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Original Article

Protective effects of pistachio hydroalcoholic extract on morphine-induced analgesic tolerance and dependence: investigating the impact of oxidative stress

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Abstract

Background and purpose: Chronic consumption of morphine (Mor) induces tolerance and dependence. This study aimed to survey the effects of pistachio extract (PX) on the induction and expression of Mor analgesic tolerance and physical dependency in mice.
Experimental approach: Animals were randomly separated into six groups (n = 7): control, DMSO, Mor (10 mg/kg), Mor + saline, Mor + PX (10 mg/kg), and Mor + PX (100 mg/kg). Mor was injected (10 mg/kg, twice a day, s.c.) for 7 days to induce tolerance. PX was administered (10 and 100 mg/kg, orally) during the examination period. On each day and 20 min after Mor administration, a tail-flick test was done to measure the analgesic response and induction of tolerance. On day 7, naloxone (5 mg/kg, s.c.) was injected into the Mor-dependent animals to evaluate dependence, and animals were monitored for 30 min for jumping. Also, malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were assessed in the brain tissue.
Findings/Results: Our results indicated that co-administration of PX with Mor for 7 days diminished the induction of Mor tolerance. PX administration for 7 days alongside Mor reduced the frequency of withdrawal signs in naloxone-injected animals during dependence induction. Also, Mor increased the level of MDA and decreased the activities of SOD and GPx. Treatment with PX (100 mg/kg) restored all of the mentioned abnormalities.
Conclusion and implications: According to the results presented in this study, chronic administration of PX forbade the induction of Mor analgesic tolerance and dependency in mice.

Keywords: Mice; Morphine; Physical dependence; Pistachio extract; Tolerance.

INTRODUCTION

Opioid drugs such as morphine (Mor) have been extensively used for the management of acute and chronic pain (1,2). Furthermore, chronic consumption of Mor induces physical dependence and analgesic tolerance in humans and animals (3,4). Mor administration can cause side effects, such as hyperalgesia (5,6).

Studies have shown that oxidative stresses play an important function in the expansion of Mor physical dependence and analgesic tolerance. On the other hand, Mor physical dependence and analgesic tolerance by blocking such oxidative stresses are reduced (7-9). Following chronic use of opioids, neuroinflammation and neuroimmune changes take place, which are thought to play major roles in the induction of dependence and tolerance (10).

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The physical symptoms of Mor withdrawal are jumping, rearing, fatigue, and mood disorders (11). These withdrawal symptoms appear when the consumption of Mor is stopped abruptly (12). Several mechanisms including activation of protein kinase C (PKC) and N-methyl-D-aspartate (NMDA) receptors in the spinal cord (12), changes in neuronal plasticity in the central nervous system (CNS) (13), and overproduction of free radicals in CNS display critical roles in the development of Mor-induced hyperalgesia and tolerance (14,15).

Recently, there has been a growing interest in the potential anti-addictive and anti-tolerance effects of natural herbs (16). However, to effectively use medicinal plants to mitigate the side effects of opioids, scientific experiments are necessary.

Pistacia vera L. (Anacardiaceae) is native to dry regions of Central and West Asia and dispersed over the Mediterranean basin (17). Its fruits (pistachios) have been cultivated for many years and are used safely (18). Pistachios are rich in antioxidants such as tocopherols, carotenes, lutein, selenium, flavonoids, and phytoestrogens. These antioxidants are important for maintaining the levels of vitamin A in our bodies. Moreover, pistachio nuts contain phenolic compounds like anthocyanins, flavan-3-ols, proanthocyanidins, flavonols, isoflavones, flavanones, stilbenes, and phenolic acids, all of which are known for their exceptional antioxidant properties (19). Three clinical studies have shown significant antioxidant effects of pistachios (20-22). Pistachios contain antioxidants that help prevent arteriosclerosis and cancers. Therefore, incorporating pistachios into your diet can contribute to better health due to their beneficial compounds such as polyunsaturated fatty acids, fiber, protein, minerals, and antioxidants (23). It along with other nuts because of high-density fatty acids, can resolve cardiovascular problems (24). According to the profile of nutrients in pistachios and the conducted clinical trials, adding pistachios to the diet of individuals with diabetes or metabolic syndrome or at risk of metabolic syndrome improves these risk factors (25).

Pistachios are reported to significantly improve endothelium-dependent vasodilation and superoxide dismutase levels and diminish serum interleukin-6 and lipid hydroperoxide levels at 20% of the daily energy intake (20). Furthermore, the same amount of consumption of pistachios significantly improved antioxidant potential and reduced malondialdehyde in the plasma (21). Phenolic compounds may have antioxidant and anti-inflammatory properties (26). In comparison with other nuts, pistachios have better antioxidant and anti-inflammatory activity (27). Due to these properties, frequent intake of pistachios, as a part of a healthy diet, is usually recommended to avoid dysmetabolic disorders thought to be the main risk factors for the onset of chronic degenerative diseases (28). In addition to their nutritional value, pistachios are also rich in phenolic compounds, including anthocyanins, flavonoids, proanthocyanidins, and stilbenes, which are uniquely found in their skin (29). Pistachios contain various food antioxidants, including polyphenols and phytosterols, which act as scavengers to neutralize reactive oxygen species and help form endogenous antioxidant defenses. Various *in vitro* and *in vivo* studies have revealed that extracts from various parts of pistachios have antioxidant activity (27).

Therefore, the present study was designed to test the hypothesis that pistachio extract (PX) could exert preventive effects against chronic Mor side effects such as the development of antinociceptive tolerance and physical dependence in mice.

MATERIALS AND METHODS

Drugs

Morphine sulfate was purchased from (Temad, Iran) and naloxone hydrochloride from (Tolidaru, Iran).

Pistachio extraction

In this study, dried pistachios from long Akbari species (genetic code: M30 and voucher No. AK212), grown in Rafsanjan, Iran, were used. To prepare the extract, the pistachios were first powdered (100 g), then macerated in 1 L of ethanol (80%) for 72 h, and finally dried in a rotary evaporator apparatus and stored at -20 °C (30). The frozen extract was freshly dissolved

in dimethyl sulfoxide 10% (DMSO; Sigma-Aldrich, Germany) for administration. Two doses of pistachio hydroalcoholic extract (10 and 100 mg/kg, p.o.) were selected from previous investigations (30).

Animals

NMRI (Naval Medical Research Institute) male mice (20-25 g) were kept in groups (3 per cage maximum) in compliance with environmental standards in a facility cared for by the staff. Animals were kept at a 12/12-h light/dark cycle at a constant 23 ± 2 °C temperature with food and water access *ad libitum*.

All experiments were carried out in strict accordance with the European Guidelines for the Care of Laboratory Animals and guidelines for the care and use of laboratory animals at Rafsanjan University of Medical Sciences, Iran (Ethical code: IR.RUMS.REC.1399.213).

Experimental design

Experiments were all performed between 10:00 a.m. and 4:00 p.m. The animals were divided into 6 groups in this study (7 each): (1) control; (2) DMSO; (3) Mor; the animals that received Mor (10 mg/kg, twice a day, s.c.) for 7 days; (4) Mor + saline, the animals that received Mor (as above mentioned) and saline daily for 7 days (for assessing the development of Mor tolerance and dependence); (5) Mor + 10 mg/kg PX, the animals received Mor (as above mentioned) and PX (10 mg/kg, orally) daily for 7 days (chronic treatment) and only once throughout the treatment protocol 45 min before the last dose of Mor on the 7th day (acute treatment); (6) Mor + 100 mg/kg PX, the animals received Mor (as above mentioned) daily for 7 days and PX (100 mg/kg, orally, and timing same as the dose 10 mg/kg).

Induction of Mor tolerance and dependence

Mor tolerance and dependence were induced in mice by repeated injection of Mor (10 mg/kg; s.c.) twice a day (10:00 a.m. and 4 p.m.) for 7 consecutive days (9,31).

Evaluation of Mor tolerance and dependence

For evaluating the effects of PX on the induction of Mor tolerance and dependence, PX (10 and 100 mg/kg) or its vehicle (10% DMSO, 0.5 mL) were administered orally during the period of examination. The nociceptive response was evaluated by the tail-flick test, 20 min after Mor injection on every experimental day. The reduction in response was used to evaluate the degree of tolerance. The tail-flick test results are expressed as the percentage of maximum possible effect (MPE). Moreover, physical dependence was assessed by the incidence of jumping following administration of naloxone (5 mg/kg, i.p.) 2 h after the last dose of Mor on the 7th day. Each mouse was placed in a Plexiglas box (35 × 35 × 45 cm), and for 30 min withdrawal sign frequency, i.e., jumping, diarrhea, defecation, head tremor, rearing, scratching, sniffing, teeth chattering, and wet-dog shake was recorded (11,32).

Tail-flick test

To evaluate the Mor analgesic, a tail-flick analgesia meter apparatus (UGO BASILE, Italy) was used (9). This apparatus was set to shine a light beam focused on the animal tail's ventral part. The heat intensity was adjusted to elicit 2-4 s baseline responses. The time between tail exposure to radiant heat and tail withdrawal was considered tail-flick latency. To avoid tissue damage (cut-off point = 10 s), the heat stimulus was discontinued after 10 s. Before the tail-flick test, the animals were habituated to laboratory surroundings. During the test, the mice were lightly restrained in a Plexiglas mice restrainer box. Through gaps located in the restrainer's interior door, the mice's tail was exposed. To reduce mice stress during the assessment, they were habituated to the test conditions for 3 days before the first trial. Results are expressed as %MPE, which was calculated according to the following equation:

$$MPE\ (\%) = \frac{T1 - T0}{T2 - T0} \times 100$$

where T0 is pre-treatment latency; T1 is post-treatment latency; and T2 is the cut-off time.

Brain tissue preparation

At the end of the study period, mice were sacrificed under deep anesthesia with CO_2 gas.

The animal (chronic treatment with PX) brain was removed rapidly under aseptic conditions and homogenized with ice-cold lysis buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, 1% NP-40, 0.1% Na deoxycholate; 2 µg each of the protease inhibitors aprotinin, pepstatin A, leupeptin, and 0.5 µmol/L PMSF, pH 7.4). The lysate was centrifuged for 20 min at 4 °C at 14000 rpm. The supernatant was collected into the new tubes. Using the Bradford method, the protein concentration in each sample was calculated. The supernatant samples were stored at -80 °C until oxidative stress analyses.

Evaluation of oxidative stress in the brain

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as the malondialdehyde (MDA) levels, as oxidative stress parameters were measured using commercially available kits (ZellBio, Germany) according to the manufacturers' guidelines (33). The kit offers a standardized tool for assessing lipid peroxidation at the MDA level using the thiobarbituric acid (TBA) method. The MDA-TBA adduct was produced through the interaction of MDA and TBA under elevated temperatures. The measurement of SOD activity involved the conversion of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2^-). The unit of SOD activity was defined as the quantity of protein necessary to catalyze the breakdown of 1 mmol of O_2^- into H_2O_2 and O_2^- within 1 min. For the assessment of GPx, the evaluation was conducted based on the enzyme's capacity to transform H_2O_2 into H_2O. The measurement unit for GPx activity is defined as the quantity of the specimen needed to facilitate the oxidation of 1 mmol of glutathione to glutathione disulfide within a minute. In the case of each of these indicators, the tissue homogenate was combined with substances supplied by the producer and subsequently incubated at 37 °C for 30 min.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by GraphPad Prism version 6.01 for Windows (GraphPad Software, USA). The results of Mor tolerance test were evaluated using a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Other obtained data were compared by one-way ANOVA followed by Tukey's multiple comparisons test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Effects of PX chronic treatment on induction of Mor tolerance

The effect of chronic treatment with PX on %MPE is shown in Fig. 1. Chronic consumption of DMSO did not cause a significant change in the analgesic effect compared with the control group. Repeated injections of Mor and Mor + saline reduced the nociception response, which confirmed the induction of Mor analgesic tolerance.

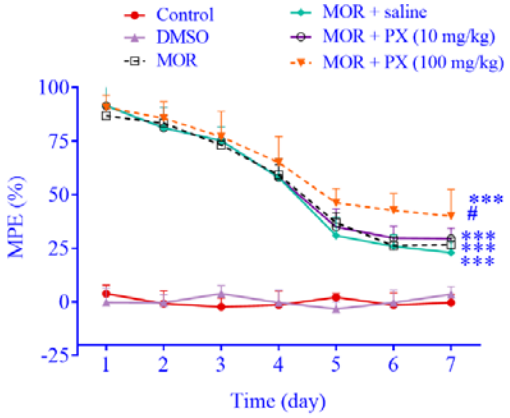

Fig. 1. Effect of chronic treatment with PX on morphine tolerance induction. Data are expressed as mean ± SEM; n = 7. ***P < 0.001 indicates significant differences compared to the control group; $^{\#\#\#}P$ < 0.001 versus the Mor group. MPE, Maximum possible effect; PX, pistachio extract; Mor, morphine.

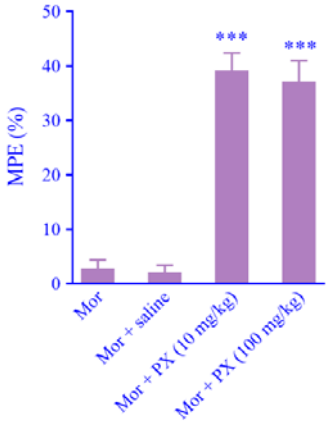

Fig. 2. Effect of acute treatment with PX on morphine tolerance expression. Data are expressed as mean ± SEM; n = 7. ***P < 0.001 indicates significant differences compared to the control group. MPE, Maximum possible effect; PX, pistachio extract; Mor, morphine.

Furthermore, co-administration of PX (10 and 100 mg/kg) with Mor significantly increased the analgesic latency on the seventh day [F (30, 210) = 18.73, $P < 0.001$] compared with the control group, and also co-administration of PX at 100 mg/kg with Mor significantly increased the analgesic latency after seven days compared with the Mor group ($P < 0.05$). Results showed that PX could prevent the induction of Mor analgesic tolerance.

Effects of PX acute treatment on the induction of MOR tolerance

The effect of acute treatment with PX on %MPE is shown in Fig. 2. To clarify the impact of acute treatment with PX in the induction of Mor tolerance (10 mg/kg; s.c., twice a day: 10:00 a.m. and 4 p.m., for 7 consecutive days), PX (10 and 100 mg/kg) administered orally only once during the period of examination, 45 min before the dose of Mor on day 7. Data indicated that PX did not prevent the expression of Mor tolerance in Mor-treated mice (Fig. 2).

Effects of PX chronic treatment on induction of Mor dependence

The animals in the control and DMSO groups showed no withdrawal signs, whereas the Mor-administered animals and Mor + saline group exhibited withdrawal signs, which confirmed the induction of Mor dependence.

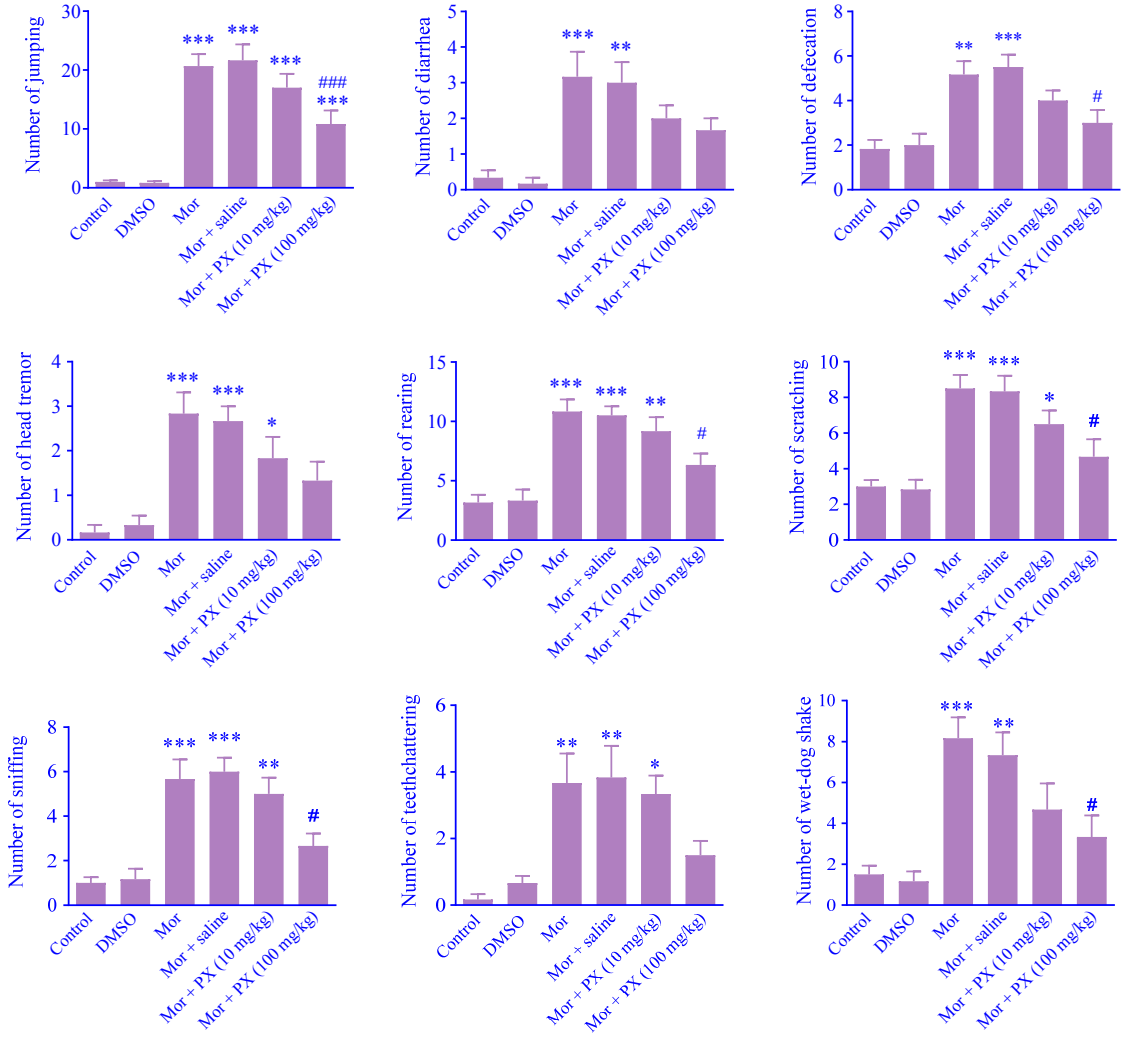

Fig. 3. Effect of chronic treatment with PX on morphine dependence. Data are expressed as mean ± SEM; n = 7. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences compared with the control group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus the Mor group. PX, Pistachio extract; Mor, morphine.

Moreover, chronic co-administration of PX (10 mg/kg) with Mor in comparison with the control group significantly increased the frequency of jumping [F (5, 30) = 39.43, $P < 0.001$], head tremor [F (5, 30) = 9.422, $P < 0.05$], rearing [F (5, 30) = 13.77, $P < 0.01$], scratching [F (5, 30) = 11.45, $P < 0.05$], sniffing [F (5, 31) = 11.88, $P < 0.01$], and teeth chattering [F (5, 30) = 7.010, $P < 0.05$], but co-administration of PX (100 mg/kg) with Mor significantly decreased all of withdrawal signs except frequency of diarrhea, head tremor, and teeth chattering compared with Mor-administered animals, which indicated that PX (100 mg/kg) could prevent the induction of Mor dependence ($P < 0.05$) (Fig. 3).

Effects of PX acute treatment on induction of Mor dependence

In order to assess the effect of PX on induction of Mor dependence, PX (10 and 100 mg/kg) was administered only once 45 min before the last dose of Mor on the 7th day of the experiment in the animals that received only Mor. The animals in the control and DMSO groups demonstrated no withdrawal signs, whereas the Mor-administered animals and Mor + saline group exhibited withdrawal signs compared with the control group, which confirmed the induction of Mor dependence. Moreover, acute co-administration of PX at both doses with Mor did not affect the frequency of withdrawal signs which indicated that PX could not prevent expression of Mor dependence (Fig. 4).

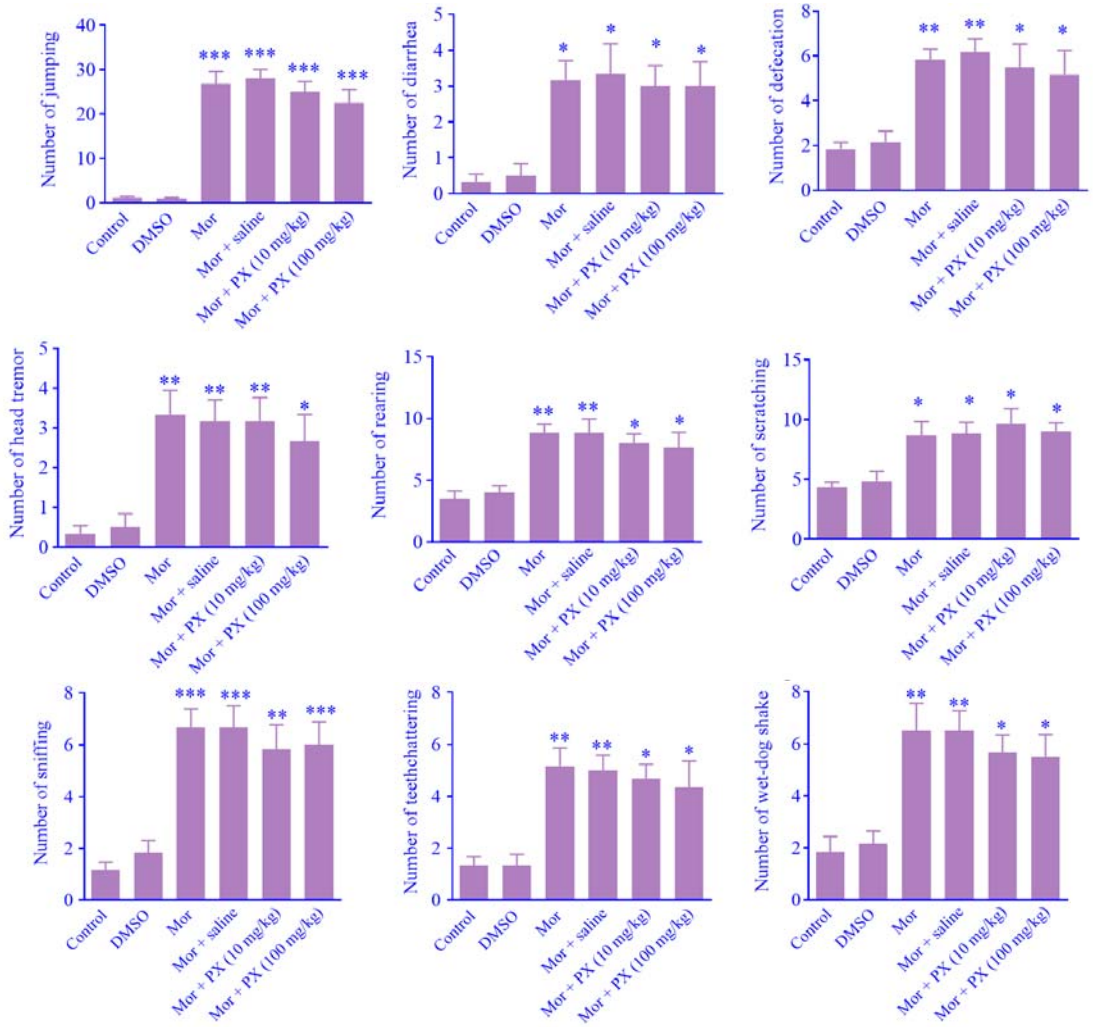

Fig. 4. Effect of acute treatment with PX on morphine dependence. Data are expressed as mean ± SEM; n = 7. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences compared with the control group. PX, Pistachio extract; Mor, morphine.

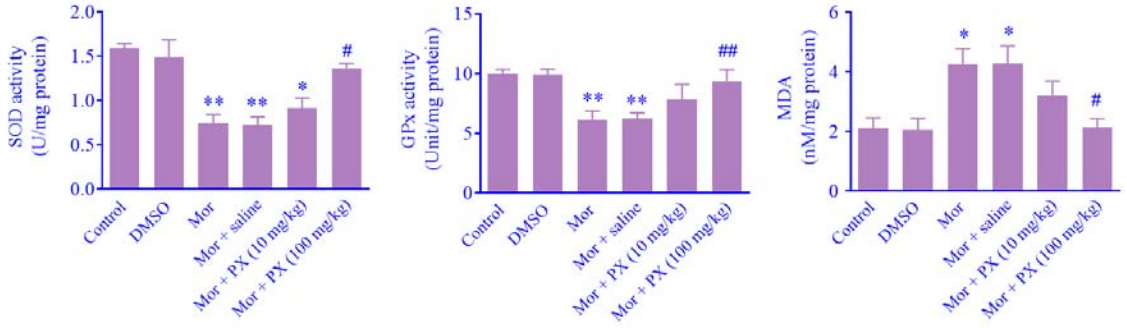

Fig. 5. Effect of treatment with PX on SOD and GPx activities and MDA level. Data are expressed as mean ± SEM; n = 7. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the Mor group. PX, Pistachio extract; Mor, morphine; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde.

Effects of PX on induction of oxidative parameters

The effect of chronic treatment with PX on oxidative parameters is shown in Fig. 5. Chronic consumption of DMSO did not cause a significant change in oxidative parameters compared with the control group. Repeated injections of Mor and Mor + saline significantly reduced SOD [F (5, 18) =12.28, ($P < 0.01$)] and GPx [F (5, 18) = 10.03, ($P < 0.01$)] activities compared with the control group, but co-administration of PX (100 mg/kg) with Mor significantly increased SOD and GPx activities compared with the Mor group ($P < 0.05$ and $P < 0.01$, respectively).

Moreover, it was observed that both Mor and Mor + saline led to a notable rise in MDA levels in comparison with the control group [F (5, 18) = 5.693, $P < 0.05$]. Conversely, administration of PX (100 mg/kg) was associated with a considerable decrease in this parameter ($P < 0.05$).

DISCUSSION

In this study, the effects of pistachio hydroalcoholic extract on the induction and induction of Mor tolerance and physical dependency in male mice were investigated. Our results showed that PX (100 mg/kg) significantly attenuated the induction of Mor tolerance and dependence in mice. Furthermore, we also found that PX at 100 mg/kg reduced MDA production as well as increased the SOD and GPx activities in brain tissue of Mor-treated animals. However, the dose of 10 mg/kg of PX, unlike the dose of 100 mg/kg, had no effect on tolerance and addiction to morphine in experimental animals. This can be due to the lower amount of antioxidant substances present in the lower dose, indicating the dose-dependent effect of PX.

Despite new developments in our knowledge about opioid tolerance and dependency, the exact underlying mechanisms of these phenomena are still poorly understood (34). The main problems related to the chronic use of opioids are induction and expression of tolerance and dependency. Therefore, the identification of compounds that can prevent these processes may have important usage in clinical practice for the management of pain (35).

Recently, the natural antioxidant properties of flavonoids and phenolic compounds extracted from plants have received much attention (36). Plants rich in these compounds are an ideal source of natural antioxidants. Torkzadeh-Mahani *et al.* showed that ginger mitigates the dependency behaviors induced by chronic consumption of morphine (37). They proposed that these effects could be attributed to the anti-inflammatory and antioxidant properties of ginger. Another study indicated that thymoquinone prevents the development of tolerance and dependence on morphine (38).

Chronic treatment with Mor could induce apoptosis and neuronal degeneration which leads to structural changes in the brain (39-42). Several investigations reveal that Mor induced these processes by increasing the production of

free radicals and oxidative stress (43,44). Interestingly, it was reported that reducing oxidative status could mitigate the tolerance and dependence induced by Mor (45-47). In this regard, our findings indicated that the activities of GPx and SOD were lower, while MDA levels were higher in the brain tissue of animals treated with Mor, in comparison with the control group. Reports have previously revealed the potent antioxidant properties of pistachios (48, 49). Treatment with PX (100 mg/kg) significantly increased GPx and SOD activities as well as reduced MDA levels in the brain tissue. Pistachios are an abundant source of campesterol, stigmasterol, and β-sitosterol, indicating that they are potent antioxidants (50). In healthy volunteers, consumption of pistachios has been shown to reduce oxidative stress (21). Ammari *et al.* showed that *Pistacia lentiscus* oil attenuated the memory impairments induced by lipopolysaccharide in rats *by* enhancing the activities of SOD and catalase in the brain tissue (51). Also, methanolic extract of pistachios has been reported to have hepatoprotective effects through ROS scavenging as well as reducing lipid peroxidation (52). These observations prove that the protective effects of PX can probably be attributed to antioxidant activity and/or increased antioxidant system capacity. Thus, it seems that PX could reduce the induction of Mor tolerance and dependence *via* antioxidant and neuroprotective properties.

CONCLUSION

Our results have demonstrated for the first time that PX has a remarkable impact on the induction of Mor tolerance and dependence. Acute administration of PX did not affect the expression of Mor tolerance and dependence. Moreover, oral administration of PX could attenuate the induction of Mor tolerance and dependence. These protective effects of pistachios might be related to their antioxidant properties. Further research is necessary to elucidate the precise mechanism behind this phenomenon.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

A. Kaeidi and I. Fatemi conceived and designed the experiments; A. Kaeidi and E. Hakimizadeh performed the experiments; A. Kaeidi and J. Hassanshahi analyzed the data; I. Fatemi and J. Hassanshahi contributed to providing the reagents, materials, and analysis tools; A. Kaeidi and E. Hakimizadeh wrote the paper. The finalized article was read and approved by all authors.

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