Protective Effect of *Flunixin meglumin* on Changes Induced by Isoproterenol in Serum Biochemical Profile, Malondialdehyde and Heart Histology of Adult Male Rats

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A B S T R A C T

Background:

Myocardial infarction (MI) is one of the most important causes of death worldwide. The effect of flunixin meglumine (FM) on MI is unknown. We investigated the protective effect of FM on the serum biochemical profile, malondialdehyde (MDA), and heart histology changes induced by isoproterenol (ISO) in adult male rats.

Methods:

Rats were randomly divided into four groups of six each as follows: Group 1, control. Group 2, was administered ISO subcutaneously at 85 mg/kg for 2 days. Group 3 was administered FM intraperitoneally at 2 mg/kg/d, for 3 days; and Group 4 was administered FM 2mg/kg/d intraperitoneally for 3 days and ISO 85mg/kg subcutaneously for two consecutive days. Blood samples were collected after anesthesia, 24h after the second ISO administration. The heart tissue was immediately removed for histopathological examination. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), Blood urea nitrogen (BUN), creatinine and serum MDA levels were measured on an autoanalyzer and spectrophotometer.

Results:

In Group 4 (FM+ISO) the levels of AST, LDH and MDA decreased compared with those for Group 2 (ISO) ($P< 0.05$). Histopathologic tests revealed that ISO induced myocardial injury in Group 3 rats, whereas the alterations of the heart architecture were improved by FM in Group 4 (FM+ISO).

Conclusions:

The results suggest that FM has a potential cardioprotective effect against an experimental model of MI in rats.

Keywords:
Cardioprotective Effect; Funixin Meglumine; Isoproterenol; MDA; Myocardial Infarction

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INTRODUCTION

Myocardial infarction (MI) is a major cause of death worldwide due to myocardial necrosis secondary to imbalances between the coronary blood supply and myocardial demand for oxygen (1). According to the WHO report (2), the morbidity and mortality rate caused by MI has now reached 16.7 million deaths per year globally. Two factors are known to play a key role in the pathogenesis of MI: the oxidative stress and the inflammatory response. It has been determined that ischemia alters antioxidant defense mechanisms and induces the production of free oxygen radicals simultaneously (3). Isoproterenol is a ß-adrenergic agonist and a synthetic catecholamine, which is widely used for the treatment of bronchial asthma, allergic emergencies, ventricular bradycardia, status asthmaticus, cardiac arrest and glaucoma (4). The administration of high doses of ISO produces cytotoxic reactive free radicals after autoxidation and leads to

the damage and necrosis of cardiac muscle (5). The subcutaneous injection of ISO has been widely used in various studies as an experimental model of MI to examine the cardioprotective effect of natural and synthetic agents (6). The infarct-like lesions induced by ISO in the rat cardiac muscle serves as a standard model for investigating MI in humans (7). Nonsteroidal anti-inflammatory drugs (NSAIDs), which are known as cyclooxygenase (COX) inhibitors, have shown beneficial effects on rhabdomyolysis in rats (8). *Flunixin meglumine* (FM) is a major NSAID, used in veterinary medicine, has anti-inflammatory, antipyretic and analgesic effects (9). Recent studies have shown that treatment with FM could be an effective approach to the management of rhabdomyolysis and tendon injuries in rats and rabbits (10, 11). The therapeutic effects of FM have been studied in the U.S. and Europe on horses and dogs. Further, studies have also been conducted in cattle (12). In this regard, there has been no study on the effect of FM against ISO-induced MI in rats. Therefore, we investigated whether FM has a protective effect on cardiac marker enzymes and the histopathology of cardiac myocytes in ISO-induced MI in rats.

MATERIALS AND METHODS

Reagents: Isoproterenol hydrochloride (ISO) was purchased from Sigma-Aldrich (St. Louis, Mo, USA). We freshly prepared ISO solution by dissolving it in 0.9% normal saline. Diagnostic kit for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatin kinase (CK-MB) isoenzyme, lactate dehydrogenase (LDH), blood urea nitrogen (BUN) and Creatinine were obtained from Zeist Chem Diagnostics, Inc. (Tehran, Iran). All other chemicals used were of analytical grades.

Animal Grouping: Twenty-four healthy adult Wistar male rats weighing 180–200g were used in this study. The Rats were kept in stainless steel cages under standard conditions (12 hr. light/dark cycle; 22 ± 3°C; 45%–55% humidity). Animals were divided in four groups of six rats each. They were allowed to acclimatize for one week before starting the study and were fed commercially available rodent food and tap water *ad libitum*. Animal experiments were approved by the Experimental Animal Local Ethics Committee of the Veterinary School of Semnan University. All animal experiments were performed according to regulations and guidelines established by this committee**.**

Rats were randomly divided into four groups (N=6) as: ***Group 1***, control, received normal saline subcutaneously for two consecutive days (4th and 5th). ***Group 2*** received isoproterenol hydrochloride (ISO) dissolved in normal saline subcutaneously at 85 mg/kg for induction of myocardial infarction on days 4 and 5 with a 24-h interval (13). ***Group 3*** was administered FM intraperitoneally at 2 mg/kg/d for three consecutive days. ***Group 4*** was administered FM 2mg/kg/d intraperitoneally for three days and ISO at 85mg/kg subcutaneously for two consecutive days.

Sample Collection: Twenty-four hours after the second ISO administration, all rats were deprived of food for 12h and were anesthetized with chloroform. Blood samples were collected from the rats in test tubes. The heart tissue samples were immediately removed from the rats, cleaned with ice cold saline, dried with filter paper for histopathological examinations and stored in 10% neutral formalin buffer.

Histopathological Examination of Heart Tissue: Cardiac tissues were isolated and washed with ice cold saline. The tissue samples were immersed and fixed in the formalin buffer as fixative for 18h, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Small pieces of cardiac tissues were sectioned serially at 4µm thick and stained with hematoxylin and eosin (H&E). The slides were examined under light microscopy (Olympus, NY, USA) and photomicrographs were taken.

Statistical Analysis: All values are presented as the means ± standard deviations (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-test by SPSS software, version 19. Statistical differences with P<0.05 were considered significant.

RESULTS

Effect of FM on Liver Enzymes: The effects of ISO on the levels of ALT, AST, CK and LDH are presented in Figures 1, 2, 3 and 4. In Group 1, the ALT level was 108.73 ± 44.42 u/ml, while in Group 2, the ALT level was significantly increased to 124.81 ± 19.16u/ml. In Groups 3 and 4, insignificant decreases were noted in the ALT levels (117.09 ± 33.84 and 117.38 ± 29.42, respectively) compared to those noted for Group 2 (Figure 1). In Group 1 (controls), the serum AST level was 115.53u/ml (± 70.27). The induction of MI by ISO caused a significant increase in the serum AST level compared to that in Group 1 (P≤0.05). As shown in Figure 2, the administration of FM at 2mg/kg significantly decreased the serum AST level (*P*<0.05). As presented in Figure 3, the serum CK levels (1744.16 ± 165.75, 1201.95 ± 599.36 and 1361.20 ± 399.96, respectively) were significantly higher (*P*<0.05) in Groups 2, 3 and 4 (ISO, FM & FM+ISO) compared to that for the control group (470.16 ± 103.2 u/ml). A significant increase in the LDH level (*P* <0.05) was observed in Group 2 compared to those in Groups 1, 3 and 4 (control, FM & FM+ISO) (Figure 4).

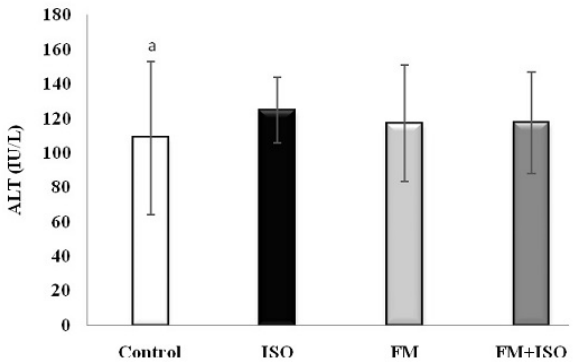

Figure 1. Effect of FM treatment on serum ALT activity in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 are compared to Group 2. ([a] P<0.05).

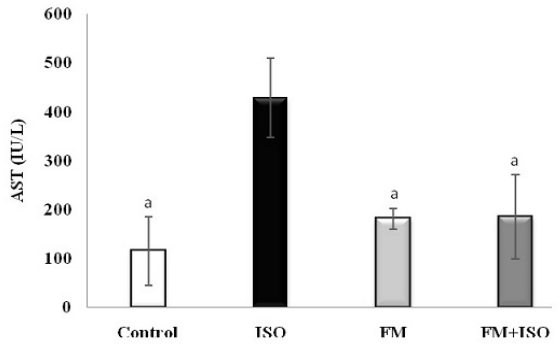

Figure 2. Effect of FM treatment on serum AST activity in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 are compared to Group 2 ([a] P<0.05).

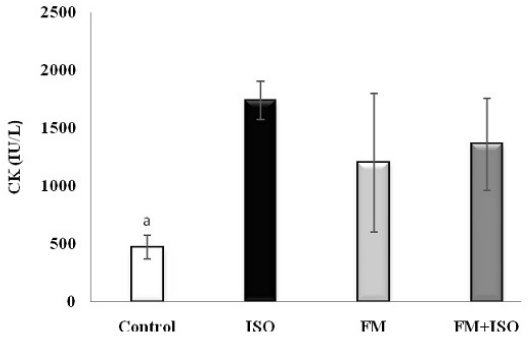

Figure 3. Effect of FM treatment on serum CK activity in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 are compared to Group 2 ([a] P<0.05).

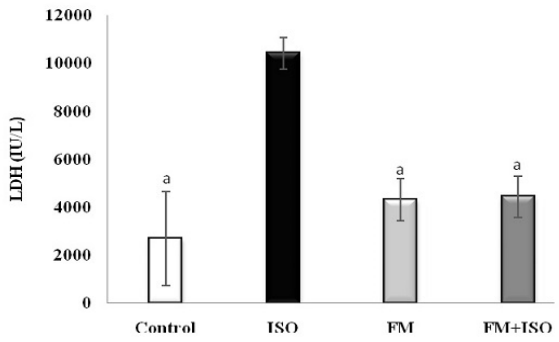

Figure 4. Effect of FM treatment on serum LDH activity in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 as compared to Group 2 ([a] P <0.05).

Effect of FM on BUN, Creatinine and MDA Levels:

The effects of ISO on the levels of BUN, Creatinine and MDA are exhibited in Figures 5, 6 and 7. In Group 2, the levels of serum BUN (28.54 ± 5.68) and Creatinine (0.49 ± 0.19) were increased compared to those in Group 1, controls (18.53 ± 3.8; 0.18 ± 0.15), Group 3, FM (20.12 ± 4.37; 0.27 ± 0.15) and Group 4, FM+ISO (20.74 ± 6.62; 0.3 ± 0.08). However; the differences were not statistically significant in Group 4 (FM+ISO) (Figures 5 and 6). A significantly higher level of MDA (P<0.05) was observed in Group 2 (0.27 ± 0.03) compared to those in Group 1, (0.18 ± 0.07), Group 3, FM (0.19 ± 0.02) and Group 4, FM+ISO (0.2 ± 0.03) (Figure 7).

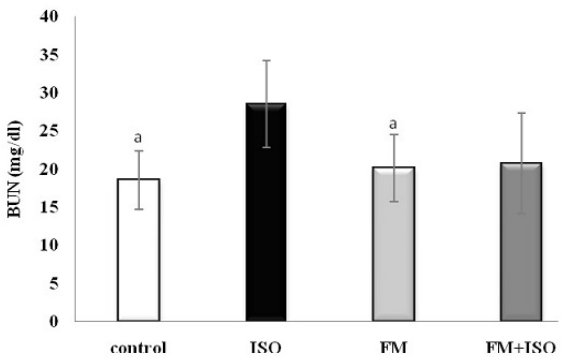

Figure 5. Effect of FM treatment on serum BUN levels in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 as compared to Group 2 ([a] P <0.05).

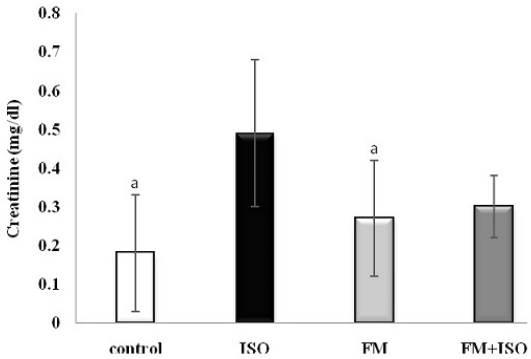

Figure 6. Effect of FM treatment on serum creatinine levels in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 compared to Group 2 ([a] P <0.05).

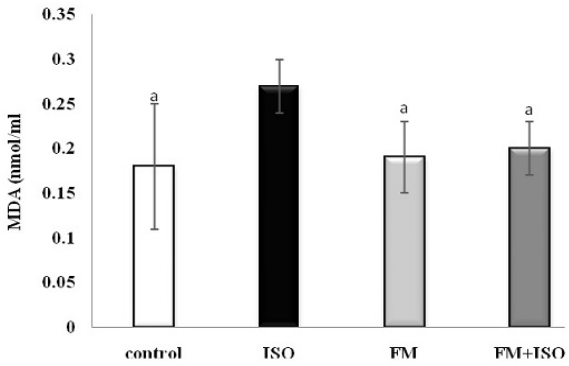

Figure 7. Effect of FM treatment on serum MDA levels in ISO-induced MI in rats. Values are means ± SD for six rats. Groups 1, 3 and 4 as compared to Group 2 ([a] P <0.05).

Histopathological Findings: The histopathological structures of the cardiac tissue in Groups 1 and 3 (controls & FM) indicated that the endocardium, myocardium and pericardium were normal (Figures 8A & 8C). Administration of ISO to rats caused extensive myocardial coagulative necrosis, interstitial edema and leukocyte infiltration in the myocardium (Fig. 8B). The mononuclear cell infiltrations in the myocardium, necrosis and hyperemia were less severe in Group 4 (FM+ISO) compared to those for Group 2 (ISO) (Figure 8D).

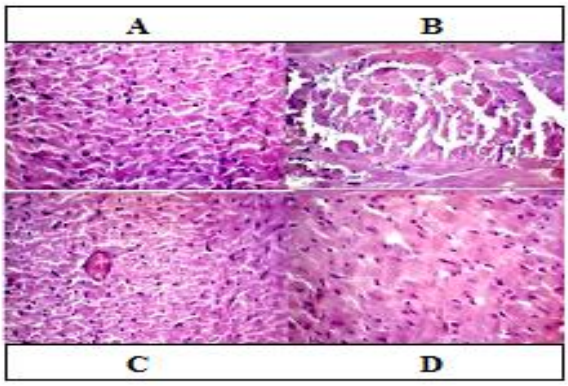

Figure 8. Effect of FM on myocardium histopathology in ISO-induced MI in rats (mag. 100×). (A) Control rats showed intact structures with no evidence of inflammation and edema; (B) The ISO-induced MI rats demonstrated a marked necrosis with infiltration of inflammatory cells compared to the control group; (C) Normal histological architectures with low hyperemia level in Group 3 (FM) were observed; (D) The mononuclear cell infiltration in the myocardium, necrosis and hyperemia were less severe in Group 4 (FM+ISO) (compared to those for Group 2 (ISO).

DISCUSSION

Induced myocardial necrosis by ISO is a well known experimental animal model for the study of the tissue morphological and pathological alterations, which are similar events in humans (14). Although the pathogenesis of acute MI remains unclear, but the study of cardiovascular pathologies induced by ISO offers insight into the involved factors. It is likely that ISO autoxidation leads to the generation of free radicals around the myocardial membranes and subsequently causes the overload of intracellular calcium leading to irreversible histological changes (15, 16). Considering the role of cytokines, growth factors and bacterial toxins in the upregulation of cyclooxygenase-2 (COX-2) and the implication of inflammation in the initial phase of MI, the use of a selective COX-2 inhibitor, such as FM, seems a logical approach to the management of myocardial injury. In veterinary medicine, FM, as a potent NSAID, has been routinely administrated to treat endotoxemia (17). There is ample evidence to suggest that FM has anti-endotoxemic property and inhibits COX independently. It has also been shown that NSAIDS have antioxidant and free radical scavenging properties in the body (18). Further, the therapeutic effects of FM on rhabdomyolysis in horse and rabbit tendon injuries have been shown in previous studies (10, 11-19). To our knowledge, the present study is the first to investigate the effect of FM against myocardial infarction induced by isoproterenol in a rat model.

There are many diagnostic marker enzymes within myocardial cells which are used to show their extracellular leakage due to alterations and damages in the membrane integrity (20). Creatine kinase is abundantly present in the skeletal muscle and is, therefore, a non-specific enzyme to evaluate MI. One of the molecular isoenzymes of CK, i.e., *CK-MB,* is known as a specific marker of MI. The sensitivity and specificity of CK-MB are known to be greater than 95%, approximately 24-36 hours after the onset of MI (21). Similarly to CK, troponin is considered a reliable biomarker for the diagnosis of MI (22). Previous studies have reported that despite the lower sensitivity and specificity than CK-MB and troponin, enzymes such as ALT, AST and LDH are used as the general indicators in the evaluation of MI severity (23). In this study, we evaluated all routine cardiac biomarkers in rats with MI that had been treated with FM for three consecutive days. The increased serum levels of ALT, AST, CK-MB and LDH in Group 2 (ISO) rats are illustrated in Figures 1-4. Such phenomena may be occurring due to an increase in the permeability or rupture of the cardiac myocyte membranes, resulting in insufficient oxygen supply or glucose to the tissue (24). The findings of present study revealed that pretreatment with FM reduced the activities of ALT, AST, CK-MB and LDH enzymes in Group 4 rats that had been treated with both FM and ISO.

One of the biochemical indicators for the assessment of lipid peroxidation in the body is the MDA levels (25). In the study of Yilmaz *et al*. (26) it was shown that FM inhibited lipid peroxidation in female dogs after the surgical removal of their ovaries and uterine. It has also been shown that the activities of antioxidant enzymes, such as catalase, GPX and SOD in rats with MI that were treated with nitroparacetamol were significantly higher than those noted in control rats (27). In this study, we showed that pretreatment with FM decreased the high MDA levels observed in Group 2 (ISO) rats. Further, improvement in the kidney function and decreased renal damages in a rat model due to rhabdomyolysis secondary to acetaminophen and FM administration, have been shown in two previous studies (8, 10). The results of our study indicated that the MI induced by ISO increased the serum levels of BUN and creatinine compared to those reported by other studies. However, the differences found between Group 2 (ISO) and Group 4 (FM+ISO) were not significant.

Our findings from the histopathological examinations demonstrated that the rat cardiac tissue in Group 1 (controls) had intact structures without any evidence of inflammation and/or edema. The rats with ISO-induced MI developed marked tissue necrosis with infiltration of inflammatory cells compared with that documented for rats in the control group. Moreover, pretreatment with FM in FM+ISO rats led to reduced necrosis and improvement of the inflammation.

CONCLUSIONS

This was a preliminary study to evaluate the effect of FM on the ISO-induced MI in rats. The experimental findings suggest that FM has a potential cardioprotective effect against MI in an experimental rat model. Also, based on our histopathological findings coupled with the assessed cardiac enzymes activities and MDA levels, it can be inferred that the cardioprotective effects of FM are derived primarily from its antioxidant properties.

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CONFLICT OF INTEREST

There was no conflict of interests whatsoever in conducting this study.

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