FABAD J. Pharm. Sci., 48, 2, 183-200, 2023
Doi: 10.55262/fabadeczacilik.1177914
RESEARCH ARTICLE

The Effect of *Troxerutin* Intraperitoneal Consumption on the Symptoms of Morphine Withdrawal Syndrome in Male Mice

Nasrin Hosseinzad MANIE[*], Ramin Ghasemi SHAYAN[**o]

The Effect of Troxerutin Intraperitoneal Consumption on the Symptoms of Morphine Withdrawal Syndrome in Male Mice

SUMMARY

*Chronic morphine use is associated with increased oxidative stress and inflammatory factors. Troxerutin is a natural bioflavonoid containing antioxidant effects that could relieve morphine withdrawal syndrome. The aim is to experiment with the effects of troxerutin on morphine dependence in mice. Troxerutin was prepared in three doses (50, 100, and 200 mg/kg) via a normal saline solution. The experiment was performed in five groups of 7 mice. One group received eight days of increasing doses (10, 20, 30, 40, 50, and 60 mg/kg) of morphine subcutaneously with normal saline (10 ml/kg) intraperitoneal, one group received only normal saline, and the other three groups received three different doses of troxerutin solved in normal saline with morphine. On the ninth day, withdrawal symptoms were recorded after the naloxone injection and blood samples were examined. Consequently, the total withdrawal score in 50 mg/kg was p<0.001***, and in the 100 mg/kg Troxerutin-morphine group was p<0.01**, significantly lower than the morphine-saline group. Antioxidant tests showed a significant increase in the level of Total Antioxidant Capacity (TAC) (p<0.001***) and a decrease in the level of Malondialdehyde (MDA) of serum (p<0.001***) in all three doses of troxerutin. In the locomotion test, no significant motility dysfunction or paralysis was observed in mice after using troxerutin. (All P>0.05). Briefly, Troxerutin reduces the symptoms of morphine withdrawal syndrome. The results of antioxidant tests declared that troxerutin possibly due to its antioxidant properties, increases the level of TAC and decreases the level of MDA in the serum of mice.*

Key Words: *Morphine, troxerutin, withdrawal syndrome, dependence, MDA, TAC*

Erkek Farelerde Trokserutinin İntraperitoneal Uygulamasının Morfin Yoksunluk Sendromu Belirtileri Üzerine Etkisi

ÖZ

*Kronik morfin kullanımı artmış oksidatif stres ve inflamatuar faktörler ile ilişkilidir. Trokserutin doğal bir biyoflavonoiddir ve morfin yoksunluk sendromunu hafifletebilecek antioksidan etkilere sahiptir. Amaç, trokserutinin farelerde morfin bağımlılığı üzerindeki etkilerini incelemektir. Trokserutin, normal salin solüsyonu ile üç doz (50, 100 ve 200 mg/kg) halinde hazırlanmıştır. Deney, 7'şer fareden oluşan beş grupta gerçekleştirildi. Bir grup sekiz gün artan dozlarda (10, 20, 30, 40, 50 ve 60 mg/kg) subkütan morfin ve intraperitoneal normal salin (10 ml/kg) aldı. Bir grup sadece normal salin aldı. Diğer üç grup morfin ile birlikte normal salin içinde çözülmüş üç farklı doz trokserutin aldı. Dokuzuncu gün nalokson enjeksiyonu sonrası yoksunluk belirtileri kaydedildi ve kan örnekleri incelendi. Sonuç olarak, toplam yoksunluk skoru 50 mg/kg'da p<0,001*** ve 100 mg/kg Trokserutin-morfin grubunda p<0,01** hesaplandı, morfin-salin grubuna göre anlamlı derecede düşük bulundu. Antioksidan testleri, her üç trokserutin dozunda da Toplam Antioksidan Kapasitesi (TAC) seviyesinde önemli bir artış (p<0.001***) ve serum Malondialdehit (MDA) seviyesinde bir düşüş (p<0.001***) gösterdi. Hareket testinde trokserutin kullanıldıktan sonra farelerde belirgin bir motilite disfonksiyonu veya felç gözlenmedi. (Hepsi P>0.05). Kısaca, trokserutin, morfin yoksunluk sendromu belirtilerini azalttı. Antioksidan testlerinin sonuçları, trokserutinin muhtemelen antioksidan özelliklerinden dolayı fare serumundaki TAC seviyesini artırdığını ve MDA seviyesini düşürdüğünü ortaya koymuştur.*

Anahtar Kelimeler: *Morfin, trokserutin, yoksunluk sendromu, bağımlılık, MDA, TAC*

Received: 21.09.2022
Revised: 17.02.2023
Accepted: 27.02.2023

[*] ORCID: 0000-0001-6895-4271, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran,
[**] ORCID: 0000-0002-7850-0756, Radiology Department, Paramedical Faculty, Tabriz University of Medical Sciences, Tabriz, Iran,

[o] Corresponding Author;Ramin Ghasemi Shayan
raminghasemi1377@gmail.com, +989145755109

INTRODUCTION

Pain is an unpleasant sensation that informs the person about the existence of injuries to body tissues and the occurrence of possible dangers, which is a natural defense mechanism. Pain is, in most cases, self-limiting. But if this condition persists and is not relieved, the pain itself is a disorder (Cappendijk, de Vries, & Dzoljic, 1993; Hosseinzadeh & Jahanian, 2010; Hosseinzadeh & Nourbakhsh, 2003; Vela, Ruiz-Gayo, & Fuentes, 1995).

Opioid drugs like morphine are derivatives of natural, semi-synthetic, and synthetic alkaloids that act on the body's major opioid receptors mu (μ), delta (δ), and kappa (κ). They express their analgesic effect and regulate the sensation of pain by acting on the N-methyl-D-aspartate (NMDA) and substance P receptors to relieve the unpleasant feeling of pain (Maldonado, Negus, & Koob, 1992).

Morphine is an alkaloid obtained from the poppy plant scientifically named *Papaver somniferum* and acts on endogenous opioid receptors (Etemadzadeh, 1993). Morphine is frequently used in surgeries and procedures and also in severe cases of several sicknesses when the patient is not responding to any painkiller. Then, the last phase of soothing pain comes with the use of morphine or other opioid drugs of this family like pethidine or oxycodone to reduce the disagreeable pain feeling. Nevertheless, the use of morphine must not be chronic since it will cause other unrepairable complications.

So far, several mechanisms have been proposed for the development of morphine dependence and the development of withdrawal symptoms (Etemadzadeh, 1993; Maldonado et al., 1992; Maldonado et al., 1997). Among the mechanisms involved in the phenomenon of dependence, an increase in inflammatory cytokines, and an increase in the amount of nitric oxide (NO) in the central amygdala, are important determined factors (Hosseinzadeh & Jahanian, 2010).

Yet, there are multiple probable mechanisms due to the references which are discussed below in the process of morphine dependence leading to withdrawal syndrome. Still, these various mechanisms should be investigated more in detail to recognize the precise operative lane of causing dependence to minimize its issues.

When there is acute use of morphine, the level of Cyclic-adenosine-mono phosphate (cAMP) decreases, while chronic use of morphine would increase the level of cAMP by negative feedback through the activation of the adenylate cyclase enzyme (Masood, Schäfer, Naseem, Weyland, & Meiser, 2020). As a result, electrical discharge of neurons occurs and morphine dependence happens. Moreover, morphine would increase the activity of the glutamatergic system; thus the obstacle of controlling stress and anxiety would disappear and this leads to dependence. Besides that, chronic use of morphine would cause down-regulation of the receptors. Finally, morphine could cause oxidative stress. There are multiple mechanisms for creating oxidative stress. One of them might be the increase of the NO synthase (NOS) hydroxyl synthesis enzyme. As a result, there will be a great climb in the level of NO (a free radical agent) which could cost oxidative stress. Furthermore, chronic morphine use would directly increase the level of superoxide (a free radical agent) in the kidney. Another mechanism would be the increase in lipid peroxidation. Oxidative stress occurs when there is an imbalance between the reactive species and the inhibitory system of these toxins. Free radicals cause neuropathic pain and behavioral disorders. Oxidative stress also disrupts cellular communication which on severe occasions, could cause necrosis.

On the other hand, chronic morphine use has been shown to increase pro-inflammatory mediators such as interleukins and TNF-α. These mediators increase the activity of cyclooxygenases, which is one of the possibilities of morphine dependence (Yamaguchi et al., 2001; Quimby & Luong, 2007).

Anyhow, *troxerutin* (tri-hydroxyl-ethyl rutin) is a natural plant bioflavonoid that prevents damage to the tissues (Maurya, Salvi, & Krishnan Nair, 2004; Sui, Zang, & Bai, 2019). *Troxerutin* inhibits the painful oxidative pathways and inflammatory cytokines and plays an important role in relieving the symptoms of morphine-induced withdrawal syndrome (Zhang et al., 2009). *Troxerutin* is originally obtained from the herbaceous plant *Sophora japonica* of the *Fabaceae* family, which is native to China and Japan and is widely distributed in Asia, Oceania, and the Pacific Islands (Adam, Pentz, Siegers, Strubelt, & Tegtmeier, 2005).

Antioxidant, anti-tumor, sedative and soothing, antimicrobial, analgesic, and anti-inflammatory properties have been reported in it (Maurya et al., 2004; Adam et al., 2005; Zhang et al., 2009; Farajdokht et al., 2017). *Troxerutin* has been used in studies to treat chronic venous insufficiency by improving capillary function, which prevents abnormal capillary leakage and capillary vascular insufficiency (Turton, Kent, & Kester, 1998; Adam et al., 2005; Zhang et al., 2009; Farajdokht et al., 2017). It is also very effective in relieving the nervous system of mice by relieving oxidative stress caused by D-galactose and improving the activity of Phosphoinositide 3-kinase / Akt (Turton et al., 1998; Maurya et al., 2004; Adam et al., 2005; Farajdokht et al., 2017).

Therefore, considering all these influences of *troxerutin*, in the present study, the effect of chronic *troxerutin* use on morphine dependence in male mice was investigated. The main option of *troxerutin* in this thesis is considered its antioxidant properties though it could regulate the oxidative stress system and maintain health, which might include dependence.

Troxerutin in the following experiment is not extracted from the plants; instead, it is in a ready and commercial capsule form. Subsequently, the changes in the levels of total antioxidant capacity (TAC) and malondialdehyde (MDA) in the serum of male mice after morphine administration have been experimented with. According to the design hypotheses, chronic use of *troxerutin* with morphine was expected to reduce the symptoms of morphine withdrawal syndrome. Furthermore, it was expected to increase the level of TAC and decrease the level of MDA in the serum of animals.

MATERIAL AND METHOD

In this design, the mice were kept in groups of 7 in transparent polypropylene cages with steel rods. First of all, disposable latex gloves and masks had to be used. To induce dependence syndrome, 10 mg/ml of morphine sulfate ampoule (Iran Pharmaceutical Company) was used based on the weight of each mouse. Naloxone ampoules (0.4 mg/ml) were injected into mice based on their weight before calculating withdrawal symptoms. For a 25 g mouse, we needed 0.125 mg of naloxone. As naloxone ampules were 0.4 mg/ml, 0.125 mg of naloxone gave us 0.3125 ml which had to be injected into a 25 g mouse. It could be arranged for each mouse owning to its precise weight. (Meybodi, Zarch, Zarrindast, & Djahanguiri, 2005; Wiebelhaus, Walentiny, & Beardsley, 2016; Lewter et al., 2022). *Troxerutin* 300 mg capsules commercially produced by Actavis Company were used. To dissolve the powder inside the capsule, as a solvent and the carrier of *troxerutin*, a 0.9% sodium chloride solution for injection was used. Then the capsule (300 mg) of *troxerutin* was opened and the powder inside was weighed accurately with electric scales (Guo, Wang, Bi, & Sun, 2005). Of course, the entire powder included not only *troxerutin* but also the other ingredients (filler, lubricant, binder, disintegrants, and…) of the capsule. However, to reach the dosages (50, 100, and 200 mg/kg) of *troxerutin*, we had to use proportionality ratios to calculate how much we have to extract from that amount of powder dissolved in saline. So it was estimated based on the weight of each mouse (Guo et al., 2005). So the entire powder was dissolved in normal saline and for each dose of *troxerutin* for each mouse, it was calculated how much of that mixture (powder-saline) should be extracted. Meanwhile,

for the morphine amount calculation, the ampules of morphine were 10 mg/ml. The adjusted increasing doses of morphine (20, 30, 40, 50, and 60) were calculated as 20 mg of morphine in 1000 gr would bring 0.6 mg of morphine for a 30 g mouse. Subsequently, 10 mg of morphine in 1 ml would cause 0.06 ml of morphine. Other dosages could be estimated based on the weight of each mouse (Alaei, Esmaeili, Nasimi, & Pourshanazari, 2005; Hassan et al., 2020). Electric scales were used to weigh the mice every day before the procedures since most of the animals had lost weight by taking chronic morphine. We used a stopwatch to increase the accuracy of the process of making signs and counting the animal's movements throughout the test. Also, after each injection, the contents of the morphine ampoule and the dissolved *troxerutin* powder were stored in the refrigerator. An insulin syringe was used to inject morphine and *troxerutin* into the animals and a 5-cc syringe was used to draw the contents of the morphine ampoule and normal saline solution. A cylindrical and glass enclosure was used to place the animals and prevent them from escaping to count and carefully examine the symptoms. Then blood samples were taken from the apex of the hearts of the animals and poured into bracket tubes using 2-cc syringes, respectively. The serum and blood cells were separated by centrifugation and the serums were stored in a freezer at -70 ° C for 24-48 hours and finally tested in a special laboratory with special kits and the results of TAC and MDA were reported (Oskuye et al., 2019; Ahmadi, Mohammadinejad, Roomiani, Afshar, & Ashrafizadeh, 2021).

Grouping

In this study, 5 groups of 7 male adult albino mice weighing 25-30 grams mice were used according to the mentioned conditions:

First Group (the Control Group (Morphine + Saline))

In a group of 7 mice, 10 ml/kg saline was first injected IP every 12 hours for 8 days. Morphine was injected SC every 12 hours for half an hour after saline injection every day. On the first day (20 mg/kg), second and third day (30 mg/kg), fourth and fifth day (40 mg/kg), sixth and seventh day (50 mg/kg), and on the eighth and ninth day (60 mg/kg) of morphine injection was performed. On the ninth day, morphine was injected only in the morning. On the ninth day, 1 hour after the morning dose of morphine, mice had been injected with a dose of naloxone (5 mg/kg) IP and showed signs of withdrawal syndrome (number of jumping, standing on feet, body grooming, genital grooming, teeth chattering, wet-dog-like shakes and abdomen writhing), which are essential parameters for assessing dependence. For 30 minutes, the number of symptoms was counted and recorded separately via filming for accuracy. The results of this group were considered as the basic responses of the animals and their comparison with other groups.

Second Group (The Control Group (Saline + Saline)

In a group of 7 male mice to evaluate the appropriateness of the dependence method, just like the previous group, but instead of morphine, normal saline was injected incrementally every 12 hours for 9 days at a dose of 10 ml/kg SC. Normal saline 10 ml/kg was also injected IP every 12 hours. On the ninth day, 1 hour after the morning dose of saline, a dose of naloxone (5 mg/kg) was injected IP, and withdrawal symptoms were recorded for 30 minutes. The results of this group indicate the behavior of the animal in this study. It should be noted that saline is considered the carrier of troxerutin.

Third, Fourth, and Fifth Groups (Groups Receiving Three Different Doses of Troxerutin with Morphine (Troxerutin Doses: 50, 100, and 200 mg/kg)

The respective groups consisted of three groups of 7 male mice that received increasing doses of morphine every 12 hours for 9 days. Troxerutin commercial capsules (Actavis Company) were opened and the powder was weighed and dissolved in the carrier of troxerutin (normal saline which was completely solu-

ble) (Elangovan & Pari, 2013; Kaeidi et al., 2020). The mice had only morphine and naloxone injections on the ninth day, and subsequently, withdrawal symptoms were recorded for half an hour.

Investigation of the Effect of *Troxerutin* on Animal Motility

A locomotion test was performed in morphine + saline groups and the groups received three doses of *troxerutin* with morphine. On the ninth day, before the morphine injection, a locomotor activity test was performed by the open-field method. A box measuring 40 × 40 cm was designed, which was divided into 25 smaller squares of the same size of 8 × 8 cm with a marker. To determine whether *troxerutin* would cause paralysis or other movement dysfunctions and abnormalities, the mice were consecutively placed in the middle of the screen of the box, and immediately after the stopwatch was activated, by checking the time, it was determined that the mice would cut the lines several times in 20 minutes. Each time the animal was crossing the lines, it was a point. At the end of the experiment, the total times it had cut the lines was estimated.

Registration of Withdrawal Symptoms

To evaluate the withdrawal symptoms after naloxone administration, the animals were placed individually under a clear glass cylindrical chamber, and withdrawal symptoms included: number of jumping, standing on feet, genital grooming, abdomen writhing, body grooming, teeth chattering, and wet dog shakes were recorded and evaluated by the camera for 30 minutes. Naloxone had to be injected at 5 mg/kg.

Calculate the Total Score of Withdrawal Syndrome (TWS)

The total score of withdrawal syndrome symptoms was calculated to summarize the symptoms and obtain an index for the set of recorded symptoms and determine the severity of withdrawal syndrome based on previous studies and a modified system of other researchers. For each index, it was divided by the standard value (Table 1).

These numbers were then summed for each mouse and averaged for each group. The sum of these symptoms was reported as the total score of withdrawal symptoms. To eliminate the differences in the animals' responses to the different withdrawal symptoms, using this relationship helps us to have a general indicator to show the effect of the drug on the onset and severity of the symptoms.(Gordon, 2004; Habibi Asl, Ahmadi, Hasanzadeh, & Charkhpour, 2007; Lammers, Kruk, Meelis, & Van der Poel, 1988; Parvizpour et al., 2013)

Table 1. Table of values given to different symptoms of Withdrawal Syndrome

Behavior (Symptom of morphine withdrawal syndrome)	**Weight factor (Value given to the desired mark)**
Jumping	4
Wet dog shake	5
Abdomen writhing	5
Head shake	5
Handshake	5
Genital rooming	5
Body grooming	10
Facial grooming	10
Teeth chattering	10
Swallowing	10
Standing on feet	20

Blood Sampling Method

At the end of the morphine and troxerutin injection courses in all groups of mice, each mouse was anesthetized with ketamine-midazolam; ketamine at a dose of 100 mg/kg and midazolam at a dose of 5 mg/kg were used. Then 1 ml of blood was taken from their heart apex. The sample was stored in a test tube without the presence of an anticoagulant at room temperature for 20 minutes until blood clotted. The clotted sample was then centrifuged at 3000 rpm for 7 minutes and after separating the blood serum, it was poured into a 1 ml microtube and stored in a -70 ° C freezer for the above tests.

Malondialdehyde (MDA)

The basis of serum MDA measurement method is based on a reaction with thiobarbituric acid (TBA), extraction with normal butane, absorption measurement by spectrophotometric method, and comparison of absorption with a standard curve.

Preparation of Solutions

1% orthophosphoric acid: This solution was prepared in a 250 ml balloon by dissolving 85% phosphoric acid and bringing it to volume using deionized water. Thiobarbituric acid 0.67%: 1.675 g of thiobarbituric acid ($C_2H_4N_2O_2S$) was collected in a 250 ml balloon with deionized water and used freshly prepared.

Test Method

500 µl of serum was dissolved in 3 ml of 1% phosphoric acid. After vortexing, 1 ml of 0.675% thiobarbituric acid was added to the test tube and after complete vortex, placed inside a boiling marijuana pan for 45 minutes. After that, the tubes were cooled under cold water and were added 3 ml of normal butanol was, and vortexed for 1 to 2 minutes. Then for 10 minutes, at 3000 rpm (round-per-minute), they were centrifuged and after separating the organic phase (supernatant), light absorption was measured at 532 nm against normal butane blank and the results were determined after transferring to the standard curve and were reported as serum MDA concentration of the samples.

Total Antioxidant Capacity (TAC)

It is a way to measure the antioxidant capacity of all the antioxidants in a biological sample.

Concentration of Reagents

The concentrations of reagents used in the present study are listed below (Table 2):

Table 2. The concentration of reagents in the TAC test

80mmol/l	Phosphate Buffered Saline pH7.4	Buffer
6.1μmol/l	Metmyoglobin	Chromogen
6.1μmol/l	2,2'-and-bis (3-ethyl benzothiazoline-6-sulphonic acid)	
250 μmol/l	Hydrogen Peroxide (in Stabilised form)	Substrate
Lot specific	6-Hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid	Standard

Solution Preparation

Chromogen: 1 vial of chromogen was mixed with 10 ml of buffer.

Substrate: 1 ml of Substrate was diluted with 1.5 ml of buffer.

Standard: 1 vial of the standard was diluted with 1 ml of deionized distilled water.

Test Method

First, MDA was measured by spectrophotometer using thiobarbituric acid (TBA) method and then the TAC test was performed. This method was performed by antioxidant capacity measuring kits, which is a simple, repeatable, and standard method. These kits work by the colorimetric method and are based on the reduction of Fe^{3+} to Fe^{2+} by the antioxidant compounds of the sample, which are paired with suitable thermogenesis and produced a colored product.

In the cuvette sample: 20 µl of the sample was mixed with 1 ml of chromogen.

In the cuvette Blank: 20 µl of DDH2O was mixed

with 1 ml of chromogen.

In the standard cuvette: 20 µl of the standard was mixed with 1 ml of chromogen.

At a wavelength of 600 nm in the Alcyon 300 at 37 ° C and in front of the air, the initial light absorption of the cuvettes was measured (A1).

Then 200 µl of substrate was added to each cuvette and after three minutes the light was absorbed again. (A2)

Calculations: (formula1)

A2 - A1 = ΔA of sample/standard/blank

Factor = $conc\ of\ standard/\ (\Delta A\ blank-\ \Delta A\ standard)$

Mmol/l = factor × $(\Delta A\ Blank\ -\ \Delta A\ Sample)$

Statistical Analysis

Statistical analysis using Sigma plot version 12.2 software was done. The results of recording analgesic effects were expressed as (Mean ± S.E.M). One Way ANOVA test was applied to emphasize just one factor (*troxerutin* effects) and the Tukey post-test was used to compare the results of more than two groups more accurately in male mice. The minimum difference between groups with p <0.05 was considered statistically significant. P <0.05 was considered the level of statistical significance. So that p <0.05 *, p<0.01 ** and p<0.001 *** were reported.

Ethical Approval Information

Under the approval of the Faculty of Pharmacy Thesis Research Council of the Iran Pharmacist Association and the Regional Ethics Committee of Pazhoohan in the Tabriz University of Medical Science, this thesis has been approved on the date of 2020.08.31 with the ethical code of (IR. TBZMED. VCR.REC.1399.167) and with the tracking code of 65384.

RESULTS AND DISCUSSION

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Jumping) in Male Mice

According to the results of this study, intraperitoneal injection of *troxerutin* significantly increased the number of jumping following naloxone administration in the group receiving 200 mg/kg *troxerutin* with morphine, with p <0.01 ** compared with the morphine-saline control group. (Figure 1)

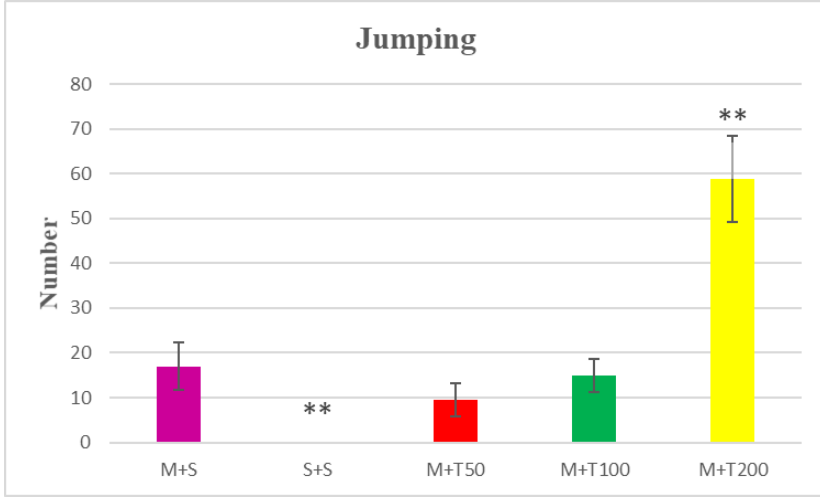

Figure 1. The effect of different doses of *troxerutin* on the number of jumping movements in male mice in comparison with the control group receiving morphine + saline and the group receiving saline + saline. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.01**) compared to the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Times Standing on Feet) in Male Mice

Based on the results of this study and statistical comparison, because the responses obtained from the groups were abnormal, the data obtained from this symptom were not used to calculate the total withdrawal score.

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Genital Grooming) in Male Mice

According to the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving all three doses of 50, 100, and 200 mg/kg of *troxerutin* with morphine, with p <0.001 ***, compared with the control group receiving morphine-saline in significantly reduced the number of genital grooming. (Figure 2)

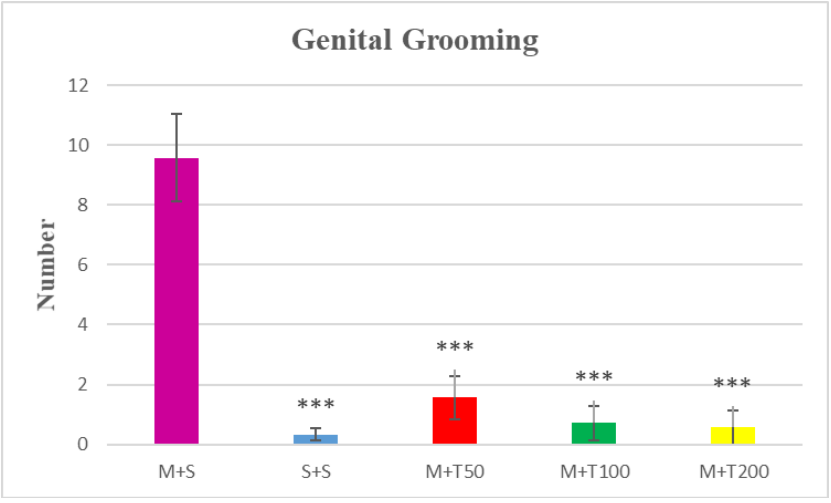

Figure 2. The effect of different doses of *troxerutin* on the number of genital grooming movements in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Body Grooming) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving 50 mg/kg and 100 mg/kg of *troxerutin* with morphine, with p <0.001 ***, and in the group receiving 200 mg/kg of *troxerutin* with morphine with p <0.01 ** compared to the control group, morphine-saline recipients significantly reduced the number of body grooming (Figure 3).

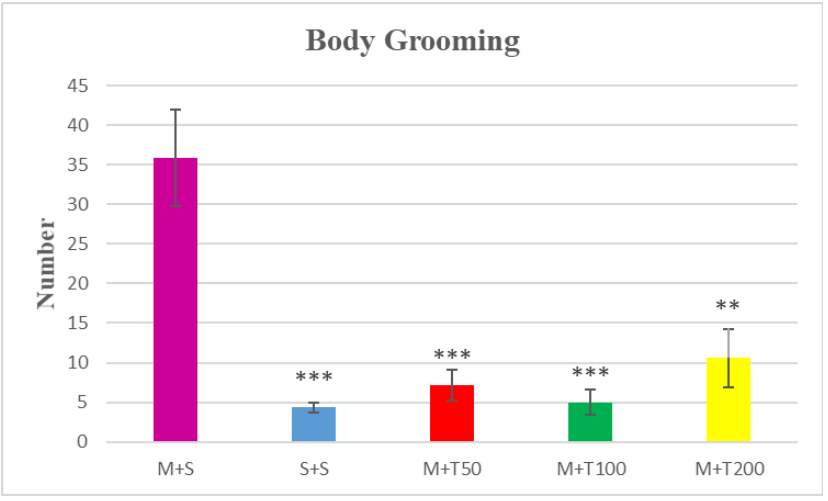

Figure 3. The effect of different doses of *troxerutin* on the number of body grooming movements in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as ($p < 0.001^{***}$) and ($p < 0.01^{**}$) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Teeth Chattering) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving 50 mg/kg and 200 mg/kg *troxerutin* with morphine, with $p < 0.001$ *** compared with the control group receiving morphine-saline significantly reduced the number of teeth chattering. Also, no significant difference ($p < 0.05$) was observed in the group receiving 100 mg/kg *troxerutin (Figure 4).*

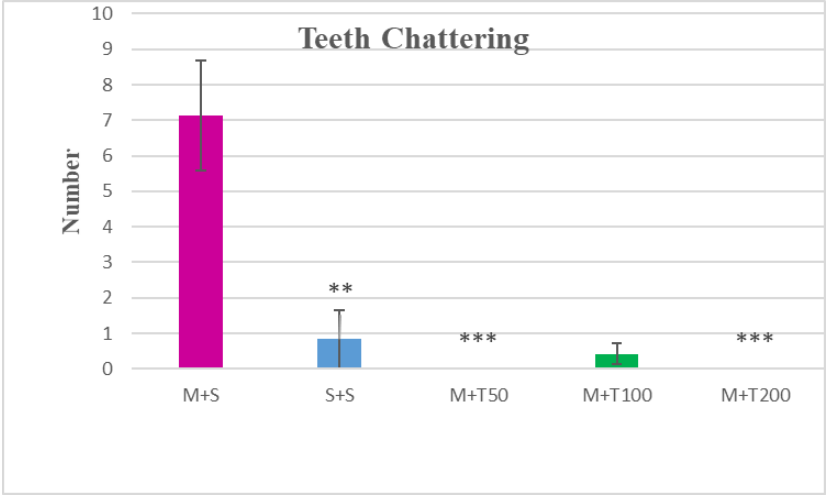

Figure 4. The effect of different doses of *troxerutin* on the number of teeth chattering movements in male mice in comparison with the control group receiving morphine + saline and the group receiving saline + saline. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as ($p < 0.001^{***}$) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Wet Dog Shakes) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving all three doses of 50, 100, and 200 mg/kg of *troxerutin* with morphine, with p <0.001 ***, compared with the control group receiving morphine-saline significantly reduced the number wet dog shake movements (Figure 5).

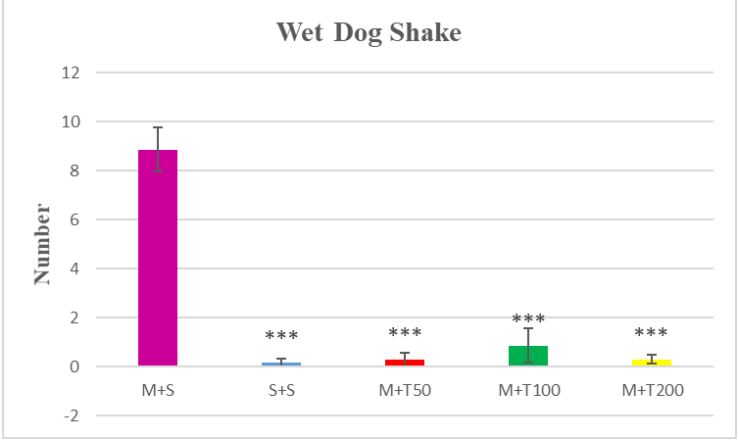

Figure 5. The effect of different doses of *troxerutin* on the number of wet dog shake movements in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Morphine Withdrawal Symptoms (Number of Abdominal Writhing on the Ground) in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the 50 and 200 mg/kg *troxerutin* dose groups with morphine, with p <0.01 **, significantly decreased the number of abdominal writhing compared to the morphine-saline control group. Also, no significant difference (P <0.05) was observed in the group receiving a 100 mg/kg dose (Figure 6).

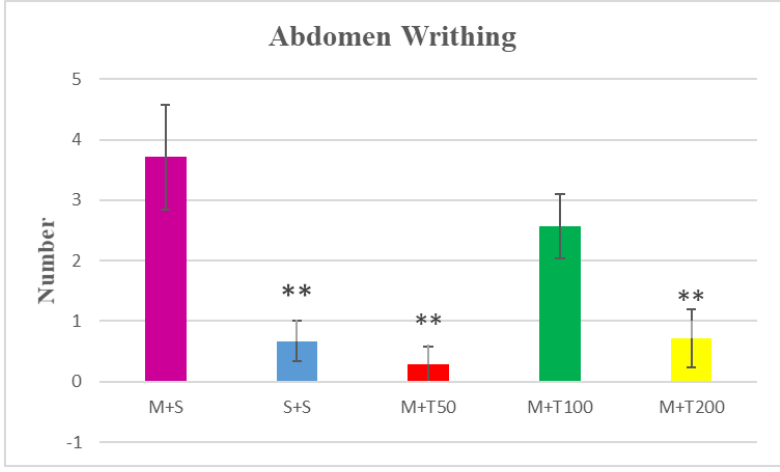

Figure 6. The effect of different doses of *troxerutin* on the number of abdominal writhing movements on the ground in male mice in comparison with the control group receiving morphine + saline and the group receiving saline + saline. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.01**) compared to the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on the Total Symptoms of a Withdrawal Syndrome in Male Mice Based on Total Withdrawal Score (TWS)

Based on this behavioral study, intraperitoneal administration of *troxerutin* with morphine at a dose of 50 mg/kg with *troxerutin* with morphine at p <0.001 *** and at a dose of 100 mg/kg with morphine at p <0.01 ** compared with the morphine-saline control group, it significantly reduced the symptoms of withdrawal syndrome (Figure 7).

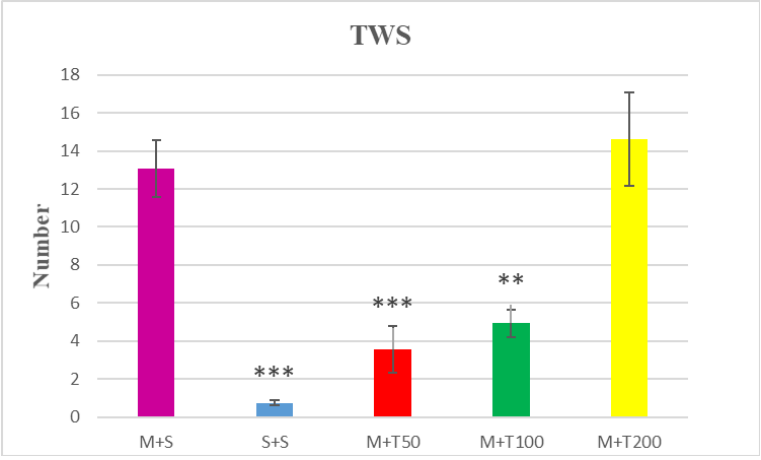

Figure 7. The effect of different doses of *troxerutin* on total withdrawal score in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) and (p <0.01**) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M = Morphine, S=Saline, T=Troxerutin

The Effect of Different Doses of *Troxerutin* on Serum TAC Levels in Male Mice

Based on the results of this study, intraperitoneal injection of *troxerutin* in the groups receiving all three doses of 50, 100, and 200 mg/kg of *troxerutin* with morphine, with p <0.001 ***, compared with the control group receiving morphine-saline significantly increased the TAC levels of serum (Figure 8).

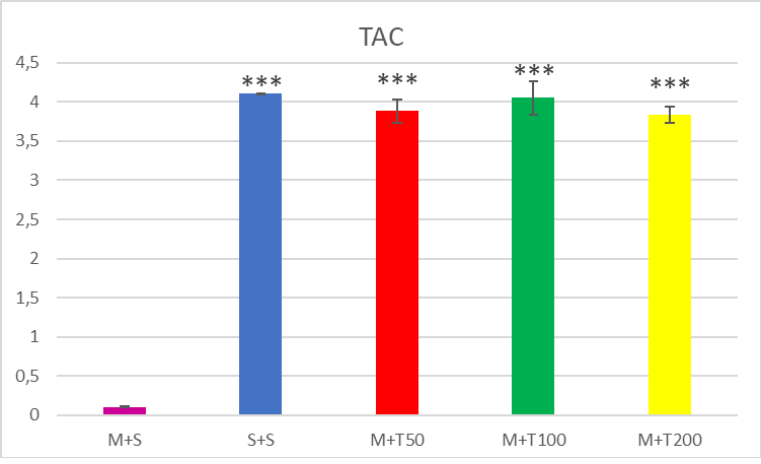

Figure 8. The effect of different doses of *troxerutin* on serum TAC levels in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M= Morphine, S= Saline, T= Troxerutin

The Effect of Different Doses of *Troxerutin* on Serum MDA Levels in Male Mice

Based on this behavioral study, intraperitoneal administration of *troxerutin* with morphine at a dose of 50, 100, and 200 mg/kg with *troxerutin* with morphine at p <0.001 *** compared with the morphine-saline control group, significantly decreased the MDA levels of serum (Figure 9).

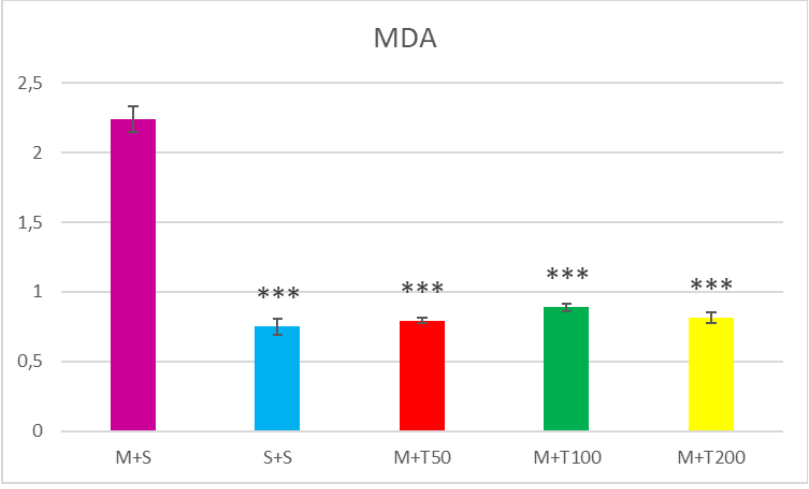

Figure 9. The effect of different doses of *troxerutin* on serum MDA levels in male mice compared with the morphine + saline control group and the saline + saline group. The results are expressed as mean ± SEM (n = 7). Statistical differences were expressed as (p <0.001***) in comparison with the morphine-saline group using One Way ANOVA and Tukey post-test.

M= Morphine, S= Saline, T= Troxerutin

The Effect of *Troxerutin* on Locomotor Factors in Locomotor Activity Testing

The results of the locomotion test show that there is no statistically significant difference between the groups receiving morphine with doses of 50, 100, and 200 mg/kg *troxerutin* with the control group receiving morphine-saline in estimating the number of entering the squares in mice. Therefore, it can be said that the effect of *troxerutin* on relieving the symptoms of withdrawal syndrome has nothing to do with its effect on the points related to the motion activities of the animals through the brain (Figure 10).

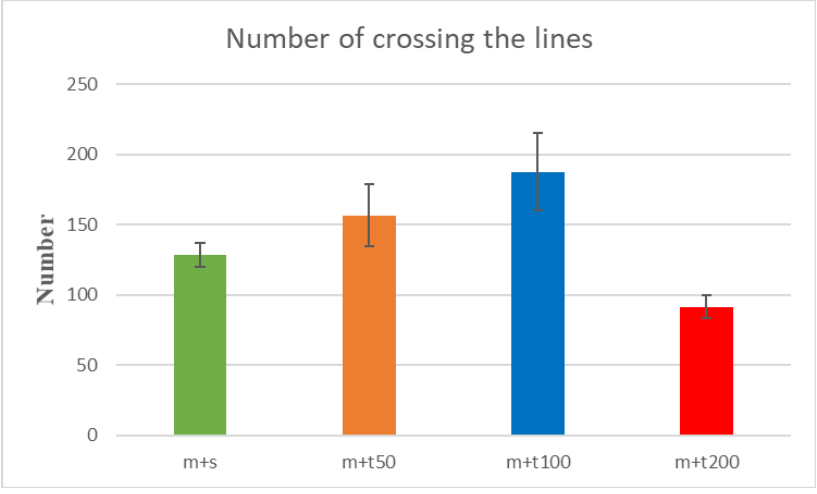

Figure 10. The effect of *troxerutin* on locomotor activity test locomotor factors in male mice compared to the morphine + saline control group. The results are expressed as mean ± SEM (n = 7).

M = Morphine, S=Saline, T=Troxerutin

The results of the present study showed that the 9-day administration of increasing doses of morphine causes dependence and subsequent administration of naloxone (5 mg/kg), causes withdrawal symptoms in mice. Also, intraperitoneal administration of *troxerutin* with morphine significantly reduces the overall symptoms of morphine withdrawal syndrome. The justification for *troxerutin* dosage selection was since dosages of 100, 200, and 300 mg/kg were practically tested previously to reach an efficient amount of the medication, so we declined the dosage for one step to do an experiment and probably reduce possible side effects of the drug to observe whether *troxerutin* would cause the same medicinal effect (Zamanian, Hajizadeh, Esmaeili Nadimi, Shamsizadeh, & Allahtavakoli, 2017;Raja, Saranya, & Prabhu, 2019; Gao et al., 2020).

Nonetheless, morphine is one of the oldest opioid drugs commonly used to relieve moderate to severe pain worldwide. It is widely used after surgery and in cancer patients. Due to its frequent consumption, side effects such as dizziness, nausea, constipation, and also physical dependence are seen in consumers (Hutchinson et al., 2008). Chronic use of morphine can lead to dependence and addiction, but the mechanisms involved are not fully understood. To understand the accurate mechanisms, it is necessary to first review the mechanisms of dependence on morphine. One of these biochemical mechanisms is the role of NMDA excitatory amino acid receptors. Glutamate is the messenger of primary stimulation in most central nervous system receptors. Almost all nerve cells are activated and depolarized with glutamate and its receptors (Wang & Wang, 2006).

In several studies, the use of NMDA receptor antagonists has been shown to prevent morphine tolerance. Another effect of stimulation of NMDA receptors by excitatory amino acids is to increase the production of NO. Some articles have also shown the involvement of the nitric oxide synthase pathway as an inhibitor of tolerance and dependence (Salehpour, Habibi Asl, Charkhpur, & Mahmoudi, 1398). In long-term use of opioids, it is seen that due to nervous adaptations, a series of behaviors such as craving, restlessness, insomnia, and high blood pressure are observed in person, and over time these behaviors become part of the mood. The use of NMDA receptor antagonists has significantly reduced many of these behaviors which are caused by addictive substances such as opioid compounds (Asl, Hassanzadeh, Khezri, & Mohammadi, 2008).

In the present study, the use of three doses of *troxerutin* (50, 100, and 200 mg/kg), reduced the withdrawal symptoms consisting of body grooming, genital grooming, and wet dog shake movements. Also, a decrease in the number of jumping movements was observed at 50 and 100 mg/kg doses of *troxerutin* compared to the control group. Additionally, a decrease in the number of teeth chattering and the abdomen writhing at a dose of 50 and 200 mg/kg *troxerutin* was observed compared to the control group. According to the results of this study, between different doses of *troxerutin* (50, 100, and 200 mg/kg) with morphine, *troxerutin* 50 mg/kg with $p < 0.001$ ***, and *troxerutin* 100 mg/kg with $p < 0.01$ **, were efficient to significantly reduce the symptoms compared to the control group. Anyhow, *troxerutin* in 200 mg/kg seemed to increase the jumping symptoms in animals significantly showing an enormous gap. The possible explanation could be illustrated this way by increasing the dose of *troxerutin*, over-stimulation of receptors in the neurologic system could have occurred; precisely whether the dosage of drugs increases illogically, it could cause toxicity. The same may have happened in this experiment, either. Perhaps the involvement of the limbic system could be another factor of occurring a serious difference (Jun Lu et al., 2012; Sharma, 2014). Nevertheless, further investigations are required.

To sum up, *troxerutin* at 50 and 100 mg/kg doses were more effective in reducing morphine dependence. Also, chronic usage of *troxerutin* significantly

increased the level of TAC and decreased the level of MDA in the serum of the mice with $p < 0.001^{***}$. Even though the dose of 200 mg/kg was effective in some symptoms, the total score was estimated and the gap in jumping symptoms prevented the efficacy of 200 mg/kg so far. Briefly, *troxerutin* has many biological properties. It can significantly reduce damage to various tissues such as the brain, liver, and kidney, and is a potential candidate for the prevention and treatment of cerebral palsy (Bruppacher, Rieckemann, Naser-Hijazi, & Wüstenberg, 1998; Zhang et al., 2015).

In the present study, the results show that *troxerutin* is a free radical scavenger, affecting the enzymes which are determined through the oxidative stress pathways, or maybe the reduction of lipid and lipoprotein peroxidation; and also involving the receptors directly by their antioxidant effects. In conclusion, it could reduce the symptoms of morphine withdrawal syndrome in mice. In recent years, *troxerutin*, especially in combination with *coumarin*, has been used in the treatment of chronic venous diseases and varicose veins (Babri et al., 2014; Farajdokht et al., 2017; Gohel & Davies, 2009; Panat, Maurya, Ghaskadbi, & Sandur, 2016). Researches show that even with a single dose of morphine, the production of Reactive Oxygen Species (ROS) increases and the level of regenerative glutathione (GSH) in brain cells decreases (Ma et al., 2019). Increased levels of glutathione-sulfide (GSSG), decreased levels of intracellular GSH, the deduction in the activity of enzymes like catalase (CAT), Superoxide Dismutase (SOD), and glutathione peroxidase enzyme (GPx) as well as overproduction of cerebral glutamate, has been reported consequently (Jun Lu et al., 2010; Zamanian et al., 2021). By increasing the activity of antioxidant enzymes containing catalase, superoxide dismutase, and glutathione peroxidase, repairing GSH deficiency, and reducing MDA and ROS levels, it can reduce morphine dependence signs. Studies have also shown that *troxerutin* not only can reduce ROS and MDA levels but also can increase the activity of GPx. By inhibiting the activation of the inflammatory pathway like c-Jun N-Terminal Protein Kinase 1/Nuclear factor- κB Kinase/Nuclear factor- κB in the hippocampus, *troxerutin* reduces the inflammatory factors of α-TNF, Interleukin (IL)-1β, and IL-6. (Saranya et al., 2020) Furthermore, it reduces the activity of NOS and the production of NO; which could be the reason why there is a noticeable increase in the level of TAC and a decrease in the level of oxidative stress-related factors like MDA inside the serum of tested animals (J. Lu et al., 2013; Medeiros et al., 2007; Najafi, Noroozi, Javadi, & Badalzadeh, 2018).

Briefly, according to the results of this study, it can be said that chronic administration of *troxerutin* in some doses can reduce the symptoms of morphine dependence in mice. Symptoms of morphine withdrawal syndrome were significantly reduced when *troxerutin* was administered at a dose of 50 mg/kg ($p < 0.001^{***}$) and 100 mg/kg ($p < 0.01^{**}$). Likewise, the level of TAC in the serum of the mice was significantly increased and the level of MDA was significantly decreased at all doses (50, 100, and 200 mg/kg) with ($p < 0.001^{***}$). Yet, the particular mechanism of action requires extra study, but it can be said that *troxerutin*, possibly by performing its effect on the immune system and inhibiting the production of inflammatory cytokines, inhibits inflammation of microglia, and also with its antioxidant effects, it inhibits oxidative stress and causes the decline of morphine dependence symptoms. Due to the increasing awareness of medicinal plants, *troxerutin* can be possibly considered a new compound to reduce the symptoms of morphine withdrawal syndrome for reducing the incidence of opioid addiction in communities. Anyhow, it might be used after supplementary studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Author contribution: Nasrin Hosseinzad Manie proposed the main idea and gathered data and Ramin Ghasemi Shayan critically revised the article.

REFERENCES

Adam, B. S., Pentz, R., Siegers, C. P., Strubelt, O., & Tegtmeier, M. (2005). Troxerutin protects the isolated perfused rat liver from possible lipid peroxidation by coumarin. *Phytomedicine, 12*(1-2), 52-61. doi:10.1016/j.phymed.2004.01.007

Ahmadi, Z., Mohammadinejad, R., Roomiani, S., Afshar, E. G., & Ashrafizadeh, M. (2021). Biological and Therapeutic Effects of Troxerutin: Molecular Signaling Pathways Come into View. *Journal of pharmaco-puncture, 24*(1), 1.

Alaei, H., Esmaeili, M., Nasimi, A., & Pourshanazari, A. (2005). Ascorbic acid decreases morphine self-administration and withdrawal symptoms in rats. *Pathophysiology, 12*(2), 103-107.

Asl, B. H., Hassanzadeh, K., Khezri, E., & Mohammadi, S. (2008). Evaluation of the effects of dextromethorphan and midazolam on morphine-induced tolerance and dependence in mice. *Pak J Biol Sci, 11*(13), 1690-1695. doi:10.3923/pjbs.2008.1690.1695

Babri, S., Mohaddes, G., Feizi, I., Mohammadnia, A., Niapour, A., Alihemmati, A., & Amani, M. (2014). Effect of troxerutin on synaptic plasticity of hippocampal dentate gyrus neurons in a β-amyloid model of Alzheimer′s disease: an electrophysiological study. *Eur J Pharmacol, 732*, 19-25. doi:10.1016/j.ejphar.2014.03.018

Bruppacher, R., Rieckemann, B., Naser-Hijazi, B., & Wüstenberg, P. (1998). Evaluation of the safety of a coumarin–troxerutin combination. *Pharmacoepidemiology and Drug Safety, 7*(S1), S37-S40.

Cappendijk, S. L., de Vries, R., & Dzoljic, M. R. (1993). Inhibitory effect of nitric oxide (NO) synthase inhibitors on naloxone-precipitated withdrawal syndrome in morphine-dependent mice. *Neurosci Lett, 162*(1-2), 97-100. doi:10.1016/0304-3940(93)90569-7

Elangovan, P., & Pari, L. (2013). Ameliorating effects of troxerutin on nickel-induced oxidative stress in rats. *Redox Report, 18*(6), 224-232.

Etemadzadeh, E. (1993). Cerebral catecholamine depletion in mice withdrawn from repeated morphine treatment and development of tolerance to the enhancing effect of morphine on noradrenaline depletion. *J Pharmacol Exp Ther, 266*(2), 749-755.

Farajdokht, F., Amani, M., Mirzaei Bavil, F., Alihemmati, A., Mohaddes, G., & Babri, S. (2017). Troxerutin protects hippocampal neurons against amyloid beta-induced oxidative stress and apoptosis. *EXCLI journal, 16*, 1081-1089. doi:10.17179/excli2017-526

Gao, Z., Ma, X., Liu, J., Ge, Y., Wang, L., Fu, P., . . . Yan, X. (2020). Troxerutin protects against DHT-induced polycystic ovary syndrome in rats. *Journal of ovarian research, 13*(1), 1-11.

Gohel, M. S., & Davies, A. H. (2009). Pharmacological agents in the treatment of venous disease: an update of the available evidence. *Curr Vasc Pharmacol, 7*(3), 303-308. doi:10.2174/157016109788340758

Gordon, C. J. (2004). Effect of cage bedding on temperature regulation and metabolism of group-housed female mice. *Comparative medicine, 54*(1), 63-68.

Guo, H., Wang, L., Bi, K., & Sun, Y. (2005). Determination of troxerutin in troxerutin tablets by monolithic capillary electrochromatography. *Journal of liquid chromatography & related technologies, 28*(5), 647-658.

Habibi Asl, B., Ahmadi, D., Hasanzadeh, K., & Charkhpour, M. (2007). Evaluation of the effect of bromocriptine and sulpiride on morphine dependence and withdrawal syndrome in mice. *Journal of Zanjan University of Medical Sciences & Health Services, 15*(59), 44.

Hassan, R., Pike See, C., Sreenivasan, S., Mansor, S. M., Müller, C. P., & Hassan, Z. (2020). Mitragynine attenuates morphine withdrawal effects in rats—a comparison with methadone and buprenorphine. *Frontiers in psychiatry, 11*, 411.

Hosseinzadeh, H., & Jahanian, Z. (2010). Effect of Crocus sativus L. (saffron) stigma and its constituents, crocin, and safranal, on morphine withdrawal syndrome in mice. *Phytother Res, 24*(5), 726-730. doi:10.1002/ptr.3011

Hosseinzadeh, H., & Nourbakhsh, M. (2003). Effect of Rosmarinus officinalis L. aerial parts extract on morphine withdrawal syndrome in mice. *Phytother Res, 17*(8), 938-941. doi:10.1002/ptr.1311

Hutchinson, M. R., Coats, B. D., Lewis, S. S., Zhang, Y., Sprunger, D. B., Rezvani, N., . . . Watkins, L. R. (2008). Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. *Brain Behav Immun, 22*(8), 1178-1189. doi:10.1016/j.bbi.2008.05.004

Kaeidi, A., Taghipour, Z., Allahtavakoli, M., Fatemi, I., Hakimizadeh, E., & Hassanshahi, J. (2020). Ameliorating effect of troxerutin in unilateral ureteral obstruction induced renal oxidative stress, inflammation, and apoptosis in male rats. *Naunyn-Schmiedeberg's Archives of Pharmacology, 393*(5), 879-888.

Lammers, J., Kruk, M., Meelis, W., & Van der Poel, A. (1988). Hypothalamic substrates for brain stimulation-induced attack, teeth-chattering, and social grooming in the rat. *Brain Research, 449*(1-2), 311-327.

Lewter, L. A., Johnson, M. C., Treat, A. C., Kassick, A. J., Averick, S., & Kolber, B. J. (2022). Slow-sustained delivery of naloxone reduces typical naloxone-induced precipitated opioid withdrawal effects in male morphine-dependent mice. *Journal of Neuroscience Research, 100*(1), 339-352.

Lu, J., Wu, D.-m., Hu, B., Cheng, W., Zheng, Y.-l., Zhang, Z.-f., . . . Wang, Y.-j. (2010). Chronic administration of troxerutin protects the mouse brain against D-galactose-induced impairment of the cholinergic system. *Neurobiology of learning and memory, 93*(2), 157-164.

Lu, J., Wu, D.-m., Zheng, Y.-l., Hu, B., Cheng, W., & Zhang, Z.-f. (2012). Purple sweet potato color attenuates domoic acid-induced cognitive deficits by promoting estrogen receptor-α-mediated mitochondrial biogenesis signaling in mice. *Free Radical Biology and Medicine, 52*(3), 646-659.

Lu, J., Wu, D. M., Zheng, Y. L., Hu, B., Cheng, W., Zhang, Z. F., & Li, M. Q. (2013). Troxerutin counteracts domoic acid-induced memory deficits in mice by inhibiting CCAAT/enhancer binding protein β-mediated inflammatory response and oxidative stress. *J Immunol, 190*(7), 3466-3479. doi:10.4049/Immunol.1202862

Ma, W., Wang, S., Liu, X., Tang, F., Zhao, P., Cheng, K., . . . Li, X. (2019). Protective effect of troxerutin and preproprotein hydrolysate injection on cerebral ischemia through inhibition of oxidative stress and promotion of angiogenesis in rats. *Molecular medicine reports, 19*(4), 3148-3158.

Maldonado, R., Negus, S., & Koob, G. F. (1992). Precipitation of morphine withdrawal syndrome in rats by administration of mu-, delta- and kappa-selective opioid antagonists. *Neuropharmacology, 31*(12), 1231-1241. doi:10.1016/0028-3908(92)90051-p

Maldonado, R., Saiardi, A., Valverde, O., Samad, T. A., Roques, B. P., & Borrelli, E. (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature, 388*(6642), 586-589. doi:10.1038/41567

Masood, M. I., Schäfer, K. H., Naseem, M., Weyland, M., & Meiser, P. (2020). Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone. *PloS one, 15*(8), e0237025.

Maurya, D. K., Salvi, V. P., & Krishnan Nair, C. K. (2004). Radioprotection of normal tissues in tumor-bearing mice by troxerutin. *J Radiat Res, 45*(2), 221-228. doi:10.1269/jrr.45.221

Medeiros, R., Prediger, R. D., Passos, G. F., Pandolfo, P., Duarte, F. S., Franco, J. L., . . . Calixto, J. B. (2007). Connecting TNF-alpha signaling pathways to iNOS expression in a mouse model of Alzheimer's disease: relevance for the behavioral and synaptic deficits induced by amyloid beta protein. *J Neurosci, 27*(20), 5394-5404. doi:10.1523/jneurosci.5047-06.2007

Meybodi, K. T., Zarch, A. V., Zarrindast, M., & Djahanguiri, B. (2005). Effects of ultra-low doses of morphine, naloxone, and ethanol on morphine state-dependent memory of passive avoidance in mice. *Behavioral pharmacology, 16*(3), 139-145.

Najafi, M., Noroozi, E., Javadi, A., & Badalzadeh, R. (2018). Anti-arrhythmogenic and anti-inflammatory effects of troxerutin in ischemia/reperfusion injury of diabetic myocardium. *Biomed Pharmacother, 102*, 385-391. doi:10.1016/j.biopha.2018.03.047

Oskuye, Z. Z., Bavil, F. M., Hamidian, G. R., Mehri, K., Qadiri, A., Ahmadi, M., . . . Keyhanmanesh, R. (2019). Troxerutin affects male fertility in prepubertal type 1 diabetic male rats. *Iranian Journal of Basic Medical Sciences, 22*(2), 197.

Panat, N. A., Maurya, D. K., Ghaskadbi, S. S., & Sandur, S. K. (2016). Troxerutin, a plant flavonoid, protects cells against oxidative stress-induced cell death through the radical scavenging mechanism. *Food Chemistry, 194*, 32-45. doi:10.1016/j.foodchem.2015.07.078

Parvizpour, A., Charkhpour, M., Habibiasl, B., Shakhsi, M., Ghaderi, M., & Hassanzadeh, K. (2013). Repeated central administration of selegiline attenuated morphine physical dependence in rats. *Pharmacological Reports, 65*(3), 593-599.

Quimby, F.W., & Luong, R.H. (2007). Clinical Chemistry of the Laboratory Mouse. T*he Mouse in Biomedical Research,* 171-216. doi:10.1016/B978-012369454-6/50060-1

Raja, B., Saranya, D., & Prabhu, R. (2019). Role of flavonoid troxerutin on blood pressure, oxidative stress and regulation of lipid metabolism. *Frontiers in Bioscience-Elite, 11*(1), 121-129.

Salehpour, M., Habibi Asl, B., Charkhpur, M., & Mahmoudi, J. (1398). *Effects of Vitamin C and Citicoline on Morphine-Inducing Tolerance In Mice*. Paper presented at the هم‌ایش بین المللی تحقیقات نانطرس 2019. https://civilica.com/doc/963401

Saranya, T., Kavithaa, K., Paulpandi, M., Ramya, S., Preethi, S., Balachandar, V., & Narayanasamy, A. (2020). Enhanced leptogenesis and oncogene regulatory mechanism of troxerutin in triple-negative breast cancer cells. *Toxicology Research, 9*(3), 230-238.

Sharma, S. (2014). Molecular pharmacology of environmental neurotoxins. *Kainic Acid: Neurotoxic Properties, Biological Sources, and Clinical Applications. Nova Science Publishers. New York. P1-47.*

Sui, R., Zang, L., & Bai, Y. (2019). Administration of troxerutin and preproprotein hydrolysate injection alleviates cerebral ischemia/reperfusion injury by down-regulating caspase molecules. *Neuropsychiatric disease and treatment, 15*, 2345-2352. doi:10.2147/NDT.S213212

Turton, E. P. L., Kent, P. J., & Kester, R. C. (1998). The Aetiology of Raynaud's Phenomenon. *Cardiovascular Surgery, 6*(5), 431-440. doi:10.1177/096721099800600501

Vela, G., Ruiz-Gayo, M., & Fuentes, J. A. (1995). Anandamide decreases naloxone-precipitated withdrawal signs in mice chronically treated with morphine. *Neuropharmacology, 34*(6), 665-668. doi:10.1016/0028-3908(95)00032-2

Wang, Z. J., & Wang, L. X. (2006). Phosphorylation: a molecular switch in opioid tolerance. *Life Sci, 79*(18), 1681-1691. doi:10.1016/j.lfs.2006.05.023

Wiebelhaus, J. M., Walentiny, D. M., & Beardsley, P. M. (2016). Effects of acute and repeated administration of oxycodone and naloxone-precipitated withdrawal on intracranial self-stimulation in rats. *Journal of Pharmacology and Experimental Therapeutics, 356*(1), 43-52.

Yamaguchi, T., Hagiwara, Y., Tanaka, H., Sugiura, T., Waku, K., Shoyama, Y., . . . Yamamoto, T. (2001). The endogenous cannabinoid, 2-arachidonic-glycerol, attenuates naloxone-precipitated withdrawal signs in morphine-dependent mice. *Brain Res, 909*(1-2), 121-126. doi:10.1016/s0006-8993(01)02655-5

Zamanian, M., Bazmandegan, G., Sureda, A., Sobarzo-Sanchez, E., Yousefi-Manesh, H., & Shirooie, S. (2021). The protective roles and molecular mechanisms of troxerutin (vitamin P4) for the treatment of chronic diseases: A mechanistic review. *Current neuropharmacology, 19*(1), 97-110.

Zamanian, M., Hajizadeh, M. R., Esmaeili Nadimi, A., Shamsizadeh, A., & Allahtavakoli, M. (2017). Antifatigue effects of troxerutin on exercise endurance capacity, oxidative stress and matrix metalloproteinase-9 levels in trained male rats. *Fundamental & clinical pharmacology, 31*(4), 447-455.

Zhang, Z.-F., Zhang, Y.-q., Fan, S.-H., Zhuang, J., Zheng, Y.-L., Lu, J., . . . Hu, B. (2015). Troxerutin protects against 2, 2′, 4, 4′-tetra-bromo-diphenyl-ether (BDE-47)-induced liver inflammation by attenuating oxidative stress-mediated NAD+-depletion. *Journal of hazardous materials, 283*, 98-109.

Zhang, Z. F., Fan, S. H., Zheng, Y. L., Lu, J., Wu, D. M., Shan, Q., & Hu, B. (2009). Troxerutin protects the mouse liver against oxidative stress-mediated injury induced by D-galactose. *J Agric Food Chem, 57*(17), 7731-7736. doi:10.1021/jf9012357