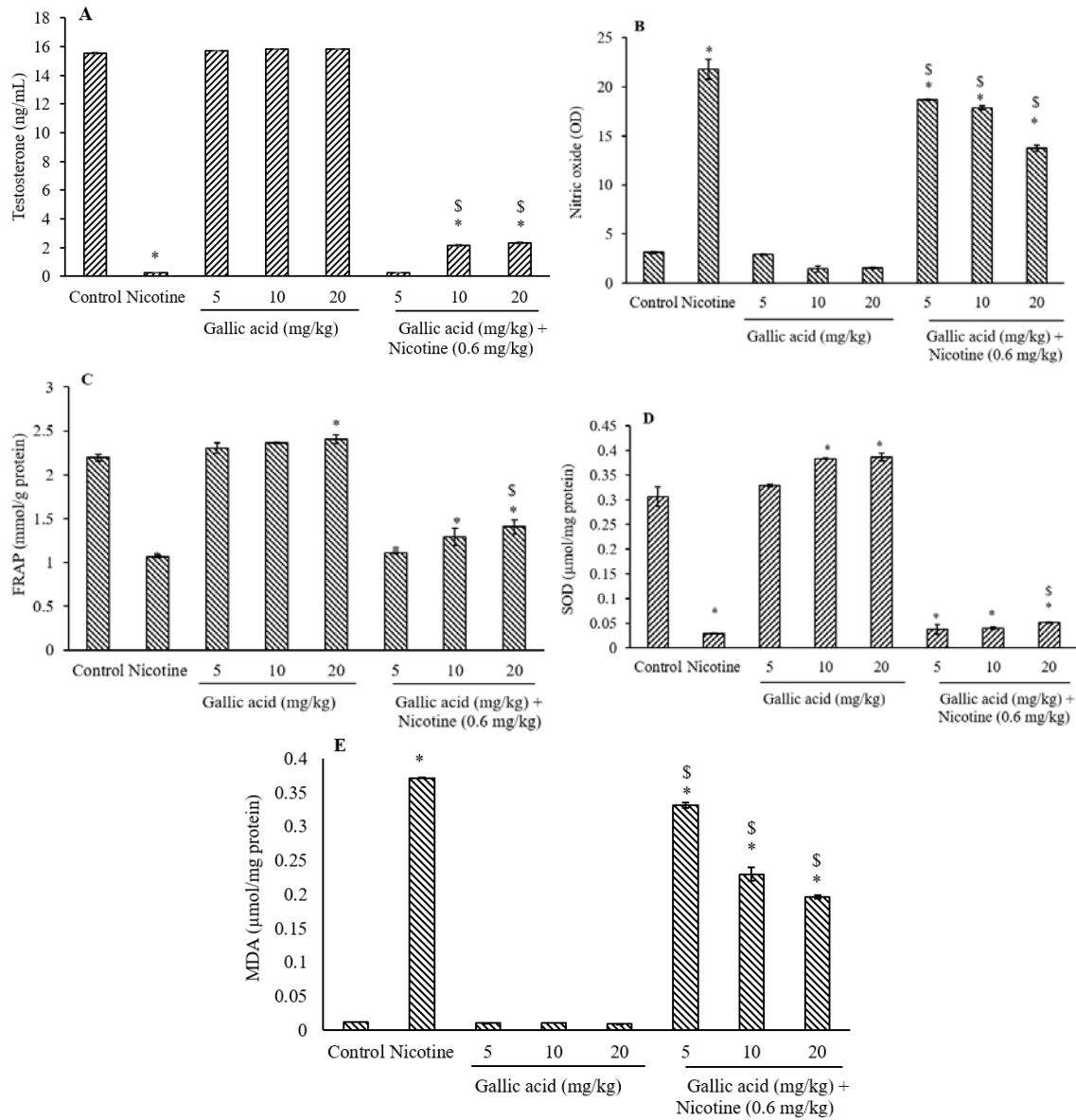
There was a significant reduction in TAC in the nicotine receiving group compared to the control group ( $P < 0.05$ ). Besides, a significant increase in TAC levels was observed in the gallic acid (20 mg/kg) receiving group compared to the control group ( $P < 0.05$ ). In the nicotine plus gallic acid (20 mg/kg) receiving group, there was a significant increase in serum TAC levels compared to the nicotine receiving group ( $P < 0.05$ ) (Fig. 4C).

There was a significant reduction in the SOD levels in the nicotine receiving group compared to the control group ( $P < 0.05$ ). There was also a significant increase in serum SOD levels in gallic acid (10 and 20 mg/kg) receiving groups compared to the control group ( $P < 0.05$ ). In nicotine plus gallic acid (20 mg/kg) receiving group a significant increase in serum SOD levels was observed compared to the nicotine receiving group ( $P < 0.05$ ) (Fig. 4D).

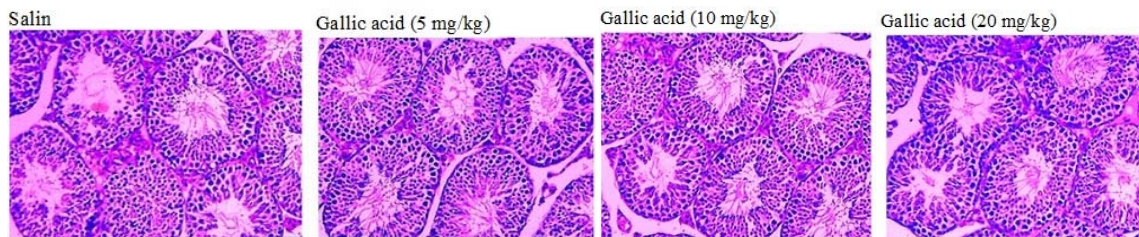
There was a significant increase in serum levels of MDA in the nicotine receiving group compared to the control group ( $P < 0.05$ ). Also, nicotine plus gallic acid (5, 10, and 20 mg/kg) receiving groups showed a significant decrease in serum MDA levels compared to the nicotine receiving group ( $P < 0.05$ ). In the gallic acid receiving groups, there was no significant difference in serum levels of MDA compared to the control group ( $P < 0.05$ ) (Fig. 4E).

### Hematoxylin and eosin staining

The microscopic structure of the seminiferous tubules of the mouse testicular tissue in nicotine and nicotine plus gallic acid receiving groups showed a decrease in germ cells as well as an epithelial height at different stages of spermatogenesis (green arrow) and an increase in lumen diameter of seminiferous. Asterisks indicate a very severe decrease in sperm in the lumen of the seminiferous tubules and irregularity in the marginal structure of the seminiferous tubules and the disruption of the structure of the seminiferous tubules (Figs. 5 and 6).

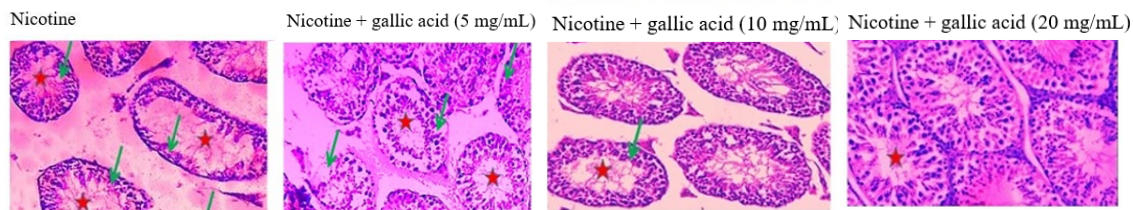


**Fig. 4.** The effect of treatment with nicotine and/or gallic acid on (A) testosterone, (B) nitric oxide, (C) FRAP, (D) SOD, and (E) MDA levels. \* $P < 0.05$  indicate significant differences compared to the control group and  $^{\S}P < 0.05$  versus nicotine group. FRAP, Ferric reducing the ability of plasma; MDA, malondialdehyde; SOD, superoxide dismutase.



**Fig. 5.** Spermatogenic tube at different stages of spermatogenesis of the control and treated groups with different doses of gallic acid (5, 10, 20 mg/kg); 100% magnification; hematoxylin and eosin staining.





**Fig. 6.** Spermatogenic tube at different stages of spermatogenesis of nicotine and nicotine plus gallic acid receiving groups (5, 10, 20 mg/kg); 100% magnification; hematoxylin and eosin staining.

## DISCUSSION

The present study investigated the possible protective effect of gallic acid on nicotine-induced disturbances such as sperm parameters, testicular tissue, NO, SOD, MDA, TAC, and testosterone levels in serum. Weight measurement showed that nicotine decreased testicular weight and body weight significantly ( $P < 0.05$ ). Testicular and body weight were increased significantly in the nicotine plus gallic acid receiving groups compared to the nicotine received group ( $P < 0.05$ ).

Nicotine is a toxic alkaloid extracted from tobacco plants (11) and comprising about 1.5% by weight in commercial cigarette tobacco and about 95% of the total alkaloid content (12). There are clear pieces of evidence that nicotine administration in animal models had some negative effects on testis and spermatogenesis. A study about the morphological and histological effects of nicotine on rat testicular showed that irregularity was observed in sperm-producing tubules with a decrease in spermatogenesis as well as the spread of luminous seminiferous tubules and gradual reduction of Leydig cells (13).

Gallic acid has antioxidant, antifungal, and antiviral properties. It is an important part of traditional medicine in some countries. The ester of gallic acid reduces cellular damage induction by oxidative stress (14). Since nicotine affects testicular tissue and the body's metabolism, it may lead to weight loss in the testicles and body. Cell proliferation and growth have been affected the weight, and protein synthesis is essential for these two events (cell proliferation and growth) (15). Nicotine reduces the levels of protein synthesis enzymes and this can be one of the reasons for weight loss by the injection of nicotine. Given that nicotine can cause the production of free

radicals, it can be argued that the production of free radicals in testicular cells, which are very sensitive, can lead to their loss and testicular weight loss. It has also been shown that reactive oxygen species production reduces the quantity and quality of semen and reduces sperm viability by increasing cell permeability. Also, one of the possible causes of testicular atrophy is unknown factors that interfere with spermatogenesis, and as a result, with the decrease in the number of sex cells, testicular weight loss occurs (16).

The results of the investigation on the number counting, motility, normal morphology, and viability of sperm and testosterone levels showed nicotine decreased all of them. In the nicotine plus gallic acid receiving group a significant increase in the number, motility, normal morphology and viability of sperm and testosterone levels was observed compared to the nicotine receiving group ( $P < 0.05$ ). Nicotine may directly affect the hormonal control of spermatogenesis in seminal glands and seminiferous tubules. Such changes in spermatogenic capacity in men may lead to infertility or mutated spermatogenesis reproduction (17). Morphological abnormalities decreased motility, and sperm density has been observed in men who smoke. Also, changes in the secretion of androgen and gonadotropin hormones have been observed in male smokers (18). Studies have shown that sperm density in smokers is greatly reduced. In general, sperm density in smokers is 22% lower than in non-smokers (19). Also, sperm motility in smokers is reduced by 20% (20). Another study reported that normal sperm morphology in smokers has decreased by 17% (21). Also, exposure to nicotine, cigarette smoke, and pollutants can lead to testicular atrophy, cessation of spermatogenesis, and changes in sperm

morphology (22). Undoubtedly, antioxidants are effective in improving sperm quality and the secretion of sex hormones and the action of their receptors. Therefore, because of the high content of antioxidants in plant products and drugs of plant origin, they are of particular importance in the treatment of infertility.

Antioxidants stabilize the blood-testicular barrier and increase sperm DNA protection from oxidative stress caused by free radical activity during puberty and migration (23).

The results of a study showed that administration of gallic acid in male Wistar rats improved body and testicular weight, increased number, motility and viability of sperms, decreased abnormal sperm morphology and prevented seminiferous tube atrophy and necrosis of the germinal and Sertoli cells after treatment with sodium valproate (24).

The present study showed that the administration of gallic acid in high doses improved sperm parameters in nicotine-treated mice. Results of a study showed that administration of this compound for 35 days in mice treated with cyclophosphamide significantly improved sperm parameters (number, motility, normal morphology, and viability), decreased apoptosis in germ cells and improved body weight (25).

The results of the seminiferous tube diameter measurement showed that nicotine decreased the diameter of seminal vesicles. On the other hand, in the nicotine plus gallic acid receiving groups, the diameter of the seminiferous tubules increased significantly compared to the nicotine receiving group ( $P < 0.05$ ). Also, the results of NO measurement showed that nicotine increased its levels in plasma. Despite the reduction in NO levels in the gallic acid receiving group, these differences were not statistically significant ( $P > 0.05$ ). Also, in the nicotine plus gallic acid receiving groups, there was a significant decrease in the serum NO levels compared to the nicotine receiving group ( $P < 0.05$ ).

NO is a free radical and plays several functions in various physiological and pathological processes, especially in vascular pathophysiology. NO levels are changed by cigarette smoking (26). The exact mechanism of adverse endothelial dysfunction associated

with cigarette smoking and changes in NO production has not yet been fully understood (27-29). Despite the beneficial effects of NO on vascular traction, immune defense modification, etc., overproduction of NO has toxic effects due to the production of active nitrogen species and protein nitrosylation (30).

Administration of gallic acid in mice treated with doxorubicin decreased NO levels in testicular tissue and epididymis (31). Also, gallic acid and curcumin reduce the growth and proliferation of MDA-MB-231 cells and decrease NO levels (32). Suppression of NO production reduces oxidative stress in animal models (33). Gallic acid has been shown to inhibit inducible nitric oxide synthase (iNOS) expression and NO production in lipopolysaccharide-activated macrophages (34). Numerous studies have shown that NO is associated with acrosome and tail in human sperm, which appear to play a role in sperm motility and acrosomal function, which are important factors in the reproductive process (35). High levels of NO destroy sperm mitochondrial membranes, thereby releasing cytochrome C, inducing caspase activity, and stimulating the apoptosis process (36). The results of a study showed that the administration of gallic acid significantly reduced NO levels in mice who suffered from spinal cord injury (37). Intraperitoneal administration of gallic acid in rats treated with gentamicin significantly decreased NO (38). Also, the administration of gallic acid in Wistar male rats treated with bisphenol-A significantly decreased serum NO levels (39).

Data showed that nicotine increased serum levels of MDA. Also, the nicotine plus gallic acid receiving groups showed a significant decrease in serum MDA levels compared to the nicotine receiving group ( $P < 0.05$ ). In the gallic acid receiving groups with different doses, there was no significant difference in the levels of MDA compared to the control group ( $P < 0.05$ ).

MDA is the final product of lipid peroxidation (40). In general, oxidative stress in the cellular environment leads to formation of unstable reactive lipid peroxides. The decomposition of unstable peroxides and derived from unsaturated fatty acids leads to the formation of MDA (41). On the other hand,



gallic acid reduces the effects of oxidative stress on cells by increasing total thiol and glutathione enzyme activity and decreasing MDA (42). The results of a study showed that the administration of gallic acid for 14 days in mice treated with cyclophosphamide significantly reduced serum levels of MDA (43). Also, the results of another study indicated that the administration of gallic acid for 16 weeks decreased serum levels of MDA in the heart tissue of the hypertension mouse model (44). A study by Turk *et al.* showed that administration of gallic acid in male mice treated with cisplatin reduced MDA levels and increased glutathione and catalase levels (45).

### CONCLUSION

The results of the present study showed that nicotine was able to induce toxicity in adult testicular tissue, and gallic acid was able to reduce or counteract the adverse effects of nicotine at high doses. Effects of nicotine on the testicular tissue of adult mice are mainly due to the induction of oxidative stress. Therefore, the use of gallic acid as a natural anti-oxidant can be a good strategy to reduce free radicals and prevent damage to the reproductive system. It can be suggested that gallic acid can be used as an anti-oxidant against nicotine-induced testicular toxicity.

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### Conflict of interest statement

The authors declared no conflict of interest in this study.

### Author's contributions

I. Rashidi designed the experiments, supervised the research and co-authored the manuscript. M. Pazhouhi and S. Davoudi performed all of the experiments and A. Ghanbari and M. Zhaleh edited the manuscript and analyzed the data. C. Jalili wrote the manuscript. The manuscript has been read and approved by all the authors, and each author believed that the manuscript represents honest work.

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