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Effects of Flunixine Meglumine, Diclofenac Sodium, Metamizol Sodium and Carprofen on Oxidative Stress in Rats Subjected to Laparotomy

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The aim of this study was to determine the effects of nonsteroid antiinflammatory drugs on oxidative stress in rats subjected to laparotomy. Rats (n=50) were divided into five equal groups. Control group (n=10, Group I) received physiological saline whereas Groups II-V (n=10 in each) received metamizole (50 mg/kg), flunixin meglumine (2.5 mg/kg), carprofen (2.5 mg/kg), or diclofenac (2.5 mg/kg) by intraperitoneal injection for 5 days following the operation. On postoperative day 6, blood samples were obtained; levels of malondialdehyde (MDA) and nitric oxide (NO) in serum, and erythrocyte glutathione (GSH), catalase levels were determined. NSAIDs caused a decrease in MDA, NO and GSH levels, whereas catalase levels increased with NSAID treatment. In conclusion, NSAIDs may have additional beneficial effects to their anti-inflammatory effects by diminishing the oxidative stress.

Key Words: *Oxidative stress, laparotomy, NSAIDs, rat*

Laparotomi Uygulanan Ratlarda Oksidatif Stres Üzerine Fluniksin Meglumin, Diklofenak Sodyum, Metamizol Sodyum ve Karprofenin Etkileri

Bu çalışmada laparatomi uygulanan ratlarda bazı nonsteroid antiinflamatuar grubu ilaçların oksidatif stres üzerine etkilerinin belirlenmesi amaçlandı. Ratlar (n=50) 5 eşit gruba ayrıldı. Kontrol grubuna (Grup I) serum fizyolojik verilirken, diğer Grup II-IV' e sırasıyla metamizol 50 mg/kg (II), flunixin meglumin 2.5 mg/kg (III), carprofen 2.5 mg/kg (IV) ve diklofenak sodium 2.5 mg/kg (V) intraperitoneal 5 gün süreyle enjekte edildi. Postoperatif 6. günde kan örnekleri alınarak serum malondialdehit (MDA) ve nitrik oksit (NO) düzeyleri ve eritrosit glutatyon (GSH) ve katalaz seviyeleri belirlendi. NSAID grubu ilaçlar MDA, NO ve GSH seviyesinde bir azalmaya neden olurken, katalaz seviyesinde artışa yol açtı. Sonuç olarak NSAID grubu ilaçların anti-inflamatuar etkilerine ek olarak değişik yollardan oksidatif stresi azaltabilirler.

Anahtar Kelimeler: *Oksidatif stres, Laparatomi, NSAID, rat*

Introduction

Peritoneal adhesions are among the most common complications of abdominal laparotomy, with high risk of morbidity and mortality (1). There have been several attempts to prevent or reduce peritoneal adhesion formation which is a consequence of an inflammatory process. Numerous defects, namely as insufficient antioxidant status, increased inflammatory mediators and ischemia may participate in pathophysiology of intra-abdominal fibrin deposition and adhesions (2, 3).

In response to injury, cells attracted to the site (neutrophils, macrophages, etc), as wells local ones, produce mediators such as cytokines, adhesion molecules, free oxygen radicals, nitric oxide (NO), and arachidonic acid metabolites to initiate healing. Elevated levels of pro-inflammatory mediators activate transcription factors such as NFκB which act as signalling mediators for a variety of signal transduction pathways and gene expression, and stimulate production of NO and reactive oxygen species (ROS) (4). Formation of elevated levels of ROS such as hydroxyl radicals, superoxides, and peroxides has been reported in various inflammatory conditions. Activated free oxygen radicals shown to be responsible for cell-membrane peroxidation which leads to arachidonic acid and free fatty acids formation, a process which then can induce generation of more ROS (5). Therefore, although inflammation is a necessary step in wound healing the persistence of inflammation as a result of prolonged exposure to stimulus can lead to tissue damage and fibrosis (6). Thus, targeting inflammation at an early stage at cellular level may allow specific attenuation of operatively induced adhesion formation via inhibiting uncontrolled production of NO, ROS, lipid oxidation, cellular damage and finally adhesion formation (2, 3).

Nonsteroidal anti-inflammatory drugs (NSAID) are among the most widely used pharmaceuticals in humans and animals for their anti-inflammatory effects. The anti-inflammatory effects of NSAIDs are thought to be mainly on the cyclooxygenases (COX) (7). COX enzymes (COX-1 and COX-2) are the first in a cascade of enzymes that

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convert arachidonic acid into prostaglandins (PG) and thromboxane (Tx), which are involved in inflammation, cancer, and embryonic development (8). NSAIDs such as aspirin, indomethacin, naproxen, diclofenac, metamizole, and flunixin inhibit both COX-1 and COX-2 non-selectively and are widely used for the treatment of various inflammatory and non-inflammatory conditions such as arthritis, cardiovascular diseases, and the management of postsurgical and post-traumatic pain in clinical and veterinary medicine (9-11). COX inhibitors such as resveratrol have been reported to have antioxidant features (13). On the other hand, antioxidant agents such as thioctic acid have been reported to be effective in acute inflammation via reduction of NO, malondialdehyde (MDA), COX-2 mRNA and induction of glutathione (GSH), IL-10 mRNA (14).

In this study, we asked the question whether NSAID treatment following laparotomic surgery would also reduce the oxidative stress in rats and wanted to evaluate the efficacy of various NSAIDs for this matter.

Materials and Methods

The study was conducted with 6-months-old, outbrade female Wistar rats (n=50) with a mean weight of 200-250 g. Rats were obtained from our animal facilities and treated in accordance with the guidelines for Animal Research from the National Institutes of Health as approved by the local committee on animal research. The animals were housed in stainless steel cages under controlled temperature and humidity conditions with 12/12-hour light/dark cycles. All rats were maintained on a standard laboratory diet with tap water ad libitum throughout the experiment. Laparotomy was performed under sterile conditions after intraperitoneal injection of ketamine (50 mg/kg) and xylazine HCL (10 mg/kg) as anesthetic agents. The rats were divided into five equal groups. The control group (Group I) received serum physiologic. We injected the drugs; metamizole (50 mg/kg), flunixin meglumine (2.5 mg/kg), carprofen (2.5 mg/kg), and diclofenac (2.5 mg/kg) intraperitoneally for 5 days to Groups II, III, IV and V, respectively. Each group also consisted of n=10 animals. On postoperative day 6, blood samples were obtained for biochemical analyses and animals were sacrificed by exsanguination.

Measurement of Serum MDA Levels: MDA concentration in serum samples was measured spectrophotometrically in terms of thiobarbituric acid reactive substances (TBARS). Serum samples were mixed with 20% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was then boiled at 95°C for 30 min, immediately followed by cooling on ice. The reaction mixture was then vortexed, following the addition of n-butanol. All vials were then centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was then measured at 535 nm. The concentration of lipid peroxidation products was calculated as MDA concentration using the extinction coefficient for the MDA-thiobarbituric acid complex of $1.56x10^5$/mol/cm (15).

Measurement of Nitric Oxide: Serum NO levels were determined indirectly by measuring the concentrations of nitrite (NO_2^-) and nitrate (NO_3^-), as the indices of NO (16). NO (nitrite+nitrate) levels were analyzed using a modification of cadmium-reduction method of Cortas and Wakid (16). Samples were deproteinized and then centrifuged at 10.000 g for 20 min at 4°C. Cadmium (Cd) granules were activated using $CuSO_4$ solution in glycine–NaOH buffer. Then, deproteinized samples and standards were added. The nitrate, reduced to nitrite with cadmium granules and the nitrite concentration were quantified by a reaction of nitrite with a mixture of naphthethylenediamine and sulfanilamide, based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm is formed. The samples were analyzed using a microplate reader and quantified against nitrite standard curve and the results were expressed as µmol/L.

Erythrocyte Lysate Preparation: Blood samples were collected in tubes containing EDTA, then centrifuged (3000 rpm 10 min at +4 °C) and plasma and buffy coat (leucocytes) were removed. The packed red cells were washed three times with 0.9% NaCl and hemolyzed with 4 volume of ice-cold deionized water to obtain stock hemolysate containing ~5 g hemoglobin/100 ml. Hemoglobin concentration in each lysate was measured using Drabkin method (17).

Measurement of Erythrocyte GSH Levels: Erythrocyte GSH concentrations were measured according to the method of Tietze, using metaphosphoric acid for protein precipitation and 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) for colour development (18). DTND is a disulfide compound, readily reduced by sulfhydryl compounds that form a coloured yellow anion. The optical density of this yellow substance is measured at 412 nm. GSH levels were expressed as µmol/ mg Hb.

Measurement of Erythrocyte Catalase Enzyme Activity: Catalase level was measured in a fresh suspension of hemolysates. Dilution, 1:1000 of the concentrated hemolysate was prepared with phosphate buffer immediately before the assay. Catalase activity was determined by the spectrophotometric/enzymatic method of Aebi (19). Buffer solution was prepared with potassium di-hydrogen phosphate and disodium hydrogen phosphate. The samples were diluted with buffer solution and the absorbance after addition of hydrogen peroxide buffer was recorded at 240 nm at the 15th second. The results were given as U/g Hb.

Statistical Analysis: Statistical analysis was performed using SPSS for Windows version 10.0 program. All data were reported as mean values±standard deviations (SD). Comparisons between experimental and control groups were performed with Mann Whitney-U test. A value of $P<0.05$ was considered significant.

Results

Effects of NSAIDs on oxidative stress markers were summarized in Table 1. Regarding the antioxidant status, there was a decrease in plasma levels of both reduced GSH and MDA. Although this decrease was not statistically significant in any of the treatment groups, change in MDA levels was statistically significant in all but flunixin treated rats (Table 1). No statistical difference was observed in NO levels between any treatment groups compared to the controls although there was a slight decrease at NO levels in rats, treated with NSAIDs. Regarding catalase levels on the other hand, an increase was observed in all NSAID treated groups only being significant in metamisole group (P=0.037) compared to its control group.

Table 1. Effects of NSAIDs on oxidative stress markers in serum and erythrocyte lysates from rats subjected to laparotomy

Groups (n= 10)	MDA (nmol/mL)	NO (µM)	Catalase (U/g Hb)	GSH (µmol/g Hb)
I	1.84±0.04	21.1±1.06	0.11±0.08	19.9±1.2
II	1.01±0.02 (0.013)	15.7±0.59 (0.031)	0.23±0.04 (0.037)	17.1±0.8 (NS)
III	0.88±0.03 (NS)	20.1±2.42 (NS)	0.39±0.13 (NS)	16.8±1.4 (NS)
IV	1.04±0.03 (0.009)	19.5±0.91 (NS)	0.34±0.11 (NS)	15.2±2.2 (NS)
V	1.00±0.01 (0.005)	17.0±0.55 (NS)	0.30±0.10 (NS)	12.4±1.8 (NS)

Control group (Group I), Animals received; 50 mg/kg metamisole (Group II), 2.5mg/kg flunixin meglumine (Group III), 2.5 mg/kg carprofen (Group IV) or 2.5 mg/kg diclofenac (Group V) via intraperitoneal injection for 5 days. Data shows Mean±SD (P value).

P value>0.05 is significant, control (Group I) vs. NSAID treatment groups (II-IV). NS: non-significant.

Discussion

Abdominal laparotomy induced peritoneal adhesions may even occur in the absence of infection (20,21). In adhesion formation, an inflammatory reaction is the first step in the common pathway, whereas oxidative stress is the triggering and/or sustaining factor of inflammation (14). Previous studies have shown that although ROS production was required for the performance of specific physiological functions, excessive ROS generation was harmful, and has been implicated in the pathogenesis of a number of diseases, including adhesion formation.

There is a complex interaction between arachidonic acid metabolites and ROS. During the oxidation step of arachidonic acid by COX and lipoxygenase (LOX), ROS may be generated as a by product, whereas ROS itself was also reported to induce cell-membrane peroxidation which leads to arachidonic acid and free fatty acids formation (5). In this study, we investigated the effects of NSAIDs with COX inhibitory effects, particularly metamisole, flunixin meglumine, carprofen and diclofenac on oxidative stress in rats subjected to laparotomy.

MDA is a by-product of increased oxidative stress due to lipid peroxidation in several inflammatory conditions. Increased concentrations of MDA in tissues and blood reflect increase lipid peroxidation, making MDA a good marker of cell membrane injury. In our study, treatment of NSAID caused a decrease in serum MDA levels in Groups II-V, compared to the control group (Table 1). This decrease was statistically significant in all but flunixin-treated rats. Our results regarding the decreased MDA levels in sera of NSAID treated rats are in agreement with previous studies (22).

Another marker of oxidative stress is nitric oxide which is needed for vasodilatation and angiogenesis during the early phases of wound repair (23). NO is also important in inflammation (24). A recent study by El-Shitany et al. (14) reported thioctic acid, an antioxidant agent and indomethacin to reduce inflammation and to decrease MDA and NO production in carrageenan-induced acute inflammation in rats. In our study, we also showed a decrease in NO levels in rats treated with NSAIDs. This decrease was statistically significant only in Group II compared to the controls (P=0.031).

The balance between generation of free radicals and antioxidants is of critical importance for functional integrity of the cells as deficiency of antioxidants such as glutathione peroxidase and catalase along with excessive production of free radicals may lead to cellular destruction. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide to water and oxygen, hence limits the formation of hydroxyl radical, the highly toxic reactive oxygen species whereas catalase is necessary for decomposition of hydrogen peroxide. In our study, an increase in catalase levels was observed in all NSAID treatment groups compared to controls but again, only in metamisole group (II) this increase was statistically significant (P=0.044). Similar results were reported by Sen et al. (22) showing lornoxicam and nitroglycerin to cause an increase in catalase activity.

GSH, a cofactor of glutathione peroxidase is another anti-oxidant that plays an important role in the cellular defence cascade against oxidative injury. The studies questioning the effects of NSAIDs on glutathione production are various and not conclusive. While several researchers describe depleted glutathione levels due to NSAID use**,** there also are studies reporting restoration of depleted GSH levels with NSAIDs (14, 25). In our study, all of the NSAIDs we used caused a decrease in erythrocyte reduced GSH levels compared to the controls. The decreases we observed were not significant in any of the NSAID treatment groups (Table 1). One explanation for this decrease may be a possible increase in utilization of glutathione by increased GPx levels in erythrocytes to counteract ROS as a consequence of NSAID treatment. To enlighten this hypothesis further studies are required. Our results regarding decreased GSH levels in NSAID treated rats

are in disagreement with the results of Shitany et al. (14) showing antioxidant agent thioctic acid and indomethasin restoring depleted GSH levels in acute inflammation and also with the results of Kirimlioglu et al. (13) showing increased GSH levels in rats subjected to partial hepatectomy and treated with NSAID resveratrol. One explanation for these differences might be the choice of NSAIDs used in these studies and our study, as well as the doses and administration ways of the drugs (26).

In conclusion, one or more of the mechanisms mentioned above may be responsible for the oxidative stress and inflammation leading to peritoneal adhesion following laparatomic operations. Our data suggest that NSAIDs are likely to diminish the oxidative stress via different routes therefore combined or alternate regimes of these drugs to enhance their antioxidative effects and to prevent the unwanted side effects due to chronic use should be the choice of therapy. Further studies are required to reach an ultimate conclusion before fully understand their pathways of action. NSAIDs seem to decrease the traumatic effects of laparotomy.

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