



## Different effects of L-arginine on morphine tolerance in sham and ovariectomized female mice\*

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**Abstract:** Objective: The roles of gonadal hormones and nitric oxide (NO) on the analgesic effects of morphine, tolerance to morphine, and their interactions have been widely investigated. In the present study, the effect of L-arginine (an NO precursor) on morphine tolerance in sham and ovariectomized (OVX) female mice was investigated. Methods: Forty mice were divided into sham and OVX groups. On the first day, a hot plate test ((55±0.2) °C; cut-off 30 s) was carried out as a base record 15 min before injection of morphine (10 mg/kg, subcutaneously (s.c.)) and was repeated every 15 min after injection. The sham group was then divided into two subgroups: sham-tolerance-L-arginine (Sham-Tol-LA) and sham-tolerance-saline (Sham-Tol-Sal) which received either L-arginine 50 mg/kg (intraperitoneally (i.p.)) or saline 10 ml/kg (i.p.), respectively, three times in a day for three consecutive days. Morphine tolerance was induced in animals by injecting 30 mg/kg morphine (s.c.) three times/day for three days. This treatment was also used for OVX subgroups. On the fifth day, the hot plate test was repeated. The analgesic effect of morphine was calculated as the maximal percent effect (MPE). The results were compared using repeated measure analysis of variance (ANOVA). Results: There was no significant difference in MPE between the OVX and sham groups. The MPEs in both the Sham-Tol-Sal and OVX-Tol-Sal groups were lower than those in both the sham and OVX groups ( $P<0.01$ ). The MPE in the OVX-Tol-Sal group was greater than that in the Sham-Tol-Sal group ( $P<0.01$ ). The MPE in the Sham-Tol-LA group was higher than that in the Sham-Tol-Sal group ( $P<0.01$ ). However, there was no significant difference between the Sham-Tol-LA and sham groups or between the OVX-Tol-LA and OVX-Tol-Sal groups. Conclusions: The results of the present study showed that repeated administration of morphine causes tolerance to the analgesic effect of morphine. L-Arginine could prevent tolerance to morphine but its effect was different in the presence of ovarian hormones.

**Key words:** L-Arginine, Ovariectomized mice, Morphine, Tolerance

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### 1 Introduction

Pain is an unpleasant sensation which results from exposure of the skin or other tissue to an injurious or potentially-injurious stimulus (Fatehi-Hassanabad *et al.*, 2005). It has been suggested that

sociocultural, psychological and biological conditions affect pain sensitivity (Wiesenfeld-Hallin, 2005; Gatchel *et al.*, 2007). There is now strong evidence for gender-dependent differences in pain perception and analgesia (Craft *et al.*, 2004; Loyd *et al.*, 2007). The prevalence of disorders such as migraine and fibromyalgia, which are accompanied by chronic pain, is higher in women than in men (Craft, 2007). These sex-dependent differences, as well as the presence of estrogen receptors in areas of the nervous system

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which contribute to pain perception, suggest that gonadal hormones such as estradiol and testosterone may affect pain perception or analgesia (Shughrue *et al.*, 1997; Craft *et al.*, 2004). The high localization of estrogen receptors in peri-aqueductal gray (PAG) matter also suggests that estrogen may affect the descending analgesia system (Loyd and Murphy, 2008). Modulation of neurotransmitters such as  $\gamma$ -amino butyric acid (GABA), serotonin and calcitonin gene-related peptide (CGRP) by estrogen, may be further evidence for its involvement in pain modulation (Saleh and Connell, 2003). It has been documented that some endocrine systems including gonadal hormones may change the development of tolerance to or dependence on analgesic drugs. Some studies have shown that estradiol administration can lead to tolerance prevention (Cataldo *et al.*, 2005). Other results showed that gonadal hormone utilization facilitates tolerance to morphine (Shekunova and Beshpalov, 2006).

Nitric oxide (NO) is a major neurotransmitter synthesized from L-arginine by NO synthase (NOS) (Pan *et al.*, 2005; Li *et al.*, 2006) and is involved in nociception processes (Janicki and Jeske-Janicka, 1998; Hosseini *et al.*, 2011). It has been reported that NO donors affect the release of neuropeptides, such as the substance P and CGRP, which contribute to pain transmission (Garry *et al.*, 1994). It has also been suggested that NO inhibition potentiates carrageenan-induced hyperalgesia (Budzinski *et al.*, 1999). The role of NO in morphine tolerance has also been reported. According to some reports, tolerance to morphine or its withdrawal syndrome is likely prevented by inhibition of NO synthesis (Toda *et al.*, 2009). However, some studies have shown that morphine tolerance is decreased by enhancement of NO synthesis (Kolesnikov *et al.*, 1992). Inhibition of NO synthesis and blocking of morphine tolerance result in an enhancement in morphine antinociception, which suggests a selective function for NO contributing to  $\mu$ -receptor-mediated tolerance and dependence (Heinzen *et al.*, 2005).

It has been well documented that estrogen affects endothelial NOS (eNOS) activity and expression as well as the release of NO in both peripheral and nervous tissues (Hishikawa *et al.*, 1995; Russell *et al.*, 2000; Stefano *et al.*, 2000; Farsetti *et al.*, 2009; Liu *et al.*, 2009; Zhang *et al.*, 2011). Estrogen also affects

neuronal NOS (nNOS) mRNA, the number of nitergic neurons, and the production of NO in the brain (Grohé *et al.*, 2004). Interactions of both sex hormones and NO with other neurotransmitters, such as GABA, acetylcholine, serotonin, and dopamine, have been widely documented (Kugaya *et al.*, 2003; Mitsushima *et al.*, 2009). In particular, it has been suggested that the interaction between estrogen and the NO system may be responsible for morphine tolerance.

Therefore, the aim of the present study was to clarify various effects of L-arginine (an NOS precursor) on morphine tolerance in ovariectomized and sham-operated female mice.

## 2 Materials and methods

### 2.1 Animals and drugs

Forty female Balb/c mice ((9 $\pm$ 1) weeks of age, and (3 $\pm$ 8) g in weight) were used. The animals were housed in ten standard cages, at room temperature ((22 $\pm$ 1) °C) on a 12-h light/dark cycle. Food and water were available continuously. Animal handling and all related procedures were in accordance with the standards of animal care approved by the Ethical Committee Acts, Mashhad University of Medical Sciences. The drugs used were L-arginine (Sigma, Chemical Co., St. Louis, MO, USA) and morphine powder (TEMAD Ltd., Teheran, Iran) dissolved in saline.

### 2.2 Nociceptive test

To assess nociceptive responses, a hot plate method was used. In this method, the mice were placed on a hot plate with the temperature setting controlled at (55 $\pm$ 0.2) °C. The cut-off time was 30 s. A nociceptive response was defined as the licking of forepaws or the moving of hind paws. The time duration between placing the animals on the hot plate and the licking of forepaws or the moving of hind paws was considered as the reaction time. The hot plate test was performed as a base record 15 min before injection of morphine (10 mg/kg, subcutaneously (s.c.)) (Ojewole, 2008) and was repeated five times, every 15 min after injection.

### 2.3 Surgery

Mice were ovariectomized under ketamine anesthesia (50 mg/kg, intraperitoneally (i.p.)). Anesthesia

was confirmed by reduced respiratory rate and no response to gentle pinching of the foot pad. The surgery was performed as the method described by Saffarzadeh *et al.* (2010). Briefly, the skin was incised in the flank region and the ovaries were isolated by ligation of the most proximal portion of the oviduct before removal. In the sham group, the ovaries were not removed.

## 2.4 Tolerance induction

Morphine tolerance was induced in animals by injecting 30 mg/kg morphine (s.c.) three times a day for three consecutive days, as described by Meng *et al.* (2008).

## 2.5 Experimental design

Forty mice were divided into two groups: the sham and ovariectomized (OVX) groups. During surgery, a few mice were omitted from the experiment. Six weeks after surgery, the hot plate test ((55±0.2) °C; cut-off 30 s) was carried out as a base record 15 min before injection of morphine (10 mg/kg, s.c.) and was repeated every 15 min after injection on the first day. The sham group was divided into two subgroups: (1) sham-tolerance-L-arginine (Sham-Tol-LA), and (2) sham-tolerance-saline (Sham-Tol-Sal), which received L-arginine 50 mg/kg (i.p.) or saline

10 ml/kg (i.p.), respectively, three times a day for three consecutive days. Morphine tolerance was induced in animals by injecting 30 mg/kg morphine (s.c.) three times a day for three days. The animals of the OVX group were also divided into two subgroups: (1) ovariectomized-tolerance-L-arginine (OVX-Tol-LA) and (2) ovariectomized-tolerance-saline (OVX-Tol-Sal), and were given the same treatments as the sham subgroups. On the fifth day, the hot plate test was repeated.

## 2.6 Statistical analysis

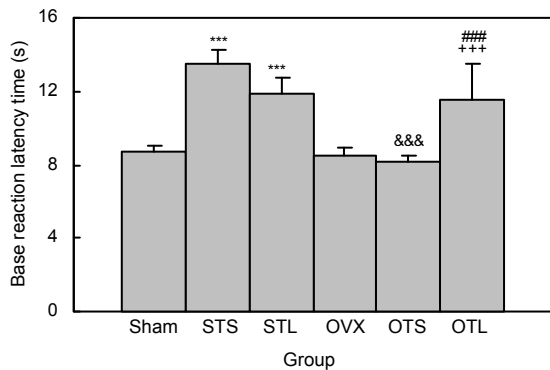
The analgesic effect of morphine was calculated as the maximal possible effect [MPE=( $t_t-t_b$ )/( $t_c-t_b$ )×100%, where  $t_t$  is test response time,  $t_b$  is basal response time, and  $t_c$  is cut-off time] (Moghaddam *et al.*, 2004; Sepehri and Shafeiee, 2006). All data are presented as the mean±standard error of the mean (SEM) of the MPE. Statistical comparisons of base reaction latency time between groups were made using one-way analysis of variance (ANOVA) and post-hoc Tukey's test or Student's *t*-test. A repeated measure ANOVA followed by post-hoc Tukey's test was used for comparison of MPE after injection of morphine. Differences were considered statistically significant when  $P<0.05$ . Table 1 shows a summary of the groups, subgroups, treatments, and tests.

**Table 1 Summary of groups, subgroups, treatments and tests in our experiment**

Group and subgroup	Treatment and test
Sham	1. Hot plate test: base record 15 min before injection; 2. Morphine injection: single dose (10 mg/kg); 3. Hot plate test: 5 times (15 min interval after injection), MPE calculation.
Sham-Tol-Sal	1. Morphine injection for inducing tolerance: 30 mg/kg, 3 times/d, for 3 d; 2. Co-treatment with morphine: saline (10 ml/kg), 3 times/d, for 3 d; 3. Hot plate test: base record 15 min before injection; 4. Morphine injection: single dose (10 mg/kg); 5. Hot plate test: 5 times (15 min interval after injection), MPE calculation.
Sham-Tol-LA	1. Morphine injection for inducing tolerance: 30 mg/kg, 3 times/d, for 3 d; 2. Co-treatment with morphine: L-arginine (50 mg/kg), 3 times/d, for 3 d; 3. Hot plate test: base record 15 min before injection; 4. Morphine injection: single dose (10 mg/kg); 5. Hot plate test: 5 times (15 min interval after injection), MPE calculation.
OVX	1. Hot plate test: base record 15 min before injection; 2. Morphine injection: single dose (10 mg/kg); 3. Hot plate test: 5 times (15 min interval), MPE calculation.
OVX-Tol-Sal	1. Morphine injection for inducing tolerance: 30 mg/kg, 3 times/d, for 3 d; 2. Co-treatment with morphine: saline (10 ml/kg), 3 times/d, for 3 d; 3. Hot plate test: base record 15 min before injection; 4. Morphine injection: single dose (10 mg/kg); 5. Hot plate test: 5 times (15 min interval after injection), MPE calculation.
OVX-Tol-LA	1. Morphine injection for inducing tolerance: 30 mg/kg, 3 times/d, for 3 d; 2. Co-treatment with morphine: L-arginine (50 mg/kg), 3 times/d, for 3 d; 3. Hot plate test: base record 15 min before injection; 4. Morphine injection: single dose (10 mg/kg); 5. Hot plate test: 5 times (15 min interval after injection), MPE calculation.

### 3 Results

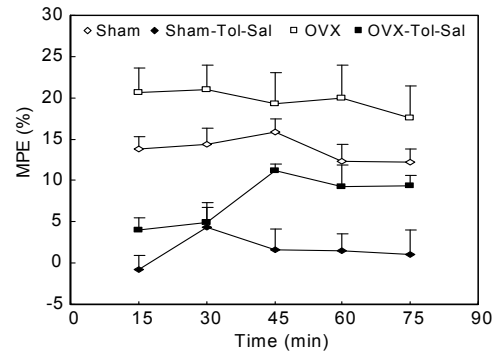
There was no significant difference in base reaction latency time between the sham and OVX groups (Fig. 1). The base reaction latency time in the OVX-Tol-Sal group was lower than that in the Sham-Tol-Sal group ( $P<0.001$ ; Fig. 1). The animals of the Sham-Tol-LA group had a higher base reaction latency time than those of the sham group ( $P<0.001$ ; Fig. 1). However, there was no significant difference between the Sham-Tol-LA and Sham-Tol-Sal groups in base reaction latency time (Fig. 1). The base reaction latency time in the OVX-Tol-LA group was higher than that in either the OVX-Tol-Sal or OVX group ( $P<0.001$ ; Fig. 1). There



**Fig. 1 Comparison of base reaction latency time between Sham, Sham-Tol-Sal (STS), Sham-Tol-LA (STL), OVX, OVX-Tol-Sal (OTS), and OVX-Tol-LA (OTL) groups**

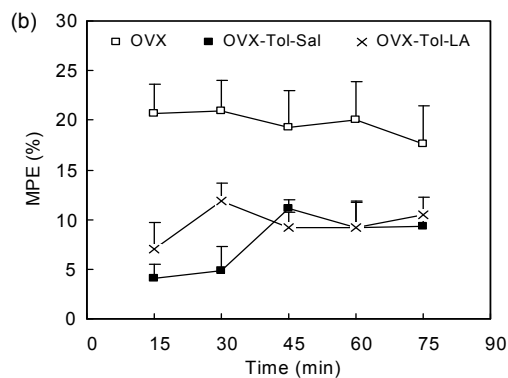
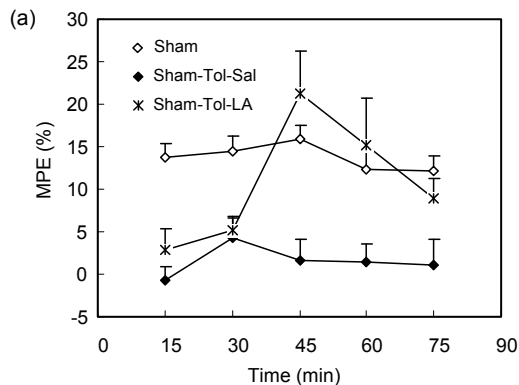
Data are shown as mean $\pm$ SEM ( $n=18$  in Sham,  $n=8$  in Sham-Tol-Sal,  $n=10$  in Sham-Tol-LA,  $n=15$  in OVX,  $n=7$  in OVX-Tol-Sal, and  $n=8$  in OVX-Tol-LA). \*\*\* $P<0.001$  compared to the Sham group, +++ $P<0.001$  compared to the OVX group, and ### $P<0.001$  compared to the OVX-Tol-Sal group, &&& $P<0.001$  compared to Sham-Tol-Sal

was no significant difference in MPE between the OVX and sham groups (Fig. 2). The MPE in the OVX-Tol-Sal group was significantly higher than that in the Sham-Tol-Sal group ( $P<0.01$ ; Fig. 2). The MPEs in both the Sham-Tol-Sal and OVX-Tol-Sal groups were lower than those in both the sham and OVX groups ( $P<0.01$ ; Fig. 2). The MPE in the Sham-Tol-LA group was higher than that in the Sham-Tol-Sal group ( $P<0.01$ ; Fig. 3a). However, there was no significant difference between the Sham-Tol-LA and sham groups. There was no significant difference between the OVX-Tol-LA and OVX-Tol-Sal groups. However, the MPE of the OVX-Tol-LA group was lower than that of the OVX group ( $P<0.01$ ; Fig. 3b).



**Fig. 2 Comparison of MPE between Sham, Sham-Tol-Sal, OVX, and OVX-Tol-Sal groups**

Data are shown as mean $\pm$ SEM ( $n=18$  in Sham,  $n=8$  in Sham-Tol-Sal,  $n=15$  in OVX,  $n=7$  in OVX-Tol-Sal). Using repeated measure ANOVA, there was no significant difference in MPE between the OVX and Sham groups. The MPEs in both the Sham-Tol-Sal and OVX-Tol-Sal groups were less than those of both the Sham and OVX groups ( $P<0.01$ ). The MPE in the OVX-Tol-Sal group was significantly higher than that of the Sham-Tol-Sal group ( $P<0.01$ )



**Fig. 3 Comparisons of MPEs between Sham, Sham-Tol-Sal, and Sham-Tol-LA groups (a) and between OVX, OVX-Tol-Sal, and OVX-Tol-LA groups (b)**

Data are shown as mean $\pm$ SEM ( $n=18$  in Sham,  $n=8$  in Sham-Tol-Sal,  $n=10$  in Sham-Tol-LA,  $n=15$  in OVX,  $n=7$  in OVX-Tol-Sal, and  $n=8$  in OVX-Tol-LA). The MPE in the Sham-Tol-LA group was higher than that in the Sham-Tol-Sal group ( $P<0.01$ ). There was no significant difference between the OVX-Tol-LA and OVX-Tol-Sal groups

#### 4 Discussion

Gender-dependent differences in nociception and opioid tolerance have been well documented (Craft *et al.*, 2004; Wiesenfeld-Hallin, 2005). It has been hypothesized that gonadal hormones may have a role in opioid tolerance (Terner *et al.*, 2005). Interaction of gonadal hormones and NO with neurotransmitters involved in pain modulation has been widely suggested (Kugaya *et al.*, 2003). It has been well documented that estrogen interacts with the NO system in both peripheral and nervous tissues (Nematbakhsh and Khazaei, 2004; Hosseini *et al.*, 2009; 2010; Saffarzadeh *et al.*, 2010; Azizi-Malekabadi *et al.*, 2011). Therefore, some actions of estradiol are probably mediated by NO (Hishikawa *et al.*, 1995; Patchev *et al.*, 1999; Russell *et al.*, 2000; Stefano *et al.*, 2000; Grohé *et al.*, 2004; Farsetti *et al.*, 2009; Shih, 2009). However, the role of NO in sex hormone-dependent changes in behavioral responses of female mice and rats has also been reported (Sadeghipour *et al.*, 2007; Hosseini *et al.*, 2011). It was shown that NOS inhibition antagonized epinephrine-induced hyperalgesia in normal male but not female rats (Dina *et al.*, 2001). We hypothesized that the NO signaling pathway may contribute to morphine tolerance differently in sham and OVX female mice. For this reason, the effect of L-arginine (the precursor of NO) on morphine-induced tolerance in sham and OVX female mice was investigated. The hot plate test used in the present study is a well known standard method for pain threshold evaluation after morphine administration (Langerman *et al.*, 1995).

The results showed no difference in MPE between the OVX and sham groups (on the first day). There is evidence that steroid hormones modulate the analgesic effects of morphine (Russell *et al.*, 2000; Cataldo *et al.*, 2005). Changes in  $\beta$ -endorphin receptors in the nervous system, functional coupling of the  $\mu$ -opioid and GABA receptors, and alteration in  $\mu$ -opioid and GABA-mediated hyperpolarization of neurons by estradiol and progesterone (Kelly *et al.*, 1992) confirm that gonadal hormones may affect the analgesic function of opioids. The association of high levels of estrogen with increased levels of proenkephalin gene expression has also been reported (Romano *et al.*, 1988). In the present study, the MPEs of both the Sham-Tol-Sal and OVX-Tol-Sal groups

(after tolerance) were lower than those of both the sham and OVX groups (before tolerance). Thus, repeated morphine administration can lead to tolerance in both OVX and sham-operated mice, as previously reported (Bourne, 2008; Hernández *et al.*, 2009). There are many controversial reports regarding the effect of estradiol on morphine tolerance (Cataldo *et al.*, 2005; Shekunova and Bespalov, 2006). The results of the present study confirmed a significant difference between the OVX-Tol-Sal and Sham-Tol-Sal groups in MPE.

NO is a potent biological messenger with a diversity of physiological functions (Moncada *et al.*, 1991; Luo and Cizkova, 2000). Our results demonstrated that co-treatment of the animals with morphine and L-arginine affects morphine-induced tolerance; the MPE in the Sham-Tol-LA group was higher than that of the Sham-Tol-Sal group. However, there was no significant difference between the OVX-Tol-LA and OVX-Tol-Sal groups. It seems that L-arginine is able to diminish tolerance in the sham group, but it does not show the ability to decrease morphine tolerance in the OVX group. There are contradictory reports regarding the role of NO in tolerance to morphine and other opioids (Kolesnikov *et al.*, 1992; Toda *et al.*, 2009). Recently, it has been reported that morphine tolerance or its withdrawal syndrome is likely to be prevented by inhibition of NO synthesis (Toda *et al.*, 2009). However, some reports claimed that morphine tolerance is decreased by the enhancement of NO synthesis (Kolesnikov *et al.*, 1992). It has also been proved that NO acutely enhances the analgesic effects of opioids (Pataki and Telegdy, 1998; Heinzen and Pollack, 2004). All of these reports show that NO has a role in morphine-induced tolerance. Therefore, the results of the present study may imply that L-arginine antagonizes the effect of morphine when it is used in combination with morphine during tolerance induction in the Sham-Tol-LA group but not in the OVX-Tol-LA group. This finding may suggest that the effect of NO on the nervous system is dependent on gonadal hormones. It has been documented that ovariectomy reduces nNOS and eNOS expression and  $\text{Ca}^{2+}$ -dependent NOS activity, which are reversed by 17- $\beta$ -estradiol (Grohé *et al.*, 2004; Ceylan-Isik *et al.*, 2009). In the present study, NOS activity reduction in OVX rats may explain why co-administration of

L-arginine (the substrate for NOS) with morphine did not affect tolerance. However, further investigation including, for example, the determination of NOS activity, is needed to elucidate the exact mechanism(s) involved.

## 5 Conclusions

It is concluded that there are potential levels of interaction between sex hormones and the NO signaling systems in the regulation of morphine tolerance.

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