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Thymoquinone Prevents Mice Pancreas Injuries Against Morphine

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ABSTRACT

Background: Morphine is a pain medication of the opiate type and one of the major risk factors for disorders of the body organs and causes disturbing effects. Thymoquinone is a phytochemical compound found in the plant Nigella sativa. It has different pharmacological effects such as anticancer and antioxidant. **Objective:** This study was designed to evaluate effects of thymoquinone against morphine damages on pancreas of mice. **Material methods:** In this study, forty-eight male mice were divided into six groups (n=8). Saline group (control), morphine group (20 mg/kg) and groups receiving thymoquinone (4.5, 9 and 18 mg/kg) and morphine plus thymoquinone (4.5, 9 and 18 mg/kg) intraperitoneally for 3 days. The diameter and number of the islets of Langerhans, the pancreas weight and serum levels of nitric oxide, glucose and insulin levels have been studied. **Result:** The results indicated that morphine administration significantly decreased pancreas weight, diameter and number of the islets and serum levels of insulin and nitric oxide and increased glucose levels to saline group ($P<0.05$). However, thymoquinone and thymoquinone plus morphine administration significantly boosted pancreas weight, diameter and number of the islets and serum levels of insulin and nitric oxide and reduced glucose levels in all doses compared to morphine group ($P<0.05$). **Conclusion:** It seems that thymoquinone can improve pancreas injuries induced by morphine in mice.

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Introduction

Blood glucose is a factor whose stability is essential for the mammals [1]. The extraordinary complexity of beta cells is due to their complicated development that may never be understood. These cells accurately regulate the production of insulin, which is the prominent mechanism for controlling the main fuel of most tissues (glucose) [2]. Opioids have long been reported to improve glucose level in the obese people with diabetes. Opioids and even their metabolites such as morphine can increase blood glucose level [3]. The increased susceptibility and number of alpha 2-adrenergic receptors in pancreatic islets lead to suppression of insulin secretion in response to a sympathetic stimulation. Morphine, probably by inhibiting the sympathetic process in peripheral tissues through post-synaptic inhibition, compensates for the increased susceptibility of alpha 2-adrenergic receptors, prevents the suppression of insulin secretion and improves the serum glucose of obese diabetic models [4]. Opioid receptors also exist in pancreas islets. The direct stimulation of these receptors and increased secretion of insulin probably provide another mechanism for improving the glucose metabolism of diabetic models [5]. Morphine is a phenanthrene derivative of opium alkaloids, with analgesic and narcotic properties. Morphine is the most important alkaloid of opium that is found abundantly in it, and more efficacy of opium is because of this alkaloid [6]. Morphine increases the production of free radicals by activating lipid peroxidation. Increased lipid peroxidation blocks the antioxidant enzymes.

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This process causes the formation of free radicals or reactive oxygen species. These radicals are able to cause the cell membrane destruction and DNA segmentation [7]. Antioxidant supplements and antioxidant-rich foods can reduce oxidative damage by decreasing free radicals in the body [8]. Thymoquinone is the major active ingredient in the aqueous extract of Nigella seeds and one of the four alkaloids extracted from this plant [9]. Anti-oxidative, anti-inflammatory, immunomodulatory and anti-histamine properties of Nigella sativa extract and oil have made this plant to have numerous pharmacologic effects such as reduction of blood glucose, lipid and hypertension, excretion of bile and uric acid, protection of the kidney, heart and liver as well as antimicrobial and antiparsitic effects [10]. The results of Rchid et al. showed that Nigella extract in the medium of Langerhans islets in mice could increase the secretion of insulin from islet cells [11]. Considering the antitoxic effects of morphine and properties of thymoquinone, and that no study has ever investigated the effects of thymoquinone on the morphine-induced injuries, the current study was conducted to evaluate the effects of thymoquinone on the morphine-induced injuries in mice with pancreatic disorders.

Materials and methods

Animals:

In this study, 48 male mice (balb /c) with a weight range of 30-27 g were purchased from Tehran Pasteur Institute and they were used in the study. For one week before the start of the experiment, they were kept in the animal house of Kermanshah University of Medical Sciences under laboratory conditions and at temperatures 20 ± 2, 12 hours in darkness and 12 hours in lighting conditions. Prior to the start of experiment, they were fed normal diet and water to get used to environment and adapt physiologically [12].

Chemicals

Thymoquinone (2-Isopropyl-5-methylbenzo-1,4-quinone; $C10H12O2$) and Morphine ($C16H19NO3$) were obtained from Sigma Chemical Company (St. Louis, USA) and were dissolved in saline (0.9%) for administration [9].

Experimental design and treatments

In this study, the harmful dose of morphine was 20 mg/kg and multiple doses of Thymoquinone were injected intraperitoneally for 3 days. The same volume of saline was administered. The mice were randomly divided into eight groups (n=6): Group 1, saline group, received 0.9% normal saline, Group 2. Morphine group, were induced by morphine. Groups 3 to 5, Thymoquinone Groups were given 4.5, 9 and 18 mg/kg Thymoquinone respectively. Mice in groups 6-8 received Thymoquinone (4.5, 9 and 18 mg/kg) plus morphine. [13,14].

Collection of blood serum and measurement of pancreas weight

All animals were anesthetized with chloroform, dissected and blood samples were taken from right ventricle by cardiac puncture. The blood samples were incubated at 37 °C to coagulate. The coagulated blood samples were then centrifuged for 15 minutes at 3000 rpm until the serum was separated. The separated serum was kept at -20 °C until the measurement of the hormone insulin, glucose and nitric oxide levels. Animals were killed and sacrificed. Pancreases were removed and weighed on a microbalance sensitive to 0.001 mg (Precisa 125A, Switzerland) and average weights of the Pancreases of mice were calculated and recorded [15].

Histological examinations

After fixing pancreas (10% formalin), the preparation process of tissues was performed by Automatic Tissue processors device and based on the common method of histology (paraffin method) that involves passing through the samples from the ascending alcohols then passing through Xylene for transparency and removing the opacity created in the process of dewatering and finally crossing the paraffin to impregnate and to fill the vacuum created in the fat pores created in the stage of passing through Xylene and then embedded in soft paraffin. Thin sections (5 mm) were cut using a microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany) and stained with hematoxylin and eosin. The preparation was examined with an Olympus BX-51T-32E01 research microscope connected to a DP12 Camera with 3.34-million-pixel resolution and Olysia Bio software (Olympus Optical Co. LTD, Tokyo, Japan) [9].

Morphometric measurements

For measurement of number and diameter of pancreatic islets (langerhans), 10 cuts were colored from each sample and they were studied separately by two people. Control samples (saline groups) were considered as natural and changes caused by morphine and thymoquinone were evaluated. After the preparation of the Lam of each group, 5 fields in 100x magnification of microscope were randomly selected. After counting the number of islets, its average was obtained. To measure the mean diameter of islets, 5 islets were selected of each lam. Then, large and small diameter of each islet was determined in micrometer and by placement it in the following formula, the mean diameter of each islet was obtained. $MD=\sqrt{L \times S \times \text{magnification}}$ (MD: mean diameter, S: small diameter of islet, L: large diameter of islet, and magnification: lens magnification) [16].

Hormone insulin and blood glucose measurement

The blood glucose level was measured at baseline and at the end of experiment. after de-freezing the serum samples placed at 20 ° C, Glucose oxidase (GOD-PAP) method was used to determine blood glucose concentration [17]. Serum insulin was measured by Monobind kit (USA, Sigma) using ELISA method. In brief, the formerly taken serum samples were defrozen (like blood glucose). The kit was exposed to room temperature; the contents of a vial were diluted with distilled water to obtain 1000 ml solution. The content of each vial was mixed with 2 ml distilled water. Fifty λ of sample was poured in the wells. One hundred λ of conjugated insulin solution was added to all wells and mixed afterwards. The surface of wells was covered with plate adhesive and was incubated for 60 min at room temperature. The content of the plate was removed and

350 λ diluted irrigation solution was added to all wells. Six λ of substrate solution was added to all wells and incubated for 15 min at dark ambient temperature. Fifty λ stop solution was added to all wells, and plates were shaken gently on the table surface for 20 seconds. After 30 minutes, plates were read by ELISA reader at 450 Nm (18).

Griess assay

Nitric oxide was measured using the Griess staining method. In sum, after de-freezing the serum samples, in this assay, sulfonamide solutions, N-(1- naphthyl) ethylenediamine dihydrochoride (NEED) and nitrite standards were prepared. Then, 100 µl supernatant was taken and 100 µl vanadium chloride, 50 µl solfanile amide and 50 µl NEDD (N-1(naphtylen) ethylenediamine dihydrocloride) were added. Standard solutions of sodium nitrate prepared with different concentrations of nitrate and the standard curve of nitrite concentration is calculated. Samples' optical density (OD) was measured by ELISA reader at the wavelength of 540 nm [8].

Statistical Analysis

For statistical analysis, SPSS software (version 16) was used and quantitative data were compared using one-way ANOVA and Tukey test and $P<0.05$ was considered significant.

Results

Weight of pancreas

The results of the investigation of pancreatic weight among the studied groups show significant decrease in pancreatic weight between group receiving morphine and the saline group ($p< 0.05$). In addition, increasing the mean weight of pancreases in the groups receiving thymoquinone and thymoquinone plus morphine in all doses was observed in comparison with the morphine group ($p< 0.05$) (Figure 1).

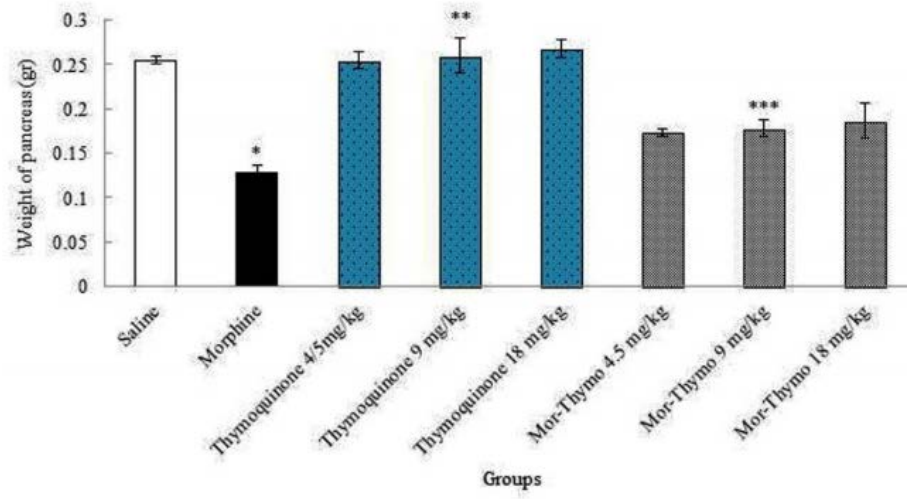

Figure 1. Effect of morphine, thymoquinone and thymoquinone plus morphine administration on weight of pancreas.*Significant decrease of weight in morphine group compared to saline group ($P<0.05$). **Significant increase in all doses of thymoquinone groups compared to morphine group ($P<0.05$). ***Significant increase in all dose of thymoquinone plus morphine groups compared to morphine group ($P < 0.05$).

Morphometric characteristics

The results of the investigating the diameter and the number of langerhans islets in treatment groups showed a significant reduction between group received morphine compared with the saline group ($p< 0.05$). Further, thymoquinone and thymoquinone plus morphine caused a significant decrease the mean diameter and the number of islets in all treated groups in comparison with morphine group administration ($p<0.05$) (Figure 2).

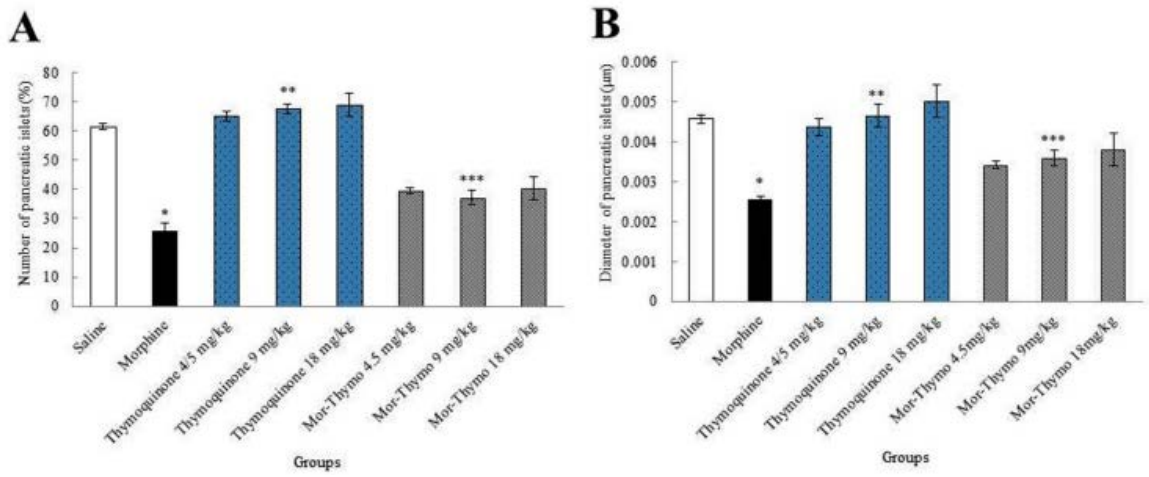

Figure 2. Effect of morphine, thymoquinone and thymoquinone plus morphine administration on the number (**A**) and diameter of islets (**B**). *Significant increase of the number and diameter of islets in morphine group compared to saline group ($P < 0.05$). **Significant decrease in all doses of thymoquinone administration compared to morphine groups ($P<0.05$). ***Significant decrease in all doses of thymoquinone plus morphine administration compared to morphine group ($P < 0.05$).

Insulin and blood glucose measurement

The results of investigating the level of insulin hormone in blood serum and the glucose levels in the experimental groups showed a significant decrease in morphine group compared with the control (saline) group($p< 0.05$). In addition, groups receiving thymoquinone and thymoquinone plus morphine in all dose showed significant increase in serum level of insulin and reduction in serum level of glucose compared to the group receiving morphine alone ($p< 0.05$)(Figure 3).

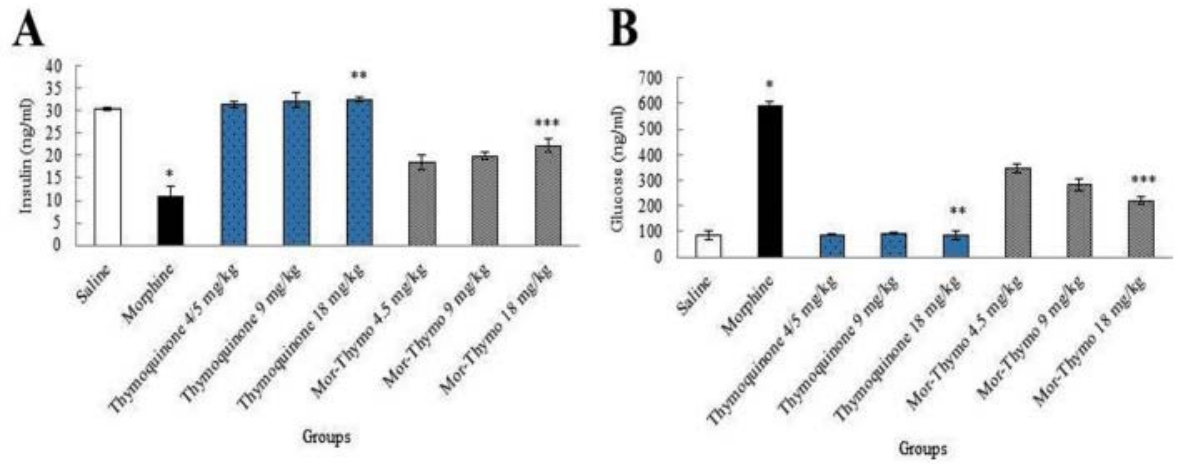

Figure 3: Effect of morphine, thymoquinone and thymoquinone plus morphine administration on insulin hormone in blood serum (**A**) and glucose levels (**B**) of Forty-eight mice was equally divided into 8 groups. *Significant increase of glucose and decrease of insulin in morphine group compared to saline group ($P<0.05$). **Significant increase of insulin and decrease of glucose in all doses of thymoquinone treated groups compared to morphine group administration ($P<0.05$). ***Significant increase of insulin and decrease of glucose in all doses of thymoquinone plus morphine treated groups compared to morphine groups ($P < 0.05$).

Nitric oxide measurement

The mean of nitric oxide in blood serum decreased significantly in thymoquinone and thymoquinone plus morphine in all doses compared to morphine group ($p<0.05$). Also, the findings of blood serum NO measurement indicated a significant increase in morphine group compared to saline group ($p<0.05$) (Figure 4).

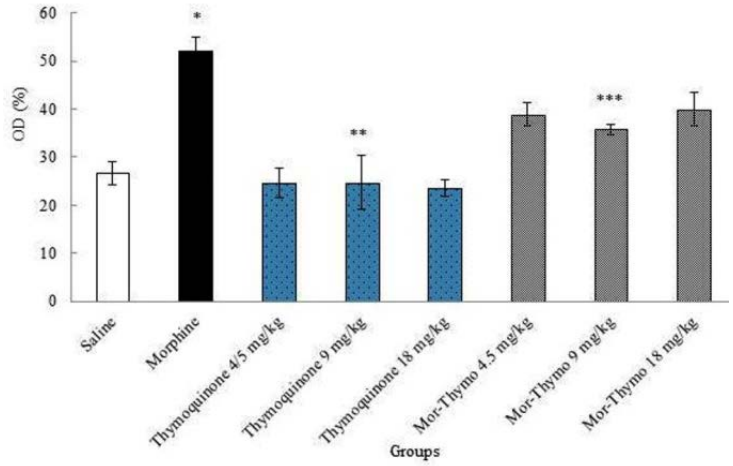

Figure. 4. Effects of thymoquinone, morphine and thymoquinone plus morphine on the mean nitric oxide levels of Forty-eight mice were equally divided into 6 groups. *Significant increase of nitric oxide in morphine group compared to saline group ($P < 0.05$). **Significant decrease in all doses of thymoquinone groups administration compared to morphine group ($P<0.05$). ***Significant decrease in all doses of thymoquinone plus morphine administration groups compared to morphine group ($P < 0.05$).

Histopathological observations

The experimental groups receiving morphine (Examination of H & E sections), the pancreas section appeared with variable changes and marked injury. These changes were evidenced by vacuolization in tissues of the pancreas, reduction in small of islet and blooding in pancreas tissue compared to saline stage. Treatment with morphine plus thymoquinone showed, thymoquinone reduced liver injury due to morphine toxicity (Figure 5).

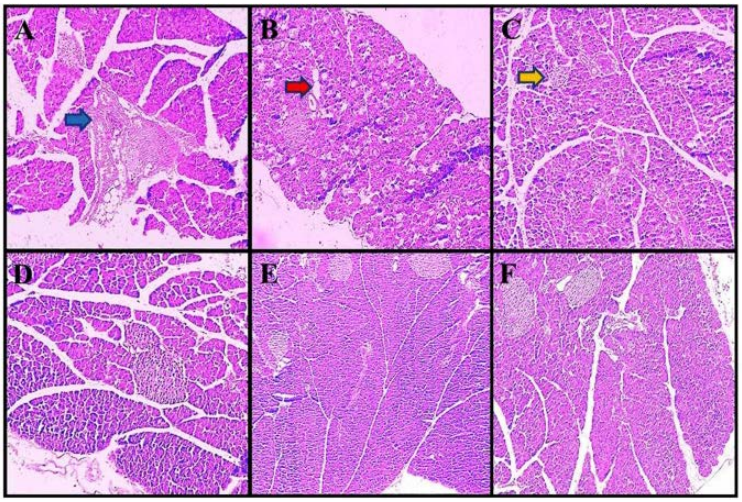

Figure 5. Histological sections of the pancreas (hematoxylin-eosin, ×100). **A, B and C;** Micrograph of pancreas section in morphine group, **D;** Micrograph of pancreas section in saline group**, E;** Micrograph of pancreas section in thymoquinone (9 mg/kg) group, **F;** Micrograph of pancreas section in morphine plus thymoquinone (9 mg/kg) group**.** The experimental groups receiving morphine, vacuolization in tissues (blue arrow), reduction of islet (red arrow) and blooding in pancreas tissue (yellow arrow) were observed.

Discussion

Morphine is currently being consumed abundantly as a potent sedative by drug addicts and for medical applications around the world [6]. Given the destructive effects of morphine on many body organs, it seems that some plant drugs can have a protective role against morphine in the body [7]. The present study assessed the protective effects of thymoquinone on the morphine-induced disorders on pancreas weight, diameter and count of Langerhans islets, glucose level, insulin level and nitric oxide secretion. The results of pancreatic weight analysis in the study groups indicated a significant decrease in pancreatic weight in the group receiving morphine than in saline (control) group. In all groups receiving morphine plus thymoquinone, the mean pancreatic weight was significantly increased compared to the groups treated with morphine alone. Morphine administration, by affecting endogenous β-endorphin, reduced the weight and food intake. Opioids can also cause lipolysis and weight reduction by exerting their effect on the metabolism of lipids and stimulation of β-lipoprotein [19]. Thymoquinone is full of nanoemulsion and lipophilic particles, which can increase the food intake and bioavailability activity. Hydrophobic and oily materials contain nanoemulsion, which increase the solubility and reduce the toxic effects of

drugs [20]. Zglinicki et al. showed that thymoquinone could increase glucose metabolism and energy production, thereby increasing the growth, confirming the results of the present study [21]. The findings of the analysis of diameter and count of Langerhans islets in the study groups revealed a significant decrease in the morphine-receiving group than in saline group. Administration of thymoquinone plus morphine significantly increased the diameter and count of Langerhans islets in all study groups than in the morphine-receiving group alone. Induction of apoptosis in the body cells and production of reactive oxygen and nitrogen species can be due to the unfavorable physiologic effects of morphine on body [6]. Increased production of free radicals and reduced antioxidant defense system lead to disordered cell performance and cell death [22]. It seems that production of reactive oxygen species and induction of oxidative stress can damage pancreatic beta cells. Impairment of beta cells can inhibit the migration of macrophages to Langerhans islets, which is called insulitis. In insulitis, the pancreatic beta cells are phagocytosed by macrophages, thereby decreasing the concentration of islets and pancreatic beta cells [23]. On the other hand, morphine seems to damage Langerhans islets by increasing nitric oxide production [7] and increasing peroxide nitrite in cytoplasm. Based on the results of Lukiati et al., peroxide nitrite can cause the destruction and death of beta cells in Langerhans islets [24]. Thymoquinone exerts its anti-inflammatory effects by activation of heme-oxygenase1expression in human and activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by reactive oxygen species [25]. Moreover, the results of Khader et al. showed that Nigella extract and thymoquinone could inhibit the expression of cyclooxygenase-2 in the pancreatic tissue of STZ-induced diabetic rats, which is in line with the findings of the current study [26]. It seems that thymoquinone prevents the morphine-induced injuries in the pancreatic tissue and its islets by inhibiting the expression of inflammatory genes and its antioxidant properties [27]. The results of insulin and glucose analysis in the study groups indicated a significant increase in glucose and a significant decrease in insulin level in the morphine-receiving groups than in saline (control) group. Administration of thymoquinone plus morphine increased insulin and reduced glucose in all study groups than in the groups treated with morphine alone. Kanter et al. reported that thymoquinone administration reduced serum glucose level and increased insulin production in the STZ-induced diabetic rats, confirming the results of the present study. In the current research, thymoquinone was considered to act as a factor protecting the beta cells of Langerhans islets [28]. It seems that insulin is reduced in non-diabetic mice three hours after morphine administration. Insulin reduction is probably due to interaction of opioid and adrenergic systems in inhibition of insulin. Also, inhibition of glucose production can be associated with the direct effect of morphine on liver and increased glucose uptake from blood by some tissues, especially skeletal muscles due to morphine, without insulin interference [29, 30]. However, Hosseini et al. reported an increase in insulin due to morphine consumption, which is in contrast with the findings of the present study [31]. Thymoquinone seems to increase blood glucose level, owing to its antioxidant properties, by exerting its effects on pancreatic beta cells. Thymoquinone can also prevent the destruction of pancreatic beta cells, decrease insulin and increase glucose level by reducing oxidative stress [32]. In the current study, there were changes in pancreatic tissue in the groups receiving morphine such as hyperemia and hemorrhage in the pancreas vessels, shrinkage of Langerhans islets, vacuolization and damage to pancreatic acinar cells. Macrophages can leave different symptoms in response to the histological injuries activated by antioxidant properties of morphine in pancreas, mostly resulting from the leakage of intracellular enzymes caused by loss of integrity and stability of the cell membrane health of the damaged cells [33]. After treatment with thymoquinone, these symptoms were minimized, which could be due to the antioxidant effects and reduced oxidative stress of thymoquinone compounds [9]. The findings of serum blood nitric oxide measurement showed that administration of morphine alone significantly increased nitric oxide compared to control group. Thymoquinone plus morphine decreased the effects of morphine in increasing nitric oxide in the study groups. The cellular mechanism shows that morphine increases the production of nitric oxide through intracellular regulation of calcium and activation of calcium/calmodulin-dependent NOS [6]. Nitric oxide is a free radical that is produced in the mammalian cells, is involved in the regulation of physiological process and is followed by many diseases while it is increased [34]. Thymoquinone can inhibit and suppress iNOS expression and can prevent the increase of nitric oxide production by inducing oxidative stress [35]. Nagi et al. reported thymoquinone could inhibit and suppress the expression of nitric oxide production in the mice under treatment with acetaminophen, which is in line with the results of the present study [36]. In general, the findings of the present study showed that administration of thymoquinone, as a potent antioxidant, to the animals in the groups receiving morphine, especially in high doses, could positively affect the performance of pancreas. Seemingly, the possible antioxidant impacts of thymoquinone have been effective in insulin and blood glucose levels. Moreover, the protective effects of thymoquinone against free radicals can improve the performance and structure of pancreas against the destructive effects of morphine.

Conclusion

The present study findings showed that potential effects of thymoquinone administration especially antioxidant effects against toxic effects of morphine administration. In addition, the results suggest that thymoquinone can significantly improve impairments resulting from the toxicity of morphine in the pancreas. However, further research in animal models is warranted to obtain more conclusive evidence for the molecular interaction between thymoquinone and morphine leading to improve pancreas damage.

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