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Original Article

Effect of *Syzygium aromaticum* (clove) extract on morphine withdrawal side effect in male reproductive system should be addressed

Maryam Moghimian, Somaye Aalami, Seyed-Hosein Abtahi-Evari, Malihe Soltani*

Department of Basic Sciences, School of Medicine, Gonabad University of Medical Sciences, Gonabad, Islamic Republic of Iran

Abstract

Introduction: To study the effect of withdrawal syndrome in morphine-dependent male rats.

Methods: Adult male rats were divided randomly into four groups: control (G1), received morphine (G2), received morphine and treated by clove (G3) and only treated by clove (G4). The rats were administered increasing doses of morphine (0.1, 0.2 and 0.3 mg/ml), each dose being administered for two days, and then a dose of 4 mg/ml was given every day for 21 consecutive days. After the last oral dose of morphine on day 21, the rats were treated daily with oral clove (4 mg/ml/kg) for 14 days. Following the treatment, the histological parameters, oxidative stress, LH, FSH and testosterone levels were measured.

Results: The histological parameters were not significantly changed. In the morphine group, it was observed that the levels of LH, FSH and testosterone decreased significantly in comparison to the control group and clove treatment could significantly increase the LH, FSH, testosterone, glutathione peroxidase and superoxide dismutase levels in G3 groups. Also, the level of malondialdehyde (MDA) increased significantly in the morphine group and treatment with clove could significantly decrease the MDA level in G3 groups.

Conclusion: Our results showed that the hormone levels (LH, FSH and testosterone) and antioxidant enzyme increased with the administration of clove after morphine withdrawal. This may be because of the antioxidant effect of clove or the direct effect of this plant on the hypothalamic–pituitary–gonadal axis, or both.

Keywords:
Syzygium aromaticum;
Morphine withdrawal;
Testosterone;
Antioxidant;
Reproductive system

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***Correspondence to:**
M. Soltani

Tel: +98-9151097612
Fax: +98-5157223814

Email:
soltani.m@gmu.ac.ir

Introduction

Morphine as one of the strongest opioids, is widely used to alleviate severe pain. Morphine and its subsequent withdrawal cause side effects on the digestive (Xu et al., 2015), nervous (Cooper et al., 2017), endocrine (Ragen et al., 2015) and reproductive systems (Ahmadnia et al., 2016; Lee et al., 2011). The effects of morphine and the withdrawal syndrome during detoxification on male testicular tissue and function were evaluated and it was found that morphine may cause adverse effects on spermatogenesis and sperm function (Amory,

2007; Jalili et al., 2016), secretory activity of the secondary sex organs, as well as serum testosterone and gonadotropin hormone levels (Ahmadnia et al., 2016; Cicero et al., 1976). These effects may occur by impairing the hypothalamic pituitary testicular axis (Amory, 2007). Another side effect of morphine withdrawal syndrome is the production of reactive oxygen species (ROS) (Abdel-Zaher et al., 2013; Jalili et al., 2016; Pancehnko et al., 2004). Therefore, antioxidants may be employed to prevent the negative impact of ROS on nociception (Rokyta et al., 2003). *Syzygium aromaticum*, commonly known as clove, from the Asiatic region (Cortés-Rojas et al., 2014), is known to be an effective and beneficial herb with antioxidant, anti-inflammatory and anti-nociceptive properties (Avila-Peña et al., 2007; Mishra and Singh, 2008). Also, the analgesic potential of clove oil and its essential oil are used for headaches, joint pains, toothache and inflammation in the mouth and throat (Khalilzadeh et al., 2016). Concentrations of up to 18% of essential oils can be found in the clove flower buds. The antioxidant components of essential oil are eugenol, phenols, flavonoids and tannins (Nikousaleh and Prakash, 2016). Some studies evaluated another effects of these compounds; for example flavonoids are known to be anti-nociceptive (Mandegary et al., 2012) and analgesic effect of eugenol has been evaluated in pain management (Lionnet et al., 2010). Also eugenol has more different kinds of biological activities, including antioxidant and antiapoptoic effect (Ma et al., 2017). These beneficial effects of clove oil as painkillers can eliminate morphine withdrawal side effect. In a previous study, we showed that use of this plant can improve antioxidant enzymes, gonadotropin and testosterone levels when exposed to oxidative stress (Moghimian et al., 2017). Several studies have shown that the clove bud can be used to treat sexual disorders in males. Clove affects the functional physiology of the male reproductive system (Choi et al., 2014; Mishra and Singh, 2013), by increasing in the testosterone level (Mishra and Singh, 2008), the motility of sperms and the secretory activities of the epididymis and seminal vesicle in lower doses (Mishra and Singh, 2013).

Since morphine increased oxidative stress, we used *Syzygium aromaticum* as antioxidants against the increased oxidative stress after morphine withdrawal. Therefore, the present study examined the effect of *Syzygium aromaticum* on seminiferous tubules, oxidative stress and hormone level after morphine withdrawal in adult male rats in order to provide a basis for human experiments.

Materials and methods

This experimental study was performed on 28 adult male Wistar rats weighing 250–300 g. All rats were housed under a 12/12-hour light/dark cycle. The room temperature was maintained at 23±2 °C and humidity at 60–70%. The rats in all groups had free access to food and water. The ethical considerations were based on the guidelines for laboratory animals provided by the Research and Technology Deputy of Gonabad University of Medical Sciences. Morphine hydrochloride and naloxone were purchased from Darupakhsh Co., (Iran). All the drugs were dissolved in saline.

In the present study rats were randomly allocated to one of the four groups: 1) control group (n=7), received normal saline; 2) morphine group (n=7), received morphine for 21 days; 3) morphine+SA (*Syzygium aromaticum*) (n=7), after the last oral administration of morphine on day 21, rats treated with *Syzygium aromaticum* (4 mg/kg) for 14 days and 4) *Syzygium aromaticum* (SA) group (n=7), treated with daily oral dose of *Syzygium aromaticum* (4 mg/kg) for 14 days.

Experimental protocol:

To induce morphine addiction in the morphine+SA and morphine groups, the rats received morphine solution in doses of 0.1 mg/ml, 0.2 mg/ml and 0.3 mg/ml. Each dose was administered for two days and then a dose of 4 mg/ml was given per day for 21 consecutive days. Also, 3% sucrose was added to the solution to omit the bitterness of morphine. For morphine withdrawal, naloxone (5 mg/ml/kg, intraperitoneal) was injected after the last oral dose of morphine on day 21. In the SA and morphine+SA groups, morphine addiction induced same as morphine group. After the last oral dose of morphine on day 21, the rats were treated with a daily oral dose of *Syzygium aromaticum* (4 mg/ml/kg) for 14 days. Finally, after the duration of treatment, the rats were anesthetized using ketamine-xylazine and one ml of blood was drawn from the vena cava inferior vein to measure the levels of LH, FSH, testosterone and

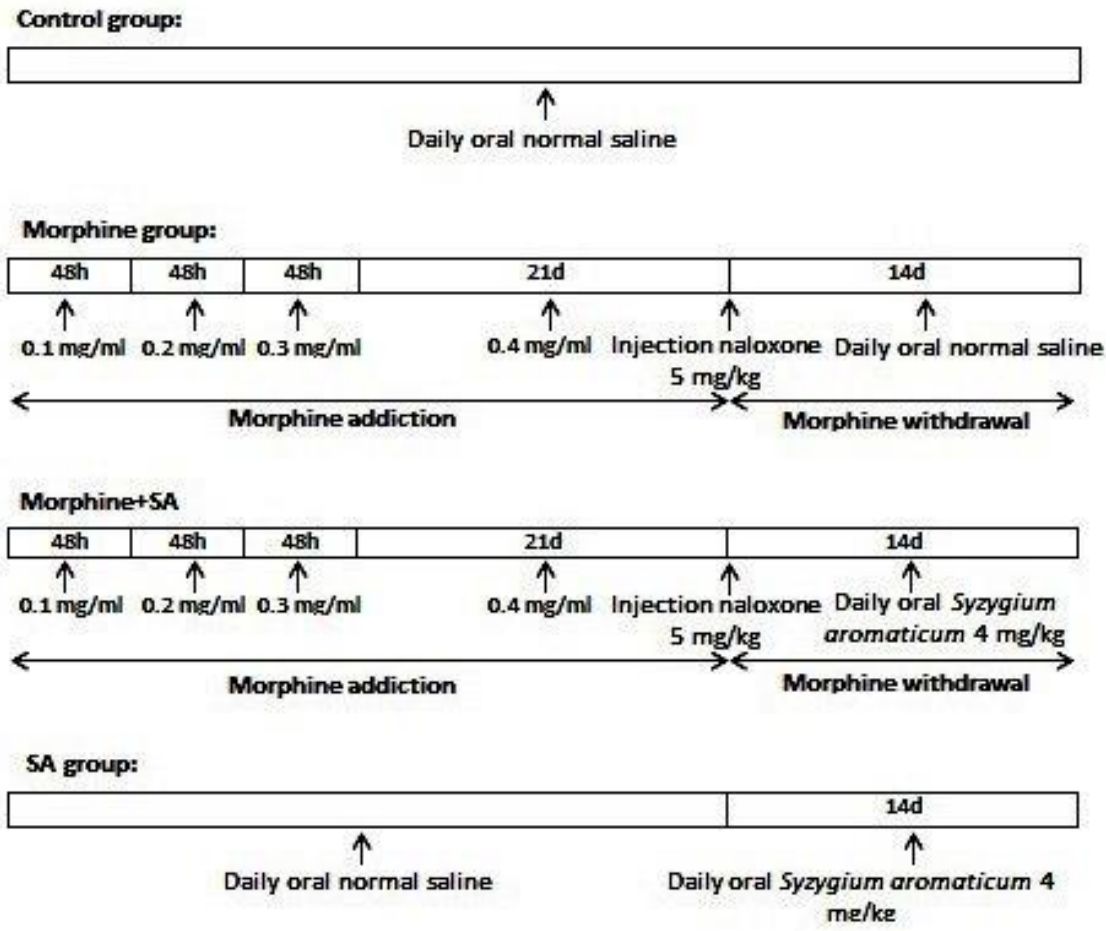

Fig.1. Illustration of the experimental protocols. Rats were administered by increasing doses of morphine (0.1, 0.2 and 0.3 mg/ml; each dose was administered for 48 hours) then a dose of 4 mg/ml was given per day for 21 consecutive days. After the last oral administration of morphine on day 21, rats treated by daily oral *Syzygium aromaticum* (4 mg/kg) for 14 days. SA=treatment with *Syzygium aromaticum*

antioxidant enzymes. Blood samples were kept at room temperature and then centrifuged at 3000 rpm for 10 minutes. Once the serum was removed from the blood cells, the serum samples were kept at −70 °C until further evaluations. The rats underwent orchiectomy on day 14 post treatment (Moghimian et al., 2017; Rahmati et al., 2016) to examine both the testis (Fig. 1).

Hydroalcoholic extract preparation of *Syzygium aromaticum*

In order to prepare the whole plant clove extract, a half kilogram of clove flower was dried at 25°C and protected from direct sunlight. For extraction after grinding the dried plants, they were dissolved in 2 liters of 96% ethanol and then kept at room temperature for 48 h. Over this period, after frequently shaking, the solution was filtered. Then, the solution was centrifuged for 5 minutes, at 3000 rpm. At the end of the process, the resulting solution was poured into an open-top container and the solvent was evaporated. Around 90 grams of a semi-solid extract was obtained from 500 g of clove powder. In order to achieve appropriate concentration, the extract was dissolved in normal saline.

Tissue fixation and preparation of specimens

After the orchiectomy, the testicles were placed in Bouin solution for 48 hours. Once the tissue was fixed, tissue passage was performed using incremental increases in ethanol, xylenol and liquid paraffin. The testicles were molded in the paraffin and slides of tissue with 5µm thickness were prepared from each specimen, stained with hematoxylin eosinophil, and studied under an optical microscope at 400x magnification.

Histological evaluation and maturation of seminiferous tubules

The Johnsen score was used to evaluate spermatogenesis in the seminiferous tubules. To do

so, seminiferous tubules were examined in each cross-section and a score of 1-10 was assigned to each cross section according to the following criteria (Johnsen, 1970): 10, complete spermatogenesis and perfect tubules; 9, many spermatozoa present but disorganized spermatogenesis; 8, only a few spermatozoa present; 7, no spermatozoa but many spermatids present; 6, only a few spermatids present; 5, no spermatozoa or spermatids present but many spermatocytes present; 4, only a few spermatocytes present; 3, only spermatogonia present; 2, no germ cells present and 1, neither germ cells nor sertoli cells present.

Morphometry of seminiferous tubules

The linear eyepiece grids on the microscope were used to characterize the morphometry of the seminiferous tubules. In each rat, 20 seminiferous tubules were randomly selected from those showing round or nearly round cross-sections and studied. Tubules that were oval or oblique in cross-section were not studied. The diameter of the seminiferous tubules, from the basement membrane on one side of the tubule to that on the other side of the tubule, was calculated at 400x magnification. The two perpendicular diameters were first calculated and the mean diameter of each tubule was then determined. The height of the germinal epithelium was calculated in micrometers (Moghimian et al., 2016).

Measurement of malondialdehyde (MDA) levels

MDA was measured by placing 0.20 ml of plasma into a test tube containing 3.0 ml of glacial acetic acid, to which 3.0 ml of 1% TBA in 2% NaOH was added. The test tube containing the mixture was placed in boiling water for 15 minutes. Absorbance of the pink-colored product was read at 532 nm after cooling. The calibration curve was constructed using malondialdehyde tetrabutylammonium salt obtained from Sigma (USA) (Gwarzo et al., 2014) .

Measurement of superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity

The activity of SOD and GPX in serum were assayed in accordance with the protocols of the kits (Randox, UK).

Measurement of LH, FSH and testosterone level

The serum hormone levels were determined using enzyme-linked immunosorbent assay (ELISA) kit (Demeditec Diagnostices, Germany).

Statistical analysis

Statistical analysis was carried out in SPSS 20 (IBM, USA). The Kolmogorov-Smirnov test was used to determine whether or not the data were normally distributed. All data were presented as mean±SD. One-way ANOVA followed by Tukey's post-hoc test was performed to compare the histopathological parameters and oxidative stress values. Statistical significance levels were determined at $P<0.05$.

Results

Testicular histological parameters

The histological parameters included the mean Johnsen score (MJS), the diameter of the seminiferous tubules (STD) and the height (thickness) of the seminiferous tubule epithelium (HE) in the experimental groups were compared. In the morphine group, decreases in the MJS, STD and HE were observed compared to the control group; however, histological parameters were not significantly different. Also these parameters were not significantly different when morphine+SA and SA groups were compared with the control group (Table 1 and Fig. 2).

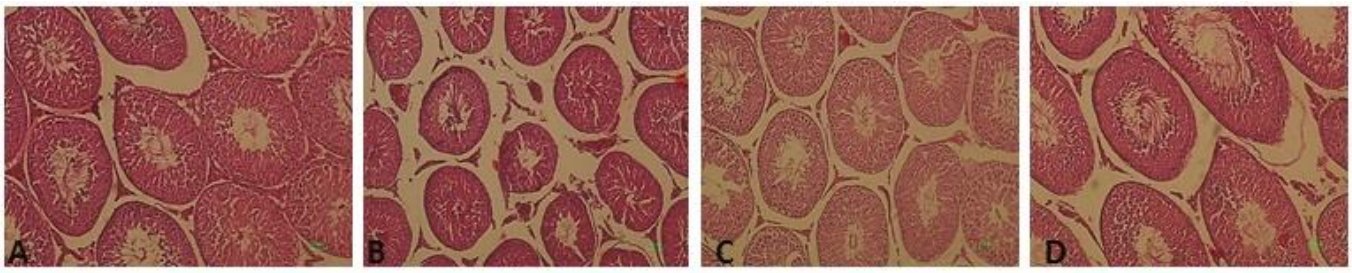

Fig.2. Histological findings (seminiferous tubule, diameter, and thickness) in control, morphine, morphine+SA and SA groups (400x). A: control, B: morphine, C: morphine+ SA and D: SA (treatment with Syzygium aromaticum).

Table 1: A comparison of the testicular Modified Johnsen Score, seminiferous tubule diameter and epithelium height in control, morphine, morphine+SA and SA groups

group	Modified Johnsen Score	STD	EH
control	9.66	261.53	73.11
morphine	7.89 (.000)	216.03 (.001)	65.76 (.009)
Morphine+ SA	8.83 (.011)	231.00 (.022)	68.82 (.014)
SA	9.77 (.001)	256.02 (.012)	70.16 (.010)

SA=treatment with *Syzygium aromaticum*. STD=seminiferous tubule diameter, EH=epithelium height. Values are mean±SD. *P*<0.05 is significant.

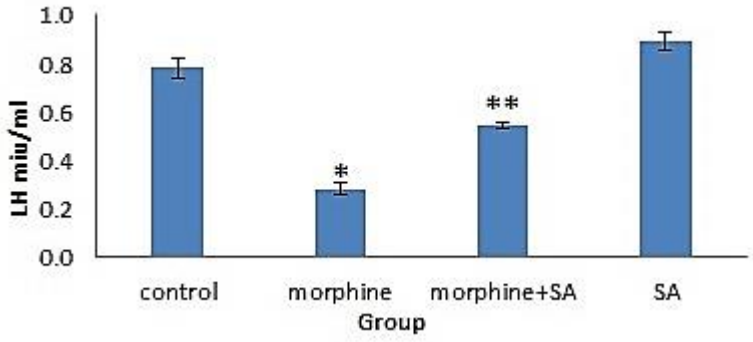

Fig.3. A comparison of the LH in control, morphine, morphine+SA and SA groups. SA=treatment with *Syzygium aromaticum*. Values are mean±SD. **P*<0.05 vs control, ***P*<0.05 vs morphine.

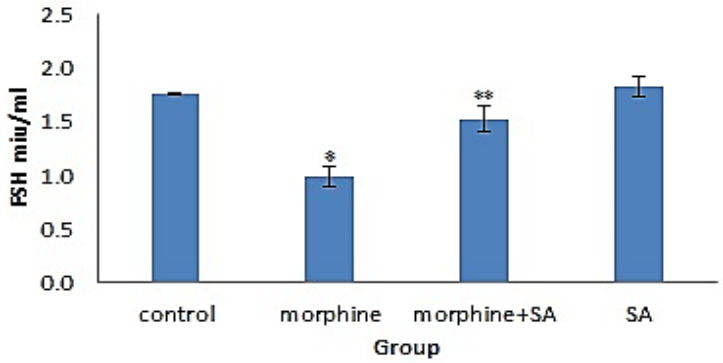

Fig.4. A comparison of the FSH in control, morphine, morphine+SA and SA groups. SA=treatment with *Syzygium aromaticum*. Values are mean±SD. **P*<0.05 vs control, ***P*<0.05 vs morphine.

Biochemical parameters

Hormonal analysis

The level of LH, FSH and testosterone significantly decreased in the morphine group when compared with the control group (*P*≤0.05). Treatment by *Syzygium aromaticum* in the morphine+SA group increased the LH, FSH and testosterone levels significantly compared to that in the morphine group (*P*≤0.05). The SA group showed no significant difference in the LH, FSH and testosterone levels compared to the other groups (Figs. 3, 4 and 5).

Antioxidant enzymes

The levels of GPX and SOD decreased significantly in the morphine group compared to the control group (*P*≤0.05). The levels of GPX and SOD increased significantly when the morphine+SA group was compared with the morphine group (Figs. 6 and 7). The level of MDA increased significantly in the morphine group in comparison to the control group

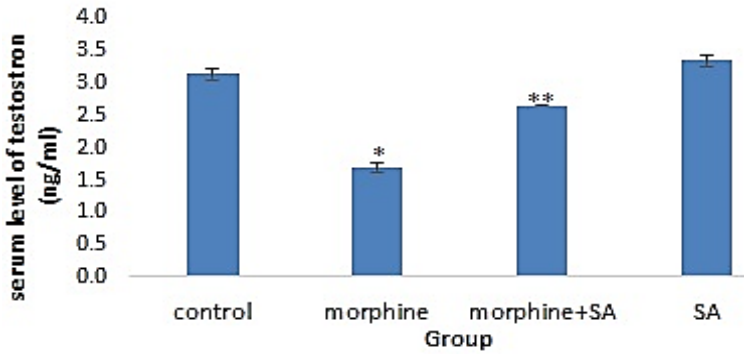

Fig.5. A comparison of the testosterone in control, morphine, morphine+SA and SA groups. SA=treatment with *Syzygium aromaticum*. Values are mean±SD. **P*<0.05 vs control, ***P*<0.05 vs morphine.

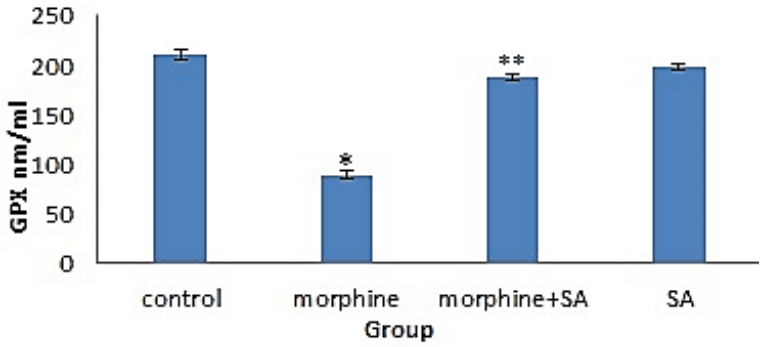

Fig.6. A comparison of the glutathione peroxidase (GPX) in control, morphine, morphine+SA and SA groups. SA=treatment with *Syzygium aromaticum*. Values are mean±SD. **P*<0.05 vs control, ***P*<0.05 vs morphine.

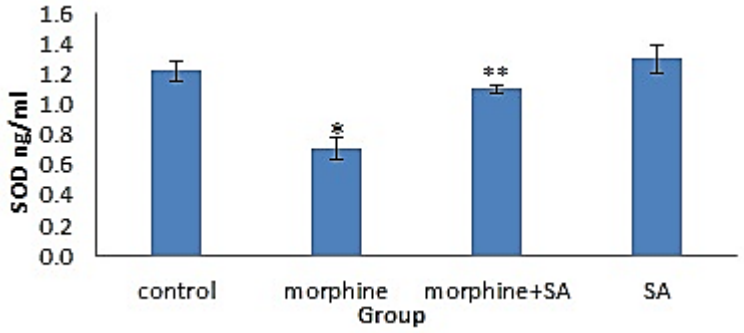

Fig.7. A comparison of the superoxide dismutase (SOD) in control, morphine, morphine+SA and SA groups. SA=treatment with *Syzygium aromaticum*. Values are mean±SD. **P*<0.05 vs control, ***P*<0.05 vs morphine.

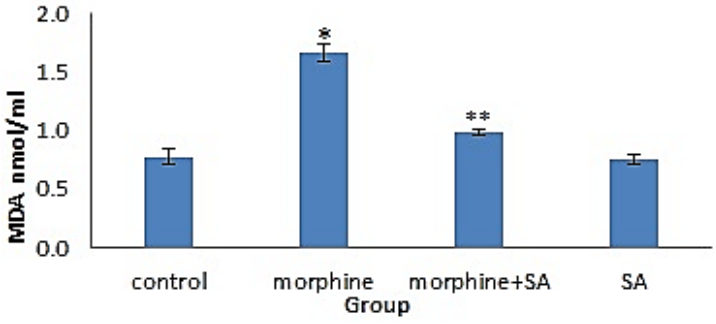

Fig.8. A comparison of the malondialdehyde (MDA) in control, morphine, morphine+SA and SA groups. SA=treatment with *Syzygium aromaticum*. Values are mean±SD. **P*<0.05 vs control, ***P*<0.05 vs morphine.

and treatment by *Syzygium aromaticum* could significantly decrease the level of MDA in the morphine+SA group (Fig. 8).

Discussion

This study aimed to determine the protective effect of *Syzygium aromaticum* on the side effects of morphine withdrawal syndrome in the male reproductive system as well as in the oxidative stress and histological parameters. Morphine as one of the strongest opioids, can lead to lasting infertility, and this may result from the functional disorder of the hypothalamus-pituitary-gonadal (HPG) axis or a direct effect on the testicular tissue (Ahmadnia et al., 2016). In contrast, we did not observe any change in the histological parameters of testicular tissue. These results are in line with those obtained from some studies which indicated that the pituitary glands or gonads were not directly affected by opioids (Zhou et al., 1990). Clinical studies suggest morphine can reduce prostates and seminal vesicles secretory activity (Cicero et al., 1975). Also, it has been shown that opioids decrease the level of gonadotropins, this effect is due to a single place in the HPG axis (Coventry et al., 2001; Saso, 2002). In this study, the levels of hormones (LH, FSH and testosterone) in the morphine group were found to be lower than those in the control group. This decrease may be due to a functional disorder of the HPG axis (Cicero et al., 1989; Gabriel et al., 1985), or it may be a result of increased oxidative stress. So that development of morphine tolerance and dependence produced an increase glutamate and MDA levels (Famitafreshi and Karimian, 2017) and nitric oxide production (Abdel-Zaher et al., 2013). These suppressive effects on antioxidant enzyme causes disruption of spermatogenesis, gonadotropin and testosterone levels (Aitken and Roman, 2008). In a previous study, we showed that testicular oxidative stress leads to a reduction in serum testosterone levels (Moghimian et al., 2016), either as a result of injury to the testicular tissue or to endocrine structures such as the anterior pituitary (Aprioku, 2013). Some studies also show that morphine withdrawal can produce ROS (Abdel-Zaher et al., 2013; Jalili et al., 2016; Pancehnko et al., 2004), It is hypothesized that the opioid treatment effects might have been induced through the overproduction of nitric oxide and oxidative stress as well as an elevation in sperm count and motility in testicular tissue (Ahmed and Kurkar, 2014). For this reason, administration of antioxidants suggested to address oxidative stress in the testes (Aitken and Roman, 2008). Clinical evaluation of *Syzygium aromaticum* suggests that it has variable but significant biological effects such as anti-nociceptive, anti-inflammatory (Chaudhuri et al., 1990) and free radical scavenging (Benherlal and Arumughan, 2007). Other studies also reported that this plant can increase the serum levels of testosterone, as well as attenuate oxidative stress and apoptosis in rats (Mishra and Singh, 2008; Shukla et al., 2014), and we showed that *Syzygium aromaticum* treatment reduces oxidative stress (SOD and GPX) and increase MDA and testosterone levels in morphine withdrawal rats. Benefit effect of *Syzygium aromaticum* can be seen in the testicular tissue examination result. The improvement of testosterone levels and testicular histological parameters in the SA group may be due to a decrease in oxidative stress, or it may be a direct effect of this plant in the testis or HPG axis or both. Some studies showed that clove extract can increase withdrawal response latencies in a dose-dependent manner (Avila-Peña et al., 2007). Also researchers have reported that this plant causes a long-lasting analgesia for pain and efficacy comparable to that produced by morphine (Taher et al., 2015). So, this effect of *Syzygium aromaticum* can be used in treating morphine withdrawal syndrome.

Conclusion

In conclusion, findings of the current study suggest that *Syzygium aromaticum* might provide a protective effect on morphine withdrawal syndrome in the male reproductive system.

Acknowledgments

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Conflict of interest

The authors declare that they have no competing interest.

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