

Original Article

Effect of long-term treatment of morphine on enzymes, oxidative stress indices and antioxidant status in male rat liver

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Abstract: In this study, biochemical changes due to long term usage of morphine in rat's liver were assessed. Twenty male Wistar rats (180-220 g) were included and divided into two groups. Normal saline was given intraperitoneally in the control group ($n = 10$). Morphine group ($n = 10$) received morphine intraperitoneally at a dose of 4, 8, and 10 mg/kg/day in the first, second and the third ten days of the study, respectively. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and liver malondialdehyde (MDA) level as well as activities of superoxide dismutase (SOD), glutathione-s-transferase (GST) and catalase (CAT) were measured. Serum levels of AST, ALT and LDH were significantly higher in the morphine group compared with the control group. The mean MDA level of liver was significantly higher in the morphine group compared with the control group ($P < 0.05$). The activities of SOD, GST and CAT were significantly lower in the morphine group compared with the control group ($P < 0.01$). Our findings pointed out the risk of hepatic damage due to long term usage of morphine via disturbance oxidant-antioxidant balance. Although morphine is showed to be effective in pain treatment, their toxic effects should be kept in mind during the chronic usage.

Keywords: Morphine, oxidative stress, liver, antioxidant, rat

Introduction

The central role of liver in drug metabolism predisposes them to toxic injury. Every drug has been related to hepatotoxicity almost mainly due to the critical role of the liver in drug metabolism [1]. Chronic opiate intoxication has been indicated to insert pathological damages in the liver in nearly 100% of cases [2]. Opioids are the most potent and effective analgesics available and have become accepted as appropriate treatment for acute, cancer and non-cancer chronic pain [3-5]. Morphine, a widely used opioid in recent years, is an effective analgesic drug for the management of severe pain, is metabolized mainly in the liver [6, 7]. While long-term treatment of morphine for chronic pain continues to be controversial [8, 9]. Published patient surveys and case-reports support the efficacy and safety of long-term

use of opioid analgesics in the chronic non-malignant nociceptive and neuropathic pain [10, 11]. However, experimental studies have reported that the chronic usage of morphine increased hepatotoxic damages [12]. Present findings point out the risk of increased serum lipid peroxidation, hepatic damage due to the long term use of morphine. These research aimed at detecting the toxicity of morphine on liver may include the measurement of several metabolites and enzymes in plasma, and no study demonstrated exactly long-term effects at liver cellular level after long term management of morphine. An imbalance between production of reactive oxygen species (ROS) and its elimination by the antioxidant defense system in the body has been implicated for liver injury. Free radicals generated from cellular metabolic processes have long been implicated in the hepatotoxicity [13, 14]. Free radicals

have been shown to cause oxidative damage to lipids (lipid peroxidation), protein, and nucleic acids [15, 16]. In order to combat the damaging effects by free radicals, cells have evolved a complex antioxidation system that includes both exogenous antioxidants and endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST). Therefore, the present study was designed to determine a possible mechanism of the liver injury after the long term treatment of morphine by measuring serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and liver malondialdehyde (MDA) level as well as activities of SOD, GST and CAT after long term treatment of morphine.

Materials and methods

Chemicals

All chemicals and diagnostic kits were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

30 Male Sprague-Dawley (SD) rats (180-200 g) were obtained from The Laboratory Animal Centre, Medical University of Mashhad. The animals were maintained in a temperature room ($22 \pm 2^\circ\text{C}$), with 12 h light/dark cycle and fed with standard rat chow and water ad libitum.

Study design

After 14 days of acclimatization, rats were randomly allocated in to two experimental groups, ($n = 10$ per group) as follows: group 1, control and group 2, received morphine. As sham-treated group, normal saline (0.9% NaCl) was given intraperitoneally to the control group ($n = 10$). Morphine group received morphine intraperitoneally at a dose of 4 mg/kg/day for the first 10 days, 8 mg/kg/day between 11-20 days and 12 mg/kg/day between 21-30 days. Rats were kept in their own cages at constant room temperature ($21 \pm 2^\circ$) under a normal 12 hr light: 12 hr dark cycle with free access to food and water. The animals were housed according to regulating the welfare of experimented animals. The study was conducted in Mashhad

Medical University Experimental Animal Research Laboratory. Protocols were approved by the Ethical Committee. At the end of the period of this study, the animals were anesthetized by ether and blood was subsequently collected from the retro orbital sinus. Blood and sera were separated by centrifugation at 3000 rpm for 10 min for ALT, AST and LDH enzymes.

Livers of rats were quickly removed, weighed, and washed in 0.9% NaCl and these samples were frozen in liquid nitrogen and kept at -80°C . The homogenates and subcellular fractions were prepared; the pellet obtained at 12,000 g containing mitochondria and the supernatant fractions were used for enzyme assays (Siddiqui et al. 2005). Whole homogenates were used for measurement of lipid peroxidation. Supernatant fraction was used for estimation of SOD, GST and CAT activities.

Measurements of enzymes

Assay of SOD: The activity of SOD was determined by the method of Marklund and Marklund 1979, using inhibition of pyrogallol autoxidation at pH 8. The specific activity of SOD is expressed as units per mg protein per minute [17].

Assay of GST: GST activity was determined in post-mitochondrial fraction of the tissues, which was separated by sequential centrifugation. In brief, tissue homogenates were centrifuged at 600 g for 10 min at 4°C to remove crude fractions. Then, supernatants were centrifuged at 10,000 g for 20 min to obtain the post-mitochondrial fraction. GST activities were measured using cumene hydroperoxide and 1-chloro-2, 4-dinitrobenzene as substrates [18].

CAT activity assay: CAT activity was assayed by H_2O_2 consumption, following Aebi's [19] method and modified by Pieper et al. [20]. Briefly, ethanol was added (1:100 v/v) to the supernatants and incubated for 30 min in an ice bath. We then added 1% Triton X-100 (1:10 v/v) (Sigma) to the homogenates. This solution was placed in an ice bath for an additional 15 min. 500 μl of this solution was placed into a glass cuvette and 250 μl of 30 mM H_2O_2 (Sigma) in a phosphate buffer was then added to start the reaction. After 15 s the absorbance at 240 nm was read every 15 s for 45 s. The first-order reaction rate (k) of H_2O_2 consumption by CAT was calculated and the results are expressed in k/g tissue. The assay was performed at 25°C .

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Table 1. Effects of morphine on liver enzyme functions in rats

Groups	ALT (U/L)	AST (U/L)	LDH (U/L)
Control	31.22 ± 3.40	119.33 ± 16.00	497.00 ± 110.21
Morphine	42.50 ± 2.91*	201.71 ± 22.10**	1322.50 ± 221.00**

Values are expressed as mean ± SD. Morphine group compared with control group: *: $P < 0.05$; **: $P < 0.01$.

Table 2. Changes in lipid peroxidation and activities of antioxidant enzymes (SOD, GST and CAT) in rat liver of control and morphine group

Groups	MDA (nmol/mg protein)	SOD (U/mg protein)	GST (nmol/min/mg protein)	CAT (k/g tissue)
Control	2.01 ± 0.30	13.50 ± 1.22	380.00 ± 22.34	18.00 ± 2.12
Morphine	5.11 ± 0.45***	8.71 ± 1.60*	311.21 ± 20.50*	12.09 ± 1.70*

Values are expressed as mean ± SD. Morphine group compared with control group: *: $P < 0.05$; ***: $P < 0.001$.

Measurement of lipid peroxidation

The formation of lipid peroxides was measured in the homogenates of the whole liver. The formation of MDA, an end product of fatty acid peroxidation was measured spectrophotometrically at 532 nm by using a thiobarbituric acid reactive substance (TBARS) essentially by the method of Genet et al. Results are expressed as nmole of MDA formed/mg protein [21].

Protein estimation

Protein was estimated in subcellular fractions by the method of Bradford using bovine serum albumin (BSA) as standard [22].

Statistical analysis

All experiments were carried out at least in duplicate. Every group consisted of ten rats. One-way analysis of variance (ANOVA) was performed and Dunnett's post hoc test was used to compare all the groups against the 2 month-old group. Statistical analyses were performed using the InStat 3.0 program. The results are expressed as mean ± SEM. The results originated from analyzing of individual livers. Linear correlation tests were also performed. Differences of $P < 0.05$ were considered significant.

Results

Serum ALT, AST and LDH levels were significantly higher in the morphine group compared with the control rats ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) (Table 1). Lipid peroxida-

tion was measured as the formation of MDA in whole homogenates of rat liver from morphine and control animals. There was an increase in Liver MDA level in the morphine group when compared with the control group ($P < 0.001$) (Table 2). Changes in the activities of SOD and GST in cytosolic fractions in the liver of control and morphine-treated rats are summarized in Table 2. In the morphine group a significant ($P < 0.05$) decrease was seen in SOD activity

when compared with the control rats. Long term treatment of morphine to animals also decreased GST activity in the morphine group, when compared with controls ($P < 0.05$). The activity of CAT was found to be significantly lower in the morphine group versus the control group ($P < 0.05$).

Discussion

Our study found that long term treatment of morphine significantly increases the serum levels of ALT, AST and LDH enzymes in the rat serum. In addition, in the present study, pro-oxidant-antioxidant balance was evaluated by measuring endogenous MDA level and enzymatic antioxidants in liver homogenate of treated rats. Increased endogenous MDA and decreased SOD, GST and CAT levels indicated that the balance changed on the behalf of pro-oxidation in the liver homogenates of rats after long term treatment of morphine. These findings propose the possible hepatotoxic effects of morphine.

The oxidative stress is implicated in a variety of biological phenomena, including aging, atherosclerosis, inflammation, diabetes mellitus, carcinogenesis and neurodegenerative disorder [23-25]. Detailed mechanisms of involvement of reactive oxygen species in the pathology of diseases are now available [23]. In this sense, monitoring oxidative stress is quite important in the morphine administration study. Although opioids are being widely used since very long time, their long-term effects exactly at cellular level are not clearly found. Present study indi-

cated increases of oxidative damage of lipid, as well as decreases of antioxidant enzymes (SOD, GST and CAT) in animals have been received morphine for long term. All these results implied there was serious oxidative stress in morphine administered rat liver.

Some data reported by others confirmed present results. A direct evidence for reactive oxygen species being involved in the process of opiate dependence is that morphine can directly induce the formation of superoxide in glomerular mesangial cells in a dose-dependent manner. Since superoxide has been indicated to cause mesangiolysis, it was proposed that reactive oxygen species may insert a essential role in the induction of mesangial injury in patients with opiate abuse [26]. Another research demonstrated that single morphine administration increased dopamine and xanthine oxidative metabolism with a consequent increase in reactive oxygen species production [27]. Also, morphine could be metabolized into free radicals [28]. A significant increase in lipid peroxidation was reported in rats receiving an acute dose of cocaine [29]. Similarly lipid peroxides were found significantly increased among chronic heroin users [30]. In another experimental study, isolated rat hepatocytes indicated a marked decrease in glutathione level when incubated with various concentrations of morphine and resulted in the over production of reactive oxygen species could lead to oxidative damage cell death [31]. Furthermore, the risk of increased the levels of lipid peroxidation in liver and hepatic damage due to sub acute use of morphine has been reported. Toxic effects of opioids at cellular level may be explained by lipid peroxidation [32]. Biological membranes contain a large amount of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by oxidants resulting in lipid peroxidation. Therefore, lipid peroxidation has been used as an indirect marker of oxidant-induced cell injury. The over production of reactive oxygen species could lead to oxidative damage. For eliminating the excessive reactive oxygen species induced by morphine, biological antioxidant enzymes including superoxide dismutase, catalase, GSH peroxidase and non-enzymatic antioxidants will react with most oxidants. This study also demonstrated that long term use of morphine led to significant decrease in the liver levels of antioxidant enzymes including SOD, GST and CAT.

These findings were supported by pervious studies that showed morphine reduced the antioxidative defense system, such as the activity of antioxidative enzymes, and the ratio of GSH/oxidized GSH. Antioxidant enzymes such as (SOD, GST and CAT) belong to multifunctional detoxification enzymes, which defend cells against a wide variety of toxic insults from chemicals, metabolites, and oxidative stress [33]. The activities of antioxidant enzymes decreased in the liver injury. Oxidative stress is postulated to be one of the most important mechanisms behind the drug abuse related decrease in SOD, GST and CAT activities in rats. However, the side effect of long-term administration of morphine was not observed in patient with chronic non-malignant nociceptive and neuropathic pain [10, 11]. But present findings pointed out the risk of increased hepatic damage through change balance between production of reactive oxygen species (ROS) and its elimination by the antioxidant defense system in the liver due to long term use of morphine. Finally, although morphine seems to be effective in pain control, their toxic effects should be considered.

Disclosure of conflict of interest

None.

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