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Original Article

Protective effect of zinc sulfate and continuous/interval training on liver oxidative stress in morphine-withdrawal syndrome in rats

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Abstract

Introduction: In this study, the effect of zinc sulfate (ZS) supplement and eight-week continuous training (CT) and interval training (IT) on liver oxidative stress of morphine-dependent rats following withdrawal syndrome (WS) were evaluated.

Methods: Seventy Wistar rats were randomly divided into seven groups: control rats, withdrawing rats (WS), withdrawing rats receiving 9mg/kg ZS orally (WS+Z), withdrawing rats under CT (WS+CT), withdrawing rats under IT (WS+IT), withdrawing rats under CT and receiving 9mg/kg zinc sulfate (WS+Z+CT), withdrawing rats under IT and receiving 9mg/kg zinc sulfate (WS+Z+IT). Animals were addicted by 0.4g/l morphine sulfate in 21 days. Animals in the training groups ran on a treadmill and received ZS 5 days/week for 8 weeks. At the end of the study, oxidative stress in liver tissue and liver enzymes were measured by spectrophotometric and ELISA methods.

Results: ZS supplement, CT/IT led to decrease DNA damage and malondialdehyde in comparison with morphine group. Also, ZS, CT and IT significantly elevated levels in superoxide dismutase, catalase activity, total antioxidant capacity and thiol groups in the liver of rats in comparison with morphine group. Additionally, it is observed that ZS and CT/IT made a significant reduction in aspartate aminotransferase levels in comparison to the morphine group.

Conclusion: CT/IT with ZS because of its antioxidant effects has the potential to be used for decreased withdrawal syndrome complications.

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Keywords:
Zinc Sulfate;
Continuous Training;
Interval Training;
Morphine;
Withdrawal Syndrome;
Liver

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Introduction

Prevalence of drug use disorders is about 31 million people worldwide. WHO has reported, roughly 450,000 people died as a result of drug use in 2015.

Opioids continued to cause the most harm, accounting for 76% of the deaths where drug use disorders were implicated (Liu et al., 1999). Morphine, the main component of opium, is perhaps the oldest drug known to man. Morphine decreases stimulation of pain nervous path neurons by connecting to μ-type receptors. Therefore, it is used as a strong anti-pain largely to relieve severe pain (Gordon et al., 1995). Nevertheless, long-term using of these drugs exerts several adverse side-effects, including addiction, tolerance, respiratory depression, immunosuppression and constipation. Addiction is one of the most important issues in human societies, which not only makes social and behavioral disorders but also impose great financial influencing (Schütz et al., 2018).

Liver is one of the vital body organs that plays a major role in detoxification of the toxic elements and chemical drugs. Liver is considered as a major tissue of morphine biotransformation where morphine exerts several adverse toxic, immunological and oncogenic effects (Salahshoor et al., 2016). Increased liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) are reported in laboratory animals after receiving morphine (Salahshoor et al., 2018).

Oxidative stress plays a crucial role in toxic liver damage (Todaka et al., 2005). Studies showed that morphine results in oxidative stress by inducing the generation of reactive oxygen species (Gach et al., 2011). Samarghandian et al. (2014) reported that morphine consumption increased induction of lipid peroxidation and decreased anti-oxidant system's power (the activity of enzymes such as superoxide dismutase [SOD] and catalase [CAT]) which is associated with liver toxicity and damage. Studies showed that morphine is converted to morphinone by morphine-6-dehydrogenase, and after conjugate with glutathione, and removed, which reduces glutathione sources (Nagamatsu et al., 1986).

The antioxidants can protect the liver tissue against oxidative damage. Exercise plays a major role in human health. The beneficial effects of regular exercise and physical activity have been known for a long time. Exercise is part of the treatment of many diseases via induces the antioxidant defense's in several tissues such as liver, heart and kidney (Simioni et al., 2018). In addition, exercise and physical activity have caused increased blood circulation in the liver and accelerated bio-transformation and they also decreased liver oxidative toxic stress (Habib et al., 2020; Trefts et al., 2015).

Trace element homeostasis is critical for antioxidant defense systems. Zinc incorporates in the function of more than 300 enzymes. Studies have shown that zinc plays a specific role in biochemical processes such as cell respiration, DNA propagation, maintaining the integrity of cell membrane and reducing free radicals (Stefanidou et al., 2006). Zinc ions defenses against free radicals by competitive role against copper and iron ions that induced producing free radicals during Haber Weiss reaction. Also, animal-based studies have shown that zinc deficiency can make a significant reduction in vitamin E serum levels. Vitamin E is a protective agent against oxidative damage and prohibits lipoproteins oxidation (Eide, 2011).

Zinc deficiency well-documented in opioid consumers (Ciubotariu et al., 2015). However, based on our studies, there is little information regarding the effect of exercise or zinc supplementation on oxidative stress markers in morphine-dependent rats following withdrawal syndrome. Hence, investigating the effect of exercise and zinc supplementation on liver oxidative stress in the opium users could be considerably beneficial for withdrawing the opium. Therefore, in the current study, we assessed the impact of interval/continuous exercise and zinc supplementation on the liver oxidative stress in morphine-dependent rats following withdrawal syndrome.

Materials and methods

In this experimental study, 70 male Wistar rats (250±20g) were used. Animals were maintained in the animal house with free access to water and food throughout 12:12 hour's light/dark cycle. All ethical considerations were confirmed by the given instructions of Hamadan University of Medical Sciences (IR.UMSHA.REC.1395.195). Animals were randomly categorized into seven groups (n=10): Group 1: healthy control rats (C); Group 2: withdrawing rats without exercise training and receiving normal saline (WS); Group 3: withdrawing rats receiving 9mg/kg zinc sulfate orally (WS+ZS); Group 4: withdrawing rats under continuous exercise training (WS+CT); Group 5: withdrawing rats under

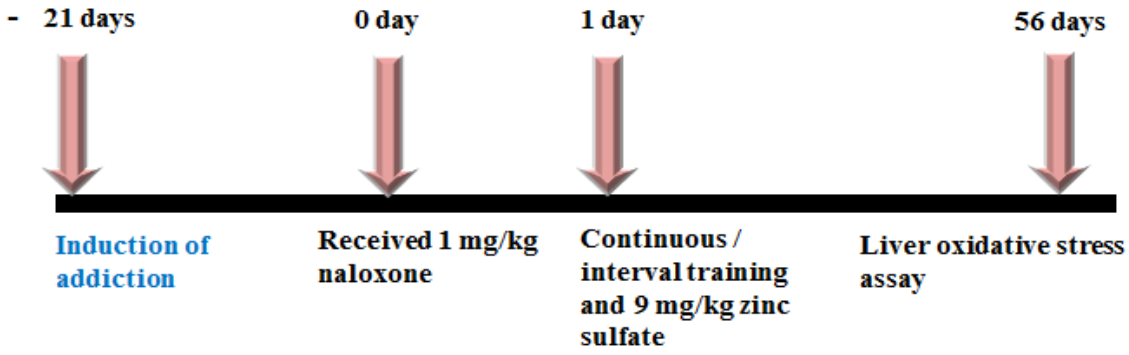

Fig.1. Experimental timeline.

Table 1: Continuous and interval training protocols

Week	Continuous group	Interval group
1	16 (12 m/min)	2 × 8 (12 m/min)
2	20 (12 m/min)	2 × 10 (12 m/min)
3	24 (13 m/min)	2 × 12 (13 m/min)
4	28 (14 m/min)	2 × 14 (14 m/min)
5	33 (15 m/min)	3 × 11 (15 m/min)
6	39 (16 m/min)	3 × 13 (16 m/min)
7	45 (17 m/min)	3 × 15 (17 m/min)
8	51 (18 m/min)	3 × 17 (18 m/min)

interval exercise training (WS+IT); Group 6: withdrawing rats under continuous exercise training receiving 9mg/kg zinc sulfate (WS+ZS+CT); Group 7: withdrawing rats under interval exercise training receiving 9mg/kg zinc sulfate (WS+ZS+IT).

Addiction

Animals in morphine-dependent groups were addicted by 0.4g/l morphine sulfate during 21 days. In addition, sucrose (40mg/m) was added to drinking water due to the bitter taste of morphine. To ensure that the morphine-induced dependency in the animals, 1-2 rats in each working group received 1mg/kg naloxone (Sigma-Aldrich, St. Louis, MO, USA, IP) (Zarrinkalam et al., 2016). After being assured of morphine, they were put on the treadmill for 1 week to get acquainted with the treadmill for 15 minutes at a speed of 13m/s. Rats that did so easily were selected as the study groups. Then animals of the training groups performed continuous/interval exercise training and 9mg/kg zinc sulfate were orally for five days in a week for 8 weeks.

Continuous training

In this protocol, rats were exercised for 8 weeks, 5 days/week. Rats had performed the training with 12m/min for 16 minutes. During 8 weeks, the training speed and duration were gradually increased and they exercised for 51 minutes with 18m/min on the treadmill (Table 1).

Interval training

The training was performed in a multi-stage phases for interval group and active recovery was considered. Training was followed with 12m/min with two 8-minute phases, which was increased to three 17-minute phases with 18m/min (Table 1) (Shabkhiz et al., 2008). At the end of treatments, rats were anaesthetized with ketamine (50mg/kg, IP) and immediately, their livers were maintained in -80°C. Also, the serum was collected and maintained for measuring liver enzymes activity (Fig .1).

Tissue collection and processing

Liver tissues of rats were excised and rinsed with ice-cold saline and grounded into a fine powder using liquid nitrogen. The homogenate was resuspended in ice cold lysis buffer (10mM HEPES, 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 0.2% triton X100, 0.5mM dithiothreitol, protease inhibitor cocktail, pH 7.9) and incubated on ice for 20min. The tissue homogenates were vortexes and centrifuged (14,000g; 10min; 4°C),

and the supernatants were retained for analysis (Goli et al., 2019).

Assay of total protein

Total protein concentration in tissue homogenates was assessed by the Bradford method using bovine serum albumin as the standard (Kruger, 1994).

Assay of lipid peroxidation

Malondialdehyde (MDA) were measured used the thio-barbitoric acid method (TBA) to assess lipid peroxidation, which its maximum absorption of the pink complex read TBA+MDA in the wavelength by 532nm. In this method the calibration curve of tetraethoxypropane standard solutions was used to determine the concentrations of TBA+MDA adducts in samples (Shateri et al., 2019).

Assay of total antioxidant capacity (TAC)

In order to evaluate TAC, we used ferric reducing ability of plasma (FRAP) method, in which it was added to major samples of FRAP containing 2, 4, 6-tripiperidyltriazine (TPTZ 6, 4,2), and maximum absorption of blue complex+Fe^{2+}TPTZ was read as 593nm in the wavelength (Ranjbar et al., 2018).

Assay of total thiol groups

Total thiol groups were evaluated as the other indicator of oxidative stress status. Thiol groups are sensitive to oxidative damages and their decrement is an important indicator for oxidative stress. It was used Hu-calorimetric method of DTNB (2.2-di-thionitrobenzoic acid, known as Ellman) in order to evaluate these parameters. So, maximum absorption was read as 412nm in the wavelength (Rahimi et al., 2018).

Assay of DNA damage

The amount of DNA damage (8-OHdG) superoxide dismutase and catalase were assessed by ELISA kit according to its protocol.

Assay of activity of liver aminotransferase enzyme

Serum levels of ALT and AST were assayed by Pars Azmoon kit.

Statistical analysis

The SPSS software, version 23.0 (SPSS Inc., Chicago, IL, U.S.A) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) were applied for statistical analysis. All data analyzed by one-way analyses of variance (ANOVA) followed by Tukey's post hoc test. Results were considered significantly different if $P<0.05$.

Results

SOD activity

The results of measuring liver tissue SOD activity in the studied groups are shown in Figure 2. SOD activity in a healthy control group was significantly more than withdrawing syndrome control groups and withdrawing groups with continuous training ($P<0.05$). Conversely, SOD activity level in withdrawing control group was significantly lower than withdrawing groups with interval training, continuous training and zinc sulphate and interval training with zinc sulphate ($P<0.05$).

CAT enzyme activity

The results of measuring liver tissue CAT enzyme activity in the studied groups are illustrated in Figure 3. In contrast, the CAT activity level in a healthy

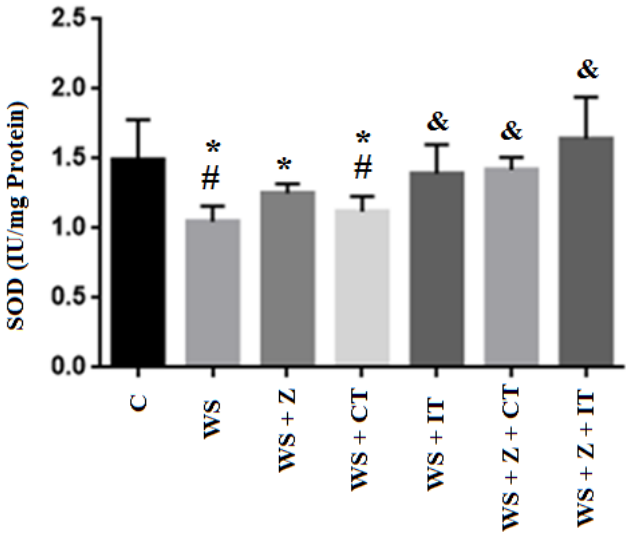

Fig.2. Comparison of superoxide dismutase (SOD) activity in the liver tissue of studied groups. Data is represented as mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). #Significant statistical difference with healthy control group, &significant statistical difference in comparison with WS group ($P<0.05$). *Significant statistical difference in comparison with WS+Z+IT group ($P<0.05$). There was no significant difference between the other groups.

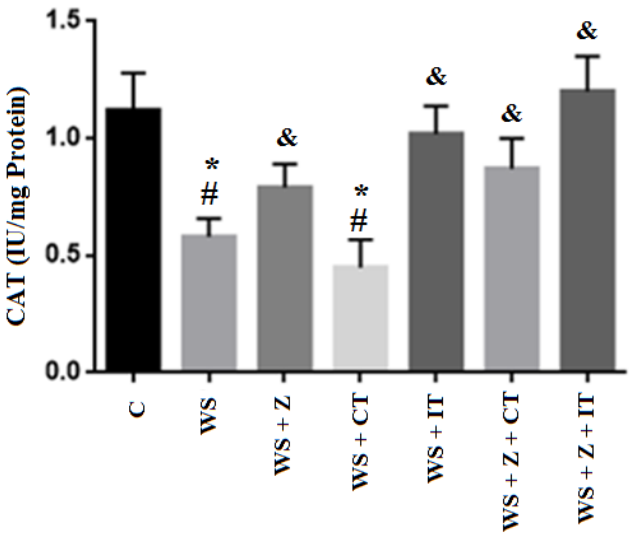

Fig.3. Comparison of catalase (CAT) enzyme activity in the liver tissue of studied groups. Data is represented as mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). #Significant statistical difference with healthy control group, &significant statistical difference in comparison with WS group (P<0.05). *Significant statistical difference in comparison with WS+Z+IT and WS+IT group (P<0.05). There was no significant difference between the other groups.

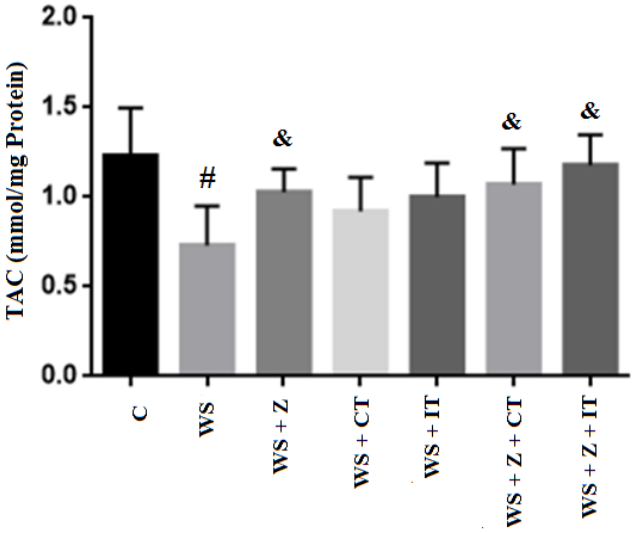

Fig.5. Comparison of total antioxidant capacity (TAC) in liver tissue of the studied groups. Data is represented as mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). #Significant statistical difference with healthy control group, &significant statistical difference in comparison with WS group (P<0.05). There was no significant difference between the other groups.

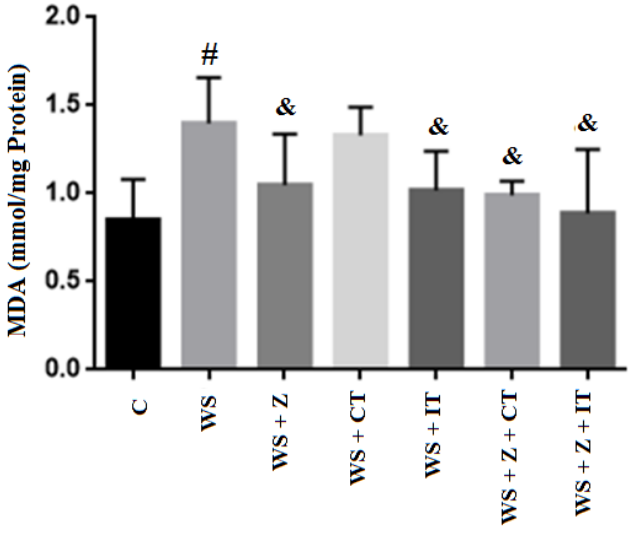

Fig.4. Comparison of lipid peroxidation level (MDA) in the liver tissue of studied groups. Data represented is as mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). #Significant statistical difference with healthy control group, &significant statistical difference in comparison with WS group (P<0.05). There was no significant difference between the other groups.

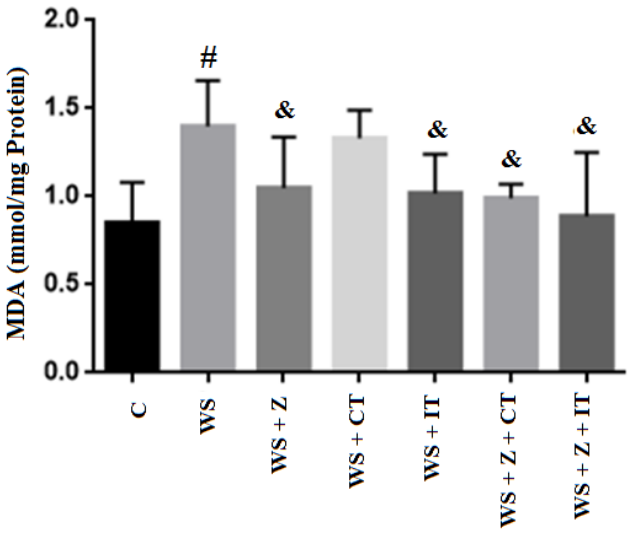

Fig.6. Comparison of alanine aminotransferase (ALT) activity level in the studied groups. Data is represent as mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). There was no significant difference between groups.

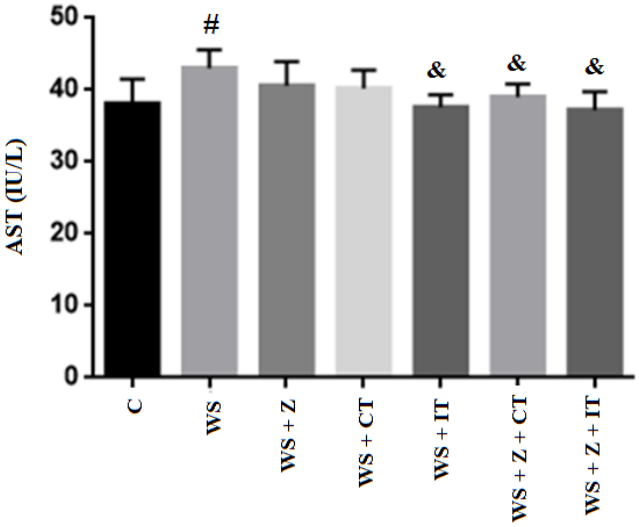

Fig.7. Comparison of aspartate aminotransferase (AST) activity in the studied groups. Data is represent as Mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). #Significant statistical difference with healthy control group, &significant statistical difference in comparison with WS group (P<0.001). There was no significant difference between the other groups.

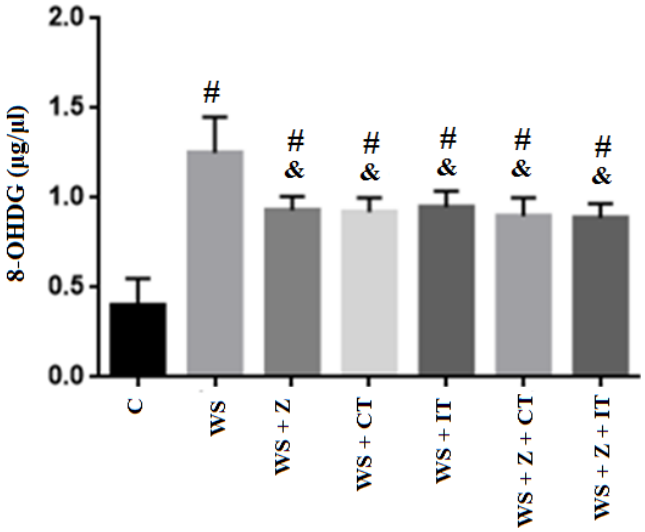

Fig.8. Comparison of DNA damage level (8-OHdG) in the studied groups. Data is represent as mean±SD. Healthy control (C), withdrawing syndrome (WS), zinc sulfate (Z), continuous training (CT), interval training (IT). #Significant statistical difference with healthy control group, &significant statistical difference in comparison with WS group (P<0.05). There was no significant difference between the other groups.

control group was significantly more than withdrawing syndrome control group and withdrawing group with continuous training (P<0.05). Conversely, the CAT activity level in withdrawing control group was significantly lower than withdrawing groups with interval training, receiving zinc sulfate, continuous training, continuous training with zinc sulfate and interval training with zinc sulfate (P<0.05).

Lipid peroxidation measurement

The results of measuring lipid peroxidation level (MDA) are illustrated in Figure 4. In contrast, lipid peroxidation level in withdrawing control group was significantly more than other studied groups (P<0.05). Significant statistical difference in other groups did not detect (P>0.05).

TAC measurement

According to the results shown in Figure 5, TAC in withdrawing control group was significantly lower than the healthy control group (P<0.05). Oral zinc sulfate, continuous exercise training with zinc sulfate and interval exercise training with zinc sulfate improved in TAC in comparison with withdrawing control group (P<0.05).

ALT and AST activity

The results of ALT activity are illustrated in Figure 6. According to these results, the studied groups did not show any significant statistical differences (P>0.05). In contrast, AST activity in withdrawing control group was significantly more than healthy control, interval training, interval training with zinc sulfate and continuous training with zinc sulfate (P<0.001, Fig. 7).

Assessing DNA damage level (8-OHdG)

Results of DNA damage level according to 8-OHdG in the liver of studied groups are illustrated in Figure 8. Level of 8-OHdG in the healthy control group in comparison with withdrawing syndrome control group was significantly lower (P<0.001). On the other hand, 8-OHdG in withdrawing syndrome control group was significantly more than other treated withdrawing groups (P<0.001).

Discussion

Opioids are considered as the most important anti-pain drugs. Morphine is an opioid analgesic drug and the main psychoactive chemical in opium. Oxidative stress and apoptosis are the main mechanisms of morphine-induced cytotoxicity. Morphine increases oxidative stress through two pathways: 1- increasing oxygen reactive species and 2- inhibiting antioxidant

system performance in the cell (Skrabalova et al., 2013). In the present study, results indicated that oxidative stress was increased in the treated group with morphine, which is similar to the results obtained from other studies. Zhang et al. (2004) reported that morphine induces oxidative stress markers in the liver of rats, such as 8-OHdG, MDA and reducing the activity of anti-oxidants enzyme including SOD, catalase and glutathione peroxidase. In addition, treating with ascorbic acid as an antioxidant substance leads to reduce oxidative stress conditions in the liver of rats. Also, it was reported that morphine decreases anti-oxidant and glutathione enzymes in the intestine and liver of rats, which is consistent with our study results (Nagamatsu et al., 1983; Salehi et al., 2018; Sumathi and Niranjali Devaraj, 2009). Studies have shown that morphine increases dopamine and xanthine oxide metabolism, which cause increased oxygen reactive species. In addition, morphine usage increased the risk of lipid peroxidation level and then liver damages due to disturbance of antioxidant/oxidant balance (Lurie et al., 1995).

In order to eliminate oxygen reactive species, biologic antioxidant enzymes such as SOD, catalase, glutathione peroxidase and non-enzymatic antioxidants react with oxygen reactive species (Samarghandian et al., 2014). In the present study, results showed that lipid peroxidation was increased and CAT and SOD antioxidant enzymes were decreased in the rats received morphine. On the other hand, treating with continuous/interval training with zinc sulfate improved the antioxidant system (SOD and CAT) and then, it decreased oxidative stress in the liver tissue. Similarly, Mallikarjuna et al. (2009) indicated that regular exercise training significantly reversed the increase MDA and decreases GSH and ascorbic acid induced by ethanol drinking. Also it was reported that endurance, resistance and concurrent exercise significantly normalized oxidative stress and the morphological changes of the intestine in withdrawal rats (Salehi et al., 2018). It is reported that continuous and interval training decreased oxidative damage in erythrocytes of the studied humans (Yunus et al., 2018). Various mechanisms are provided for describing antioxidant enzyme responses to exercise training. It is well described that following exercise training especially severe endurance training, free radical production increases. Subsequently, MDA as an indicator of lipid peroxidation increases, then cell defensive system such as antioxidant enzymes induce and activate in order to meet produced oxidative stress (Jahani et al., 2010).

It was shown that swimming training decreased oxidative damage, fat mass and protein oxidation in high-fat diet animals. Also, it has improved their metabolism characteristics in comparison with the control group (Zacarias et al., 2017). Following exercise training, it seems that cell defensive system attempts to provide the balance and/or enhancement of antioxidant enzymes against oxidative stress. Interestingly, our findings is in agreement with previous studies which showed that, continuous and interval training and zinc sulfate decrease oxidative stress level in morphine-dependent rats in withdrawing syndrome.

Liver injury causes changes in liver enzyme levels and secretion soluble cytosolic enzymes into the circulation. Aminotransferase (ALT, AST and ALP), bilirubin (total and direct) are the most sensitive and the most widely used for the detection of liver injury (Khazaei et al., 2016). Releasing these enzymes from liver cytosol to blood is reported during toxicities with morphine (Atici et al., 2005). This study also demonstrated that serum ALT and AST levels decreased after treatment with continuous and interval training and zinc sulfate.

It was reported in the study by Samarghandian et al. (2014) that the levels of liver enzymes AST and ALT in the serum of treated rats with morphine were significantly more than the healthy control group which is consistent with the results of the present study. Also, Nabizadeh Haghighi and Shabani (2016) reported that exercise training significant reduction in AST and ALT enzymes activity in fatty liver patients. Significant decreasing of AST and ALT enzymes due to exercise training are probably related to the increased liver oxidation rate, decreased activity of lipogenic enzymes and decreased liver fatty acids (Shamsoddini et al., 2015).

Finally, this study demonstrated that, the effect of zinc with continuous and interval training on the provided stress in morphine-dependent rats was considered as synergistic. Various mechanisms were mentioned for zinc anti-oxidant effects. Zinc increases the synthesis of metallothionein –cysteine-rich protein – which acts as a free radical inhibitor.

Also, zinc is incorporated in the structure of the most body enzymes, for example, SOD which is considered as the major antioxidant enzyme (Stefanidou et al., 2006). Another important effect of zinc is its competitive role against iron and copper ions. Free ions of iron and copper convert H_2O_2 to free radicals of OH through Haber–Weiss reaction and increase oxidative stress through this reaction. Zinc, as a competing ion in this interaction, restricts that and reduces oxidative agents. Also, animal studies have shown that zinc deficiency may significantly decrease vitamin E serum level. Vitamin E is a protective factor against oxidative damage and inhibits lipoproteins oxidation (Bunk et al., 1989; Eide, 2011). Lack of histological tests, gene expression and molecular tests and short study period are some of the limitations of the present study. On the other hand, the simultaneous use of zinc supplements and continuous/interval training on oxidative stress of liver tissue was performed for the first time in morphine-withdrawal model, which is one of the strengths of the study. Also, different factors of oxidative stress, enzymatic activity in tissue were measured.

Conclusion

Our findings pointed out the risk of increased oxidative stress and hepatic damage due to morphine. Although morphine is reported to be effective in pain management, their toxic effects should be kept in mind during usage. Results showed that interval/continuous training with zinc sulfate supplementation decreased oxidative stress in morphine withdrawing syndrome rats. Based on the results of this study, we conclude that IT, ZS+CT or ZS+IT exert similar effects on liver oxidative stress. However, further studies are needed in order to examine and verify the molecular pathways underlying the effects of continuous and interval training with zinc sulfate supplementation in the liver tissue of morphine-dependent animal models.

Acknowledgments

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Conflict of interest

The authors have declared no conflicts of interest.

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