Effects of gallic acid on rat testopathy following morphine administration: an experimental study

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ABSTRACT

Introduction: Morphine (MOR) as a psychoactive agent in opium family causes free radicals accumulation which leads to failure in spermatogenesis. Gallic acid (GA), a polyphenolic acid, is found in various plants with antioxidant, anti-fungal, anti-viral, and anti-allergic activities. The purpose of this study was to evaluate the effects of GA against MOR-induced damage to the reproductive parameter of rats.

Methods: Sixty-four male Wistar rats (8 weeks, 220-250 g) were categorized into 8 groups by random (n=8/each); normal control and MOR control groups; GA groups (5, 10, 20 mg/kg) and MOR + GA groups (5, 10, 20 mg/kg). Treatments were administered intraperitoneally (i.p), daily for 4 weeks. The sperm parameters, spermatogenesis index (SI), total antioxidant capacity, testosterone level, and seminiferous tube diameter (STD) were assessed.

Results: All sperm parameters reduced significantly in MOR control group than to the normal control group (P< 0.01). All parameters were significantly improved in GA and GA + MOR treatment groups compared to the MOR control group (P< 0.01).

Conclusion: MOR caused a detrimental effect on male reproductive parameters. Also, no significant modifications were observed in all doses of GA treatments in comparison with the normal control group. GA compensates the toxic effect of MOR on reproductive parameters. Hence, GA administration is beneficial in MOR users.

Implication for health policy/practice/research/medical education:
Gallic acid can significantly reduce the morphine related damages in male reproductive parameters through antioxidant properties. Hence, its consumption is recommended in morphine consumers.

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neurons, glial cells, hepatocytes, immune and epithelial cells (10). This material increases the level of free radicals by pro-oxidant activity. Lipid peroxidation increases the rate of anti-oxidant enzyme deformation. This process leads to accumulation of ROS. ROS has harmful effects on cell membrane and DNA (11). However, the cellular defense system is not fully capable of preventing free radical damage, especially under acute conditions. Thus, the use of antioxidant agents helps to reduce the damage and prevent the disease (12). Trihydroxybenzoic acid, or gallic acid (GA), is a phenolic acid with a molar mass of 170.12 g/mol. This compound is found in the form of red crystals and exists in various plants including oak, tea, Rhus (sumac), Vitis (grape) seeds and apples (13). The salts and esters of GA are known as Gallate. GA has an important place in traditional medicine due to the antioxidant, antifungal and antiviral properties. The biochemical group of ester available in GA prevents cellular damage by a reduction in cellular oxidative stress (14). GA (3,4,5-trihydroxy benzoic acid) also is a polyphenolic compound found in grape juice and green tea containing free acids, esters, catechin derivatives and hydrolyzable tannins (15). In addition, this compound has free radical scavenging activity. A study by Punithavathi et al has shown that GA has antihyperglycemic, antioxidant, peroxidative and anti-lipid properties in streptozotocin-induced diabetic Wistar rats (16). Therefore, the goal of this study was to evaluate the protective effects of GA on male reproductive dysfunction of rats induced by MOR administration. This preliminary study is the first investigation in protective effect of GA on male reproductive parameters induced by MOR in rats.

Materials and Methods

Animals

Sixty-four male Wistar rats (200-250 g, 8 weeks) were purchased from the Pasteur Institute (Tehran, Iran) and transferred to the animal house in medical school. The animals were kept under standard conditions including 12:12 hours light/dark cycle and 22 ± 2°C, in special cages and on a straw bed. Water and food (plate and treated municipal water) were freely available to all animals.

Study groups and treatment of animals

Sixty–four male Wistar rats were randomly divided into 8 groups (8 rats). The first group (normal control group) received normal saline equivalent to the amount of experimental groups. Animals in the second group (MOR control group) were administered by injection as follow; 10 mg/kg once daily in the first day, on the days of 2–28, the MOR doses increased to 20 mg/kg/d at 9.45 AM. Third to fifth groups (GA administration groups) in which each animal received 5, 10 and 20 mg/kg of GA daily (On days 1–28) for 4 weeks at 10 AM, respectively. In sixth to eighth groups (MOR + GA administration groups) each animal was treated with MOR in order to induce reproductive parameters damage at 9.45 AM, then they received 5, 10 and 20 mg/kg of GA i.p daily (On days 1–28) at 10 AM, respectively (7,13). All experimental treatments were applied i.p.

Animals dissection and sampling

After 28 days of treatment all rats were anesthetized by intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (10 mg/kg). Blood was taken from the heart without thoracotomy. The samples were kept in a 37°C incubator for 20 minutes and then centrifuged at 255 g in 15 minutes. The blood serum was isolated and part of the serum was kept at -70°C for measuring total antioxidant capacity, nitric oxide, and testosterone levels. The tail epididymis was isolated and placed in DMEMF12/FBS 5% culture medium. The testicles were removed from the abdominal cavity and fixed in a 10% formalin solution (7).

Sperm cells collection

The caudal part of the epididymis was used for sperm cell parameters assessments and the left testis was applied for histological evaluations. Both cauda epididymis was crushed and conserved in a warmed petri dish (37°C) containing 10 ml Hank’s balanced salt solution. After 15 minutes, the cauda was removed and the suspension was slightly shaken to be normalized and observed by a light microscope (400×) (10).

Sperm viability

The eosin staining was used to identify the living sperm from the dead cells based on the absorption of stain by dead cells and their disposals by the membrane of living cells. At the end of the given time, about 20 μL of the medium containing semen fluid was collected from each dish, and mixed with an equal volume (about 20 μL) of eosin stain solution. 2-5 minutes later, a part of the mixture was poured onto a neobar slide culture. The living sperm cells lacked color and dead sperm cells became pink. The prepared slide culture was examined with a magnification of 40×. At least 100 sperm cells were calculated from each random sample from the 10 fields of imagining and the percentage of live sperm cell was documented (8).

Sperm progressive motility

Four degrees of sperm motility were calculated according to WHO guidelines (2010), class A: progressive motility. The progressive motility of the sperm cells was examined by an optical microscope with a magnification of 40 in 10 fields of view. For this purpose, 50 μL of the semen liquid culture medium was prepared and placed on a slide culture cleaned with alcohol. Then, the slide culture was placed there and examined by the microscope. Sperm cell counting was performed through a cell count device (100 sperm cells were counted in each sample). The count was...
Repeated in all experimental and control groups (12).

**Sperm count**
To analyze the sperm number, 400 μL of the sperm suspension was diluted through formaldehyde fixative (Sigma; USA). Approximately, 15 μL of which was placed on a hemocytometer which was located into a Petri dish with dampened filter paper and allowed to stand for 10 minutes. The stable sperms were counted and assessed per 250 small squares of the hemocytometer using a ×40 objective. The amount of sperm per mm³ equated the number of sperm counted (10).

**Sperm cells morphology**
This parameter was assessed through sperm smears extracted from the right cauda epididymis. An aliquot of the sample was used to make the smears to appraise the malformations in the spermatozoa. Eosin/nigrosine stain was used to guesstimate the normal spermatozoa morphology. A drop of eosin was added to the suspension and was mixed slightly. The slides were then observed underneath a light microscope at 400× magnification. A total of 400 spermatozoa was studied on the respective slide (4000 cells in each group) for assessment of the head and tail irregularities (11).

**Semeniferous tube diameter (STD) measurement**
After testes fixation, the routine tissue processing was applied including dehydration, clearing and embedding. 5 μm sections were ready and hematoxylin and eosin staining was applied. Of each block, more than 30 segments were organized. A Motic camera and software (Moticam 2000; Spain) were hired for STD measurement (7).

The ferric reducing ability of plasma (FRAP) method
This method was hired to measure serum total antioxidant capacity. In this technique, the ability of the plasma to reinstate ferric ions was measured. This process requires a large amount of FeIII. A blue stain was formed when the FeIII-TPTZ in acidic pH is changed to the FeII and absorption at the maximum wavelength of 600 nm. Total antioxidant capacity values were strategized using the standard curve with diverse concentrations of iron sulfate (12).

**Testosterone measurement**
The collected blood was centrifuged (5000 g) in 23°C for 15 min. The serum samples were then kept in a freezer (-18°C). The serum testosterone level was examined through ELISA (Abcam 108666, USA) technique (9).

**Spermatogenesis index (SI)**
Via Johnson’s score, the testis tubules were evaluated according to the SI value. Based on the previous score, the grades of 1 to 10 (no cell to complete spermatogenesis) were given to each cross-section tubule (12).

**Statistical analysis**
The Kolmogorov–Smirnov test was conducted to confirm data compliance of the normal distribution. One-way analysis of variance (ANOVA) and Tukey post hoc test were used for statistical analysis and determination of the differences. Statistical Package for the Social Sciences 16 (SPSS Inc., Chicago, IL) was used for data analysis, and the results were expressed as mean ± standard error, and P < 0.05 was considered as significant.

**Results**
Sperm viability, progressive motility, count and normal morphology
MOR caused a significant reduction in viability, progressive motility, count and normal morphology compared to the normal control group (P < 0.01). No significant variations were detected in GA groups compared to the normal control group (P > 0.05). Also, these parameters in whole GA and MOR + GA groups increased significantly compared to the MOR control group P < 0.01 (Table 1).

Seminiferous tube diameter
A significant reduction was seen in STD by MOR consumption compared to the normal control group (P < 0.01). No significant alterations were observed in

| Groups           | Mean of sperm count (10⁶) | Sperm progressive motility (%) | Sperm viability (%) | Normal sperm morphology (%) |
|------------------|---------------------------|-------------------------------|---------------------|----------------------------|
| Normal control   | 85.37±1.06                | 19.6±11.32                    | 75.53±11.16         | 81.37±2.46                 |
| MOR control      | 31.16±4.05                | 1.8±11.40                    | 40.83±3.05          | 36.53±3.1                 |
| GA 5 mg/kg       | 85.75±2.43                | 21.12±1.21                   | 76.62±2.09          | 81.75±4.09                |
| GA 10 mg/kg      | 86.12±5.07                | 20.87±1.74                   | 76.55±5.04          | 81.37±4.54                |
| GA 20 mg/kg      | 85.25±4.07                | 20.50±0.67                   | 75.05±1.07          | 82.52±2.19                |
| GA + MOR 5 mg/kg | 49.50±2.50                | 7.12±1.33                    | 54.35±5.08          | 55.12±1.49                |
| GA + MOR 10 mg/kg| 51.36±3.17                | 8.87±1.53                    | 56.37±2.09          | 57.12±4.02                |
| GA + MOR 20 mg/kg| 55.25±4.23                | 8.75±1.10                    | 57.21±3.51          | 59.87±4.55                |

Data are presented as mean ± SEM, n = 8 for each group. * P < 0.01 compared to the normal control group. † P < 0.01 compared to MOR control group. ¶ P < 0.01 compared to the MOR control group. MOR: morphine; GA: gallic acid.

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GA groups compared to the normal control group (P > 0.05). STD in all GA and MOR + GA groups enhanced significantly compared to the MOR control group (P < 0.001) (Figure 1). The histological changes of seminiferous tubes are shown in Figure 2.

**Total antioxidant capacity**

Serum level of total antioxidant capacity was reduced significantly in the MOR control group compared to normal control group (P < 0.001). A significant increase caused by GA consumption was observed in total antioxidant capacity levels in treated rats of whole doses compared to the MOR control groups (P < 0.01). The total antioxidant capacity level was improved significantly in all MOR + GA groups compared to the MOR control group (P < 0.001) (Figure 3).

**Testosterone**

MOR caused a significant decrease in testosterone hormone level compared to normal control group (P < 0.01). No significant alterations were detected in GA groups compared to the normal control group (P > 0.05). Furthermore, the testosterone hormone level in all GA and MOR + GA groups improved significantly compared to the MOR control group (P < 0.01) (Figure 4).

**Spermatogenesis index**

MOR caused a significant decrease in the SI compared to the normal control group (P < 0.01). No significant changes were observed in all GA groups compared to the normal control group (P > 0.05). Moreover, SI in all GA and MOR + GA groups showed a significant increase compared to MOR control group (P < 0.01) (Figure 5).

**Discussion**

Present study showed that the values of viability, motility, count and normal morphology of sperms in MOR control group reduced significantly compared to normal control group. Also, it decreased the levels of total serum antioxidant capacity. MOR induced oxidative stress in testicular tissue. It was demonstrated as growth in the levels of ROS and lipid peroxidation and a reduction in antioxidant enzymes activity like total antioxidant capacity (17). In a study MOR-induced hepatocarcinogenesis in male rats and reduced serum levels of total antioxidant capacity significantly, which confirms the results of the
present study (18). It seems that the ROS affects the DNA and RNA synthesis and inhibits the mitochondrial function in sperms (19). It is also possible that the oxidative stress in germ cells also acts in the same way and disrupt the cellular divisions and differentiation. In this way, a number of spermatogonia available on the basement membrane may be affected.

Also, the number of primary and secondary spermatocytes, spermatids and adult sperms reduced significantly (20). Oxidative stress can disrupt spermatogenesis in which the defective gametes with remodelled chromatin will be formed. These are susceptible to the free radicals in the case of reduction in the number of spermatogonia, spermatocytes, spermatids, and spermatozoa (21). In agreement with the results of the present study, Khan et al showed that arsenic-induced oxidative stress in male rats, imposed an increase in ROS production, deformity of sperm and reduction in motility, number of sperms, and testosterone level (22). The first consequence of the ROS attack to the structure of membrane is occurrence of cell peroxidation within the cellular and organelles membrane. Since a large amount of sperm cytoplasm will be lost following the spermatogenesis, it is reported to be more sensitive to the presence of ROS than somatic cells due to the lack of antioxidant systems (23). It seems that high levels of ROS can reduce the spermatozoal motility because of the effect on Ca++ channels and reduction in spermatozoa ATP reserves (24). Also, the reduction in glutathione levels can reduce the spermatozoal motility (25). It seems that the plasma membrane of sperms is susceptible to oxidative damage due to the presence of large amounts of unsaturated fatty acids. This phenomenon can result in reduced motility and viability of sperms (7). Salahshoor et al showed that the oxidative stress in male rats caused by nicotine consumption induced ROS, sperm deformity, DNA fragmentation, reduced fertility index, motility, number of sperm and level of testosterone (26).

Administration of MOR in male rats caused a significant reduction in sperm motility index and survival rate compared to control group, which confirms the results of the present study (10). In addition, the results of present study showed that GA or MOR + GA administration significantly increased the viability, motility, count and normal morphology of sperm cells compared to the MOR control group. GA is a methylxanthine derivative that can increase sperm motility by enhancement of intracellular calcium concentration and membrane penetration of cAMP analogs to inhibit phosphodiesterase (27). Due to low amount of cytoplasmic enzymes, no potential is found for regeneration of oxidative damage. Hence, antioxidants and antioxidant enzymes are highly necessary for the semen fluid to protect against oxidative damage (8). According to the studies by de Oliveira et al GA administration reveals the renal and hepatic anti-oxidative effects on biochemical and histological contents in diabetic mice (28). It has been shown that a number of GA derivatives has antioxidant effects to prevent neurodegenerative detrimental changes through free radicals and liposomes scavenging and by anti-apoptosis property in human SH-SY5-Y cell line (29). GA similarly exerts its anti-inflammatory activity by suppressing p65-NF-Kβ and IL-6/p-STAT3 pathways (30). Also, GA has anti-inflammatory properties, which seems to reduce the level of LPO and prevent damage to cells (31). It was shown that the proximity of sperm with GA induced motility in immobile sperm (32). Also, in the line of our study, Punithavathi et al found the per-oxidative and antioxidant effects of GA on streptozotocin-induced diabetic Wistar rats (16). Another study suggests that GA by initiation the pathway of receptor-alpha co-activator alpha has a crucial role in the Adenosine 3’,5’-monophosphate-activated protein kinase cellular pathway and regulation of mitochondrial function (33). However, we found that the serum level of total antioxidant capacity was improved significantly in the GA and GA + MOR groups compared to the MOR control
group. The reduction in total antioxidant capacity level in this study shows the effects of oxidative stress of MOR on reproductive parameters. The present study showed that the STD and testosterone levels in the MOR control group were reduced significantly compared to the normal control group. In the GA and MOR + GA groups, a significant increment was observed in the normal morphology of sperm cells, the STD and testosterone level compared to the MOR control group. MOR can induce an increased level of oxidative stress, DNA damage, peroxidation lipid, as well as formation of additional protein compounds by producing ROS such as superoxide and hydrogen peroxide (8). An increased state of differentiation was reported in seminiferous tubules in the MOR control group in which the increased diameter of the tubules is probable (23). In addition, increasing state of ROS with increasing lipid peroxidation leads to induction of tubules’ atrophy and apoptosis of the germ cells (9). It seems that a significant relationship is found among the production of oxygen species in sperms and disruption in morphology of sperms. The increased amount of free radicals results in destruction of surrounding cells, damage to Sertoli cells, the collapse of cytoplasmic bridges in which a reduction in the number of sperms and a decrease in sperm motility are probable (10). MOR administration causes a reduction in testosterone level due to the presence of oxidative stress (7). The study of Oyagbemi et al consistent with the present study showed that the administration of GA increased the STD and testosterone level significantly (32). It seems that, along with antioxidant properties, the vasodilatation and increased blood supply of GA can be represented as factors in increasing the production of testosterone in the present study (16). Moreover, the results of this survey showed that the MOR decreases the SI value. Similarly, a significant increase was detected in SI in all GA and GA + MOR groups compared to the MOR control group. Thus, the SI shifted from level 8 (few spermatozoa) to 5.5 (no spermatozoa and many spermatocytes) during the treatment by MOR, and also administration of GA increased the spermatozoa. Our results are supported by the findings of Roshankhah et al which showed that administration of crocin might increase SI in diabetic rats (12). ROS can affect the DNA and RNA synthesis in sperm cells (8). Therefore, it was assumed that GA raises the count and SI in the treated groups through improving the anti-oxidant protection of the body (34). A limitation of this study was the lack of information on the exact mechanism about the action of the protective effect of GA. Therefore, future studies are warranted to follow it.

Conclusion
The outcomes of this study demonstrated that MOR could generate defects in some male reproductive parameters and that GA has an antioxidant and defensive effect. GA was able to elevate quality of spermatozoa and improve normal morphology, SI, sperm viability, germlinal layer seminiferous tubules height, TAC, motility, and sperm count. GA can be a valuable agent for treatment of infertile men and for enhancement of male fertility. The antioxidant properties of GA could be the main reason for its optimistic outcome on reproductive parameters. Supplementary studies are essential to explain its careful mechanism of action.

Authors’ contribution
CJ conceived the research idea. MRS designed the work. SR wrote the first draft of the manuscript. AA and MRS carried out the literature search. CJ and SR carried out the statistical analysis. All authors read and approved final manuscript.

Conflict of interests
All authors declare no conflict of interest.

Ethical considerations
The protocol for this study was confirmed by Ethical Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1397.500) and the authors of this manuscript observed ethical issues. Animals were handled according to the International Guidelines for Care and Handling of Experimental Animals.

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Gallic acid and morphine testopathy

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