

## **The Effect of Swim Stress on Morphine Tolerance Development and the Possible Role of Nitric Oxide in this Process**

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### **Abstract**

It has been shown that stress and chronic pain could prevent the development of tolerance to morphine analgesia, which appears to be related to the activation of hypothalamus–pituitary–adrenal (HPA) axis, activation of neuroendocrine systems and changes in neurochemical levels. Moreover, the involvement of nitric oxide (NO) in the development of tolerance to morphine analgesia has been implicated. In the present study, we have tried to investigate the effect of swim stress, as a painless kind of stress, on the development of tolerance to find out whether the inhibition of tolerance is mediated by the direct effect of pain on the pain conduction pathway, or by its stress aspect. Besides, we evaluated the probable interactions between swim stress, nitric oxide level and the development of morphine tolerance. Adult male Wistar rats, weighing 180-220 g, were used in all these experiments. The experimental groups received chronic morphine (20 mg/kg, i.p), swim stress in 20°C water bath (4 min), or a combination of swim stress and chronic morphine (20 mg/kg, i.p), each for 4 days, while the first control group received saline (1 ml/kg, i.p) for 4 days. On the 5<sup>th</sup> day, all the experimental and control groups received a single dose of morphine (10 mg/kg i.p). The second control group received saline for 5 days. The intact group received only one single dose of morphine (10 mg/kg, i.p). All the mentioned groups were subjected to tail-flick and formalin tests on the 5<sup>th</sup> day. Other experimental groups were subjected to the assay for measuring nitrite as an indicator of NO, using the Griess method. Our results showed that co-administration of swim stress with chronic morphine prevented the development of morphine tolerance and the level of NO increased in the presence of swim stress ( $p < 0.001$ ). The combination of morphine and swim stress significantly decreased NO production in comparison with the chronic morphine administered group ( $p < 0.001$ ). These data suggest that the activation of HPA axis and consequently the suppression of (NO) production induced by chronic morphine, lead to the inhibition of morphine tolerance.

**Keywords:** Morphine; Swim stress; Tolerance; Nitric oxide; Analgesia.

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### **Introduction**

It has been reported that chronic opioid treatment could lead to the development of

antinociceptive tolerance in both human and laboratory animals (1), which is one of the major problems of using morphine as an analgesic drug. Clinical studies, however, have indicated that tolerance and dependence are not a major concern when opiates are used to control pain (2, 3). For instance, chronic morphine administered

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in the presence of formalin-induced pain does not lead to the development of analgesic tolerance in rats (3- 6).

There are some evidence indicating that the hypothalamic-pituitary-adrenal (HPA) axis is involved in the development of tolerance to morphine analgesia and could respond to stressful stimuli such as formalin induced pain (7-9). For example, it has been found that adrenocorticotropin hormone can prevent the development of tolerance to morphine analgesia (10) or stress produced by the same effect on intact mice, but not on the adrenalectomized mice (11). Both adrenalectomy (12) and hypophysectomy have been shown to potentiate the opiate tolerance and interestingly, the effects of hypophysectomy were reversed by the replacement of ACTH (13). These results raise the possibility that blockage of tolerance development by formalin-induced pain is related to the activation of HPA axis, following formalin injection. Supportive data by Vacarrino and Couret (3) showed that genetical differences in stress induced HPA activity, may contribute to differential development of tolerance to morphine analgesia during pain. The inhibition of tolerance development by formalin-induced pain depends on corticosterone activity, such that an increase in corticosterone triggered by the stress fullness of pain, acts to attenuate the tolerance development (14). Moreover, it has been demonstrated that chronic administration of opioids leads to the development of tolerance to their stimulatory effects on the HPA axis (15-17). The effect of opioids on the HPA axis is currently thought to be mediated, directly or indirectly, by the release of corticotropine releasing factor (CRF) (18).

Recent studies have suggested that opioid tolerance may also be mediated by increased production of nitric oxide (NO). NO has also been implicated in nociception processing (19). It has been shown that nitric oxide synthase (NOS) inhibitors attenuate tolerance development following chronic morphine administration (20-23). It has also been reported that increased NO production potentiates morphine analgesia and enhances the development of morphine tolerance in mice (24). NO production is one of the mechanisms that has been shown to be

involved in the development and maintenance of morphine tolerance, in addition to the activation of NMDA receptors, translocation and activation of protein kinase C (PKC) (25).

The localization of constitutive NOS (cNOS) in hypothalamic cells, which regulates the activity of the HPA axis, suggests that NO may play a physiological role in HPA axis activation following different stimuli. The role of endogenous NO in the regulation of the HPA axis activity remains controversial, because in different preparations contradictory results have been reported. Both stimulatory and inhibitory effects of NO on the stimulated release of CRH were observed in a short-term -culture of the hypothalamus tissue (26). On the other hand, stress induced NO production in the adrenal cortex, attenuates the adrenal corticosterone release stimulated by stress-induced ACTH release, or facilitates recovering of the increased corticosterone to the basal level (27). In support of this hypothesis, Pasternak et al. (28) reported the implication of NO in the development of tolerance and dependence to mu receptor agonists. They demonstrated that a NOS inhibitor blocked the development of tolerance to morphine (28, 29). NO may modulate the release of corticosterone from the adrenal cortex *in-vivo* and it has been found that immobilization induced stress, provoke a marked increase in neuronal NOS (nNOS) mRNA expression in the adrenal cortex (27, 30).

Therefore, considering the inhibitory effect of chronic pain on the development of tolerance to opioids, which is accounted by the stress aspect of pain and activation of HPA axis, the present study was designed to study the effect of swim stress, as a non painful stress, on the development of tolerance. In parallel, we also measured the amount of nitrite, as a metabolite of NO, to investigate the probable correlation between NO production and swimstress induced inhibition of morphine tolerance.

## Experimental

### Animals

Male Wistar rats (Pasteur Institute, Iran) weighing 180-220 g, which 3-5 rats housed per cage, were fed with a pellet diet and tap water

ad libitum. Environmental conditions were standardized ( $22 \pm 2^\circ\text{C}$  and 12 h artificial lighting per day). Experimental procedures confirmed to national and international protocols regarding animal welfare. Eight rats were used for each treatment group.

### Reagents

The chemicals used in this study were as follows:

Morphine Sulfate (Temad, Iran), N-(1-Naphthyl) Ethylene Diamine Dihydrochloride (Sigma, Germany); 5-Sulfosalicylic acid dihydrate; Sodium hydroxide; Natrium Nitrite (Merck, Germany), Ammonium chloride; Phosphoric acid; (Fluka, Switzerland)

### Methods

#### *Procedure of swimming stress*

The swim stress test was carried out in a cylindrical plastic container, 28 cm in diameter and 44cm in height. The level of water ranged between 30-35 cm above the floor, so that in all cases escape from the cylinder was impossible. The water temperature was monitored carefully and maintained at  $20 \pm 1^\circ\text{C}$ . To examine the effect of swim stress on the development of tolerance to morphine analgesia, rats were individually made to swim daily for 4 min in water for 4 days (31).

#### *Morphine Tolerance induction*

Rats were rendered tolerant by daily injections of morphine (20 mg/kg), dissolved in physiological saline and administered intraperitoneally (i.p) for 4 days (3).

#### *Assessment of morphine analgesia*

Twenty-four hours after the final injection for tolerance induction, pain sensitivity was assessed using the tail-flick and formalin tests as described previously (32), after administration of a single dose of morphine (10 mg/kg, i.p) on the 5<sup>th</sup> day.

#### *Measurement of nitrite*

Animals were decapitated to collect their blood. The serum samples were separated and stored at  $-70^\circ\text{C}$  until the measuring time. NO was measured using Griess reaction (33, 34).

### Test groups

Saline: These animals received saline for 5 days. Sal/M: These animals received saline for 4 days and a single dose of morphine (10 mg/kg, i.p) on the 5<sup>th</sup> day. M: These animals received morphine for 4 days (20 mg/kg, i.p) and a single dose of morphine (10 mg/kg, i.p) on the 5<sup>th</sup> day. SS/M: These animals received swim stress for 4 days and a single dose of morphine (10 mg/kg, i.p) on the 5<sup>th</sup> day. SSM/M: These animals received swim stress combined with morphine (20mg/kg) for 4 days and a single dose of morphine (10 mg/kg, i.p) on the 5<sup>th</sup> day. Intact: These animals received only a single dose of morphine (10 mg/kg, i.p) on the 5<sup>th</sup> day.

### Statistical analysis

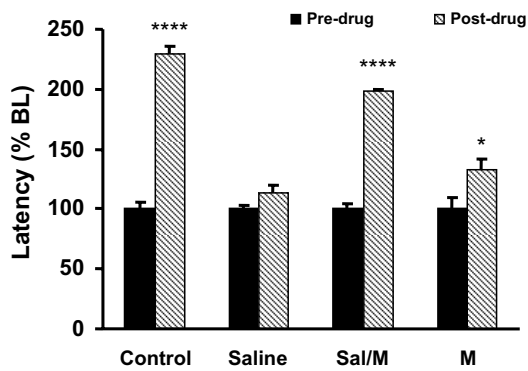
All the data were analyzed using paired and unpaired t-tests and the one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparison.  $P < 0.05$  was considered as significant.

## Results and Discussion

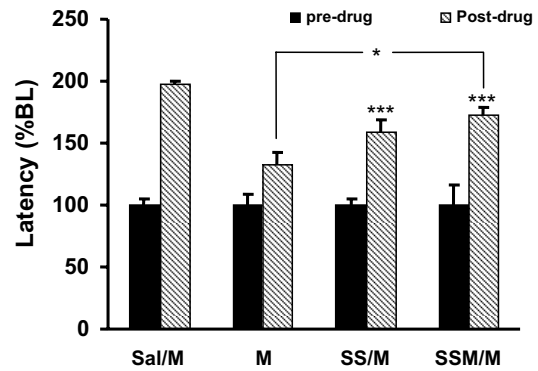
As shown in Figures 1 and 2, a significant tolerance to morphine (10 mg/kg, i.p) was induced in rats receiving chronic morphine (20 mg/kg, i.p for 4 days) in the absence of stress, but not in the rats receiving the similar doses of morphine in the presence of swim stress ( $p < 0.05$ ). There was no significant difference between the animals that received a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day (intact rats) and those which received swim stress for 4 days and a single dose of morphine on the 5<sup>th</sup> day.

The results of the formalin test indicated that in acute phase, the rats that received swim stress and those which received chronic morphine combined with the swim stress, both showed significant analgesia comparing to the animals that received saline or chronic morphine ( $p < 0.01$ ) (Fig. 3).

In chronic phase of the formalin test, the animals which received only swim stress or the combination of swim stress and chronic morphine, showed significant decrease in the development of tolerance in comparison with those that received chronic morphine ( $p < 0.01$



**Figure 1.** Repetitive administration of morphine (20 mg/kg) for 4 days results in tolerance development to the analgesic effect of morphine (10 mg/kg) in tail flick test. Control=received a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day, Saline=received saline (1 ml/kg) for 5 days, Sal/M=received saline (1 ml/kg) for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> day, M=received morphine (20mg/kg) for 4 days and a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day.  
\*P<0.05 \*\*P<0.01 \*\*\*\*P<0.001 \*\*\*\*P<0.0001



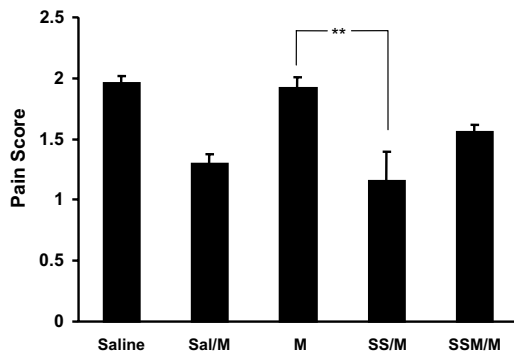
**Figure 2.** Swim stress delayed the development of tolerance to analgesic effect of morphine (10 mg/kg) in the tail flick test. Sal/M=received saline (1 ml/kg) for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> day, M=received morphine (20 mg/kg) for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> day, SS/M=received swim stress for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> day, SSM/M=received a combination of swim stress and morphine (20 mg/kg) for 4 days and a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day.  
\*P<0.05 \*\*P<0.01 \*\*\*\*P<0.001

and p<0.05, respectively) (Fig.4).

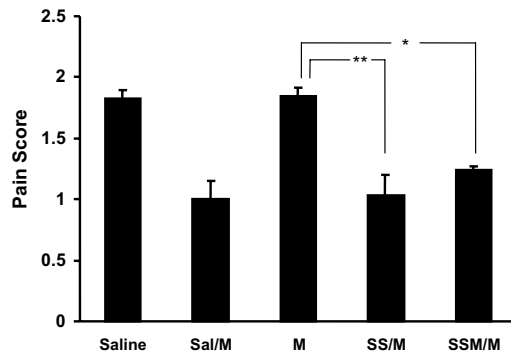
Our results showed that NO production increased significantly in the animals that received chronic morphine, compared with the control group (p<0.001). Moreover, NO production increased in the rats that received swim stress and those that received a combination of chronic morphine and swim stress, but this increase was significantly lower than that of the animals which received only chronic morphine (p<0.01, Fig.5).

It has been reported that chronic pain inhibits

the development of morphine tolerance and this effect is mediated through the stress aspect of pain and the activation of HPA axis. Chronic morphine administration can suppress HPA axis and leads to the development of tolerance. It should also be noted that stress could activate the opioid system (14). The inhibitory effect of foot shock and psychological stresses on the tolerance development has been demonstrated to be mediated through the Vasopressin–Arginine system (35). Stress, through adrenal



**Figure 3.** Swim stress delayed the development of tolerance to analgesic effect of morphine (10 mg/kg) in the first phase of formalin test. Saline=received saline (1 ml/kg) for 5 days, Sal/M=received saline (1 ml/kg) for 4 days and a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day, M=received morphine (20 mg/kg) for 4 days and a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day. SS/M=received swim stress for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> dy, SSM/M=received a combination of swim stress and morphine (20 mg/kg) for 4 days and a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day.  
\*P<0.05 \*\* P<0.01

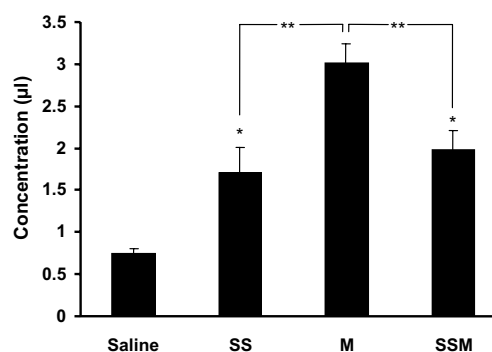


**Figure 4.** Swim stress delayed the development of tolerance to the analgesic effect of morphine (10 mg/kg) in the second phase of formalin test. Saline=received saline (1 ml/kg) for 5 days, Sal/M=received saline (1 ml/kg) for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> day, M=received morphine (20 mg/kg) for 4 days and a single dose of morphine (10mg/kg) on the 5<sup>th</sup> day. SS/M=received swim stress for 4 days and morphine (10 mg/kg) on the 5<sup>th</sup> day, SSM/M=received a combination of swim stress and morphine (20 mg/kg) for 4 days and a single dose of morphine (10 mg/kg) on the 5<sup>th</sup> day.  
\*P<0.05 \*\*P<0.01

glucocorticoids and ACTH, can prevent the development of tolerance to morphine analgesia (10, 11) and adrenalectomy or hypophysectomy enhances the development of analgesic tolerance (12, 13). The effects of hypophysectomy could be eliminated by the administration of ACTH (13). Therefore, it is possible that stress induced HPA activity contributes to the inhibitory effect of pain on the development of morphine tolerance (3). Finally, it has been shown that low doses of  $\beta$ -endorphin and morphine increase the secretion of corticosterone, while higher concentrations of opiates (e.g. the chronic administration of morphine) decreases it (36, 37). The inhibitory effect of morphine on the corticosterone secretion in pups exposed to chronic morphine reflects both decreased release of ACTH and direct inhibition of steroid synthesis caused by the chronic morphine regimen (38).

Our results showed that swim stress can attenuate the development of morphine tolerance in both tail flick (Fig. 1 and 2) and formalin tests (Fig. 3 and 4).

Treatment of rats with swim stress and the combination of chronic morphine and swim stress resulted in an increased NO production, which was significantly lower than that produced in the animals that received only chronic morphine (Fig. 5). These results indicate that swim stress could attenuate NO production probably through activation of HPA axis, which consequently results in the increased secretion of ACTH. It has also been reported that the activation of ATP sensitive potassium channels following NO production participates in development of tolerance to morphine analgesia (39). NO inhibitors could prevent the development of morphine tolerance (40) and administration of N-methyl-D-aspartate receptor antagonist and NO synthesis inhibitors, can suppress the development of morphine tolerance. Moreover, in the spinal cord of the rats rendered tolerant to morphine, the expression of nNOS increases (41). These data suggest that NO plays a role in the development of morphine tolerance. Bugajski et al. (26) suggested that NO may play a physiological role in the response of HPA axis to different stimuli. The role of endogenous NO in regulating the activity of the HPA axis remains controversial, as contradictory results



**Figure 5.** Nitric oxide levels in different groups treated for 4 days with saline 1 ml/kg (Saline), Morphine 20 mg/kg (M), Swim stress (SS) and a combination of swim stress and morphine 20 mg/kg (SSM). Nitric oxide was subjected to the assay 24 h after the last treatment.

\* $P < 0.05$  \*\* $P < 0.01$

have been reported in different preparations.

Blockage of NO synthesis significantly impairs the release of ACTH in response to a mild electroshock and water avoidance stress, which causes rapid activation of the HPA axis (26). There are some reports that the inhibition of NOS can potentiate continuous cold water swimming (CCWS) antinociception, which indicates that the inhibition of NOS could probably affect a selective form of pain inhibition (19). N-nitro-L-arginine (L-NA) or its methyl ester (L-NAME) could potentiate antinociception elicited by the continuous cold water swimming (19, 42).

NO antagonists have been reported to reduce the development of morphine antinociceptive tolerance (22, 23). Hence, the effects of swim stress on morphine tolerance are probably the result of the inhibition of NO production. Although a moderate increase in NO production was observed following the swim stress, there was no change in morphine analgesia in this group of animals. As a result, it seems that a moderate increase in NO production alone is not sufficient for tolerance development. Chronic administration of morphine produced a significant increase in NO production, which reduced to a moderate level when morphine was combined with the swim stress. Therefore, large increases in NO levels may be responsible for tolerance development and its inhibition, even to a moderate level by the swim stress, could delay the tolerance process.

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