ORIGINAL ARTICLE

Non steroidal antiinflammatory drugs may be harmful to normal kidneys: experimental surgery model*

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Abstract
Background and aim: The exact effect of analgesics on normal kidneys is not known yet. We aimed to evaluate the impression of non steroidal antiinflammatory drugs (NSAID) used post-operatively on kidneys, in rat (tracheotomy) model.
Methods: Twenty-five non-uremic male wistar albino rats were included. For 18 rats, tracheotomy was performed and divided into two groups. First group, NSAID (diclofenac 10 mg/kg/day intramuscular (im)) (NSAID, n=8); second group isotonic (im)(Control, n=10) were administered for a week. For third group (Histological control,n=7) in order to evaluate normal histology neither surgery nor medication were applied. At the end (7th day), 24 hours urine collected then, blood samples were taken by intracardiac punction and were sacrified. One of the kidneys fixed for histological evaluation, the other was preserved for the measurements of tissue enzyme levels. Lipid peroxidation products and antioxidant enzyme levels were measured both from plasma and renal tissues. Histologically inflammation, regeneration, degeneration assessed semiquatitatively and immunohistochemical dyes were applied.
Results: Hemoglobin thiobarbituric acid reactive substance level indicating the increase of lipid peroxidation in NSAID group was higher than control group (673±204 vs.373±27nmol/gHb respectively, $p>0.05$). Superoxide dismutase (one of the antioxidant enzymes responsible for reduction of reactive oxygen substances) and serum nitrate levels were lower in NSAID groups (700±68 vs.1371±164U/gHb and 26±4.4 vs.50.8±6.8 μmol/mL respectively, $p<0.05$).Although tissue levels were parallel to plasma levels but the difference wasn't significant. In histological assessment degeneration was present only in NSAID group (1.3±0.6 vs.0.0±0, $p<0.05$). Inflammation were lower than the control group (0.8±0.4 vs.1.2±0.2, $p>0.05$). Cyclooxygenase-2 expression was disappeared in NSAID group.
Conclusions: NSAIDs mostly used post-operatively for analgesia, may cause unfavorable effects on kidneys by oxidative stress. Hippokratia. 2012; 16 (2): 160-165

Keywords: analgesic nephropathy, cyclooxygenase, oxidative stress

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Introduction

An outstanding number of prescriptions (over 100 million) are written every year for NSAIDs, besides those which are available as nonprescription agents. They are potent and popular analgesics and often used clinically for the short-term alleviation of postoperative pain, dysmenrrhoea.[1–3]. They exert their therapeutic effects through inhibition of cyclooxygenase (COX)[4] a key enzyme in the formation of prostaglandins. A few study related to NSAID-induced renal injury were presented, this injury has two components, one or both of which may be present in a given patient: acute interstitial nephritis, with an interstitial infiltrate composed primarily of T lymphocytes; and the nephrotic syndrome due to minimal change disease[5-7]. The latter may be due to release of a toxic lymphokine from the activated T cells. Although the underlying pathophysiologic mechanism of renal injury under the treatment of NSAID is not clearly

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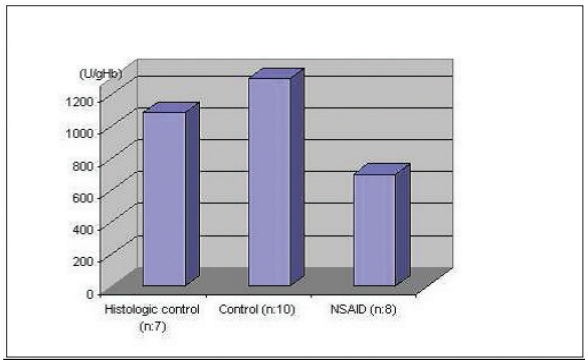

Figure 1: Plasma Nitrat Levels NSAID v.s Control, (p< 0.05).

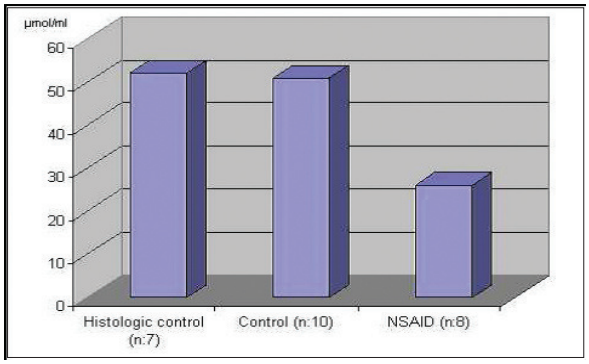

Figure 2: Erythrocyte Superoxide Dismutase Levels NSAID v.s Control, (p< 0.05).

known, there are some hypotheses such as an elevation in oxidant stress and an inhibition of cycloxygenase enzyme resulting with renal vasoconstriction and a consequent decrease in renal blood flow and (Glomerular filtration rate) GFR[8]. Reactive oxygen species (ROS) may play a key intermediary role in the pathophysiologic processes of a wide variety of clinical and experimental renal diseases ranging from acute to chronic injuries[9]. ROS have been demonstrated to be capable of degrading glomerular basement membrane and inducing glomerular injury, characterized by impaired glomerular filtration and sieving function[10, 11]. In order to eliminate toxic ROS, cells are equipped with various antioxidant defense systems. Therefore, the development of tissue injury depends on the balance between ROS generation and tissue antioxidant defense mechanism[12]. The glomerular antioxidant enzymes are suggested to play an important role in the functional derangement induced by the ROS[13].

Previously it has been shown that toxic doses of NSAID damaged to renal tissue, we have no enough information on the therapeutic doses. In this study we aimed to investigate the possible harmful effect of NSAID in a therapeutic dose which used for analgesic effect postoperatively on renal tissue. We focused on the oxidative stres and antioxidant enzymes in order to determine the mechanism underlying of the injury that is evaluated by histopathologically

Materials and methods

Study protocol

Non-uremic Wistar-Albino male rats (n= 25; weight 175-200g) which housed in polycarbonate cages under 24°C room temperature with 12 hour light/dark cycle and feeding with standard laboratory diet were divided into three groups. The Animal Ethics Committee of Ege University Hospital approved the study design. The three groups of rats; for 18 rats, tracheotomy was performed and they were divided into two groups. One of the groups, NSAID (2-[(2,6 diclorophenyl)amino]phenylacetate which is a phenylacetic acid NSAID) (diclofenac 10 mg/kg/day intramuscular (im))(Diclomec® 100 mg ampul (Mecom Sağlık Ürünleri San. ve Tic. A.S. Zincirlikuyu – Istanbul) (NSAID, n=8); second group isotonic (im) (Control, n=10) were administered for a week. For third group (Histological control, n=7) in order to evaluate normal morphology and histology neither surgery nor medication were applied. At the end (7th day), 24 hours urine collected then, ketamine HCL anesthesia (60 mL/kg body weight) was applied and immediately blood samples collected through direct cardiac puncture and were sacrified.

Functional Parameters

Blood samples with heparin were obtained and plasma were immediately separated, erythrocytes were analyzed after washing process.

Erythrocyte analyses

Preparation of haemolyzates; After separation of plasma, the packed erythrocytes were washed two times with 9 g/l NaCl solution and haemolysed with ice-cold water (1/5, v/v). eTBARS, levels, eSOD and eCAT activities were determined immediately in haemolyzates. The haemoglobin values were measured by Drabkin's method[14].

a) eSOD activities were measured based on the inhibition of autoxidation of epinephrine by SOD at 492 nm, with with a plate reader. The assay was calibrated by using purified SOD and one unit of enzyme was defined as the amount of enzyme, which inhibits 50% of autoxidation of epinephrine[15].

b) eCAT activities were determined as described by Sozmen et al.15 in which the degradation of hydrogen peroxide is recorded spectrophotometrically at 240 nm. One unit of CAT was defined as the amount of enzyme, which decomposes 1µmol H2O2/min under specific conditions.

c) Determination of eTBARS

After the dilution in required amount of erythrocyte lysates, eTBARS measurements were performed by incubation with TBARS solution (0.12 M TBA in 15% TCA and 1% HCl) for 30 min at 95°C[16] TBARS levels were calculated using 1,1,3,3, tetramethoxypropane standard curve.

Preparation of Tissue Homogenates

Right kidneys were separated for the measurement

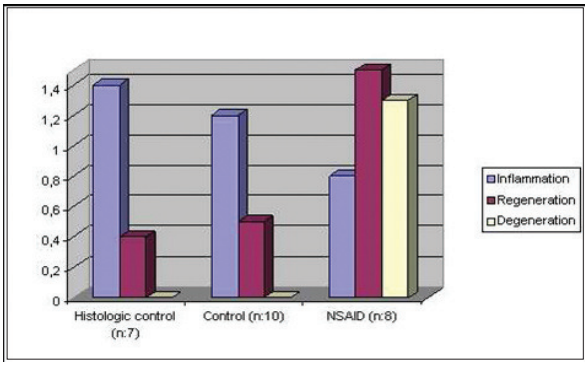

Figure 3: Histological assessment. NSAID v.s Histologic control and NSAID v.s Control, ($p< 0.05$).

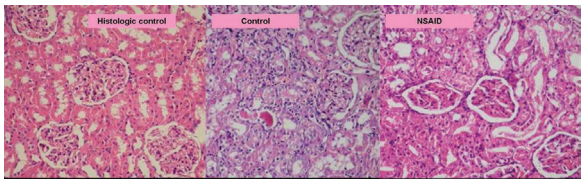

Figure 4: Renal Histopathological Sections
Representative micrographs of renal histopathological sections stained with hematoxylin–eosin under light microscopy. (Kidney section of Histologic control and Control groups showing almost normal morphology unlike NSAID group.)

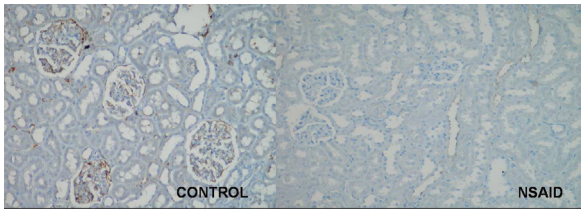

Figure 5: COX-2 Staining of the Histopathological Sections.

of tissue lipid peroxidation products and antioxidant enzyme activities.

Tissue samples were homogenized in phosphate buffer (0.5M, pH=7.0), (1/10 : w/v). The homogenate was centrifuged for 5 min at 700xg at 4°C to sediment unbroken cells and cellular debris. The determination of SOD and CAT activities, TBARS and nitrite/nitrate contents in supernatants were carried out by the same methods previously explained for ertythrocytes.

Measurement of Protein Content

Protein concentrations were measured using bovine-serum albumin as standard by the method of Lowry et al[17].

Determination of nitrite/nitrate levels

Tissue homogenates were deproteinised equally volume with 1N NaOH and 5% ZnSO4 solution. Nitrite and nitrate levels were determined colorimetrically based on the Griess reaction[18] in which nitrite is reacted with sulfanilamide and N-(1-naphthyl) ethylendiamine to produce an azo dye detected at 546 nm while nitrate is first reduced to nitrite by nitrate reductase via a reaction in which it is coupled to the oxidation of β-NADPH and detected at 340 nm[19] Sodium nitrite and nitrate solutions were used for standard measurements. Tissue nitrite and nitrate levels were expressed as nmol/mg protein.

Measurement of MPO activity[20]

Tissue MPO activities were measured according to the modified method of Grisham et al. Briefly, following homogenization of tissue, homogenates were centrifuged at 10 000 rpm for 15 min. Pellets were rehomogenized in 0.5% HETAB (hexadecyltrimethyl ammonium bromide) in phosphate buffer (50 mM, pH=6.0). Following three freeze and thaw cycles, samples were centrifuged 10 000 rpm for 10 min. Supernatants were added to reactive solution containing 0.5 M o-dianisidin (in phosphate buffer). After addition of hydrogen peroxide solution (20 mM), absorbancies of samples were recorded at 492nm with a microplate reader during 3 minutes with 15 second intervals. MPO activities were calculated using a standard curve.

Plasma total nitrite levels

Nitrite and nitrate levels were determined spectrophotometrically based on the Griess reaction (Bories and Bories 1995) where nitrite reacts with sulfanilamide and N-(1-naphthyl) ethylendiamine to produce an azo dye detected at 540 nm, while nitrate is first reduced to nitrite by nitrate reductase (EC 1.6.6.2.) via a reaction in which it is coupled to the oxidation of β-NADPH and detected at 340 nm. Sodium nitrite and nitrate solutions were used for standard measurements. Total nitrite levels were calculated by summing of nitrate and nitrite levels[19].

Structural Parameters

Left kidneys were fixed in 4% formalin and embedded in paraffin wax. Parafin blocks were divided into sections in size of 5 micrometer in thickness and then stained with hematoxylin-eosin and Masson trichrome. All samples were examined by the same pathologist who was unaware of the nature of the samples originated from groups. Histologically inflammation, regeneration, degeneration assessed semiquatitatively were measured with an ocular micrometer per high power field at 400x magnification and immunohistochemical dyes were applied for COX-2.

Statistics

Study results are presented as mean ± standart error of the mean (SEM). The statistical analyses were performed using ANOVA, unpaired t-test, and the Mann- Whitney U test. A p value of less than 0.05 was considered significant.

Results

The results as mean ± standard error of mean are given in the Table–1. Serum urea (59 ± 23.5 vs 42.7±2.1 vs 46.4±1.8 mg/dl) and creatinine levels (0.51±0.04,

Table 1: Results

		Histologic control (n:7)	Control (n:10)	NSAID (n:8)
Plasma	Creatinine (mg/dl)	0.4±0	0.5±0	0.5±0
	Nitrate (μmol/ml)	52.1±15.1	50.8±6.8	26±4.4 [b]
Erythrocyte	TBARS (nmol/g Hb)	379±59	373±27	673±204
	SOD (U/g Hb)	1085±153	1371±164	700±68 [b]
	CAT (U/g Hb)	3908±612	3220±268	3326±420
Renal tissue	TBARS (nmol/g protein)	569±44	606±23	502±68
	Nitrate (μmol/g protein)	1253±258	1302±205	1049±312
	MPO (U/mg protein)	0.3±0	0.3±0	0.3±0
Renal histology	Inflammation	1.4±0.4	1.2±0.2	0.8±0.4
	Degeneration	0.0±0	0.0±0	1.3±0.6[a,b]
	Regeneration	0.4±0.2	0.5±0.2	1.5±0.6

TBARS: Thiobarbituric acid reactive substance, SOD: Superokside dismutase, CAT: Catalase, MPO: Myeloperoxsidase, NSAID: Non steroidal anti inflammatory drug.
a: NSAID v.s Histologic control, b: NSAID v.s Control, ($p< 0.05$)

0.52±0.03 vs 0.42±0.02 mg/dl, respectively).in NSAID group were same as naive and histologic control groups. The amount urine of twenty-four hours in NSAID group was not lower than histologic control group (3.32±0.43 vs 5.3±0.41 respectively) and hourly urine level was same as control group and histologic control group (0.14±0.02, 0.11±0.02 vs 0.22±002 ml/min respectively). Estimated GFR in NSAID group were same as control group and histologic control group (0.17±0.03, 0.15±0.04 vs 0.23±0.03 ml/100gr) ($p>0.05$).

While eTBARS level as an indicator of lipid peroxidation was higher ($p>0.05$), eSOD and serum nitrate levels were lower in NSAID groups ($p<0.05$) (Figure 1 and 2) compared to controls. eCAT activities showed no statistically significant change between the groups. There was no significant change in TBARS levels (569.4 vs 606.3 and 502 nmol/mg protein), Nitrate levels (1253.5 vs 1031.7 and 1048.7 μmol/mg protein), and myeloperoxidase (MPO) activities (0.32 vs 0.29 and 0.27 U/mg protein) in kidney tissues between the groups

Histological assessment showed a degeneration only in NSAID group (1.3±0.6 vs. 0.0±0, $p<0.05$). Inflammation was lower in NSAID group than the control group insignificantly (0.8±0.4 vs. 1.2±0.2) (Figure 3 and 4). Cyclooxygenase–2 expression was disappeared in NSAID group immunohistochemically (Figure 5). Thyroidisation was not seen in any group.

Discussion

Although analgesics are used quite widespread all over the world, it hasn't known its exact effect on renal tissue and function yet. Lee et al. determined the effects of NSAIDs on postoperative renal function in a large population of adults and found there was no significant difference in serum creatinine in the early postoperative period between patients receiving diclofenac, ketorolac, indomethacin, ketoprofen or etodolac[21].

In the present study, we investigated the effect of NSAID in therapeutic dose on the renal tissue and renal function in tracheotomised rats. Our data showed that NSAID might be detrimental for kidneys even in therapeutic doses. The renal injury has been shown in tissue level althought clinically there were no increase in serum urea, creatinine levels, and no decrease in 24 hours urine, estimated GFR levels, on the contrary histologically degeneration was present in kidney tissue of rats which treated by NSAID for a week.

Besen at al. also determined that the treatment with diclofenac sodium (2.5mg/kg/day for 7 days) caused non significant increase in serum urea and creatinine levels[22].

In our study serum nitrate levels were lower in NSAID groups. Increase in the production of and/or a decrease in the production of NO in the kidney primarily determine the condition of oxidative stress. Under normal conditions, an oxidative balance in the kidney is maintained by optimal production of NO. Any condition that leads to an increase in -O2 production and/or a decrease in NO generation tilts the balance towards oxidative stress and modulates renal function[23].

In the current study, diclofenac produced alterations in the activities/levels of oxidative stress biomarkers in erythrocyte of male rats. While animals treated with NSAID showed a significant decrease in erythrocyte SOD level, eTBARS levels (lipid peroxidation marker) were increased than histologic control group. Erythrocyte CAT activity was increased also but the difference didn't reach the statistical significance. In accordance with our data, it has been shown that acute renal failure is associ-

ated with depressed SOD and elevated NADPH oxidase expression, which can contribute to oxidative stress by increasing superoxide anion[24] ROS have been implicated in several renal and hepatic diseases induced by some antibiotics and anti-inflammatory drugs[25-27]. In turn, ROS are capable of initiating and promoting oxidative damage as lipid peroxidation (LPO)[28]. LPO is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerisation of polysaccharide, as well as protein cross-linking and fragmentation[29]. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism[30]. SOD accelerates the dismutation of superoxide radicals (O2•−) to H2O2, which can be considered to be a primary defense, whereas CAT catalyzes the breakage of toxic H2O2 produced in the cell to O2 and H2O[31]. Depletion of SOD activity accompanied by the increase of TBARS levels suggest an induction of radicals diclofenac sodium administration, but time and/or dose of diclofenac sodium failed to affect catalase activty.

Palmen et all have shown that while compounds with known oxidative capacity induced increased TBARS formation in erythrocyte haemolysates no significant change was observed in hepatocytes. These findings in concordance with our results may suggest that interaction with haemoglobin also, induces free radicals which give way to more severe lipid peroxidation in comparison to other hepatocytes[32]. It is known that when ROS generation overloads the antioxidant defense, the free radicals can then interact with endogenous macromolecules and alter the cellular function[33]. It has shown that various biochemical abnormalities are produced in the kidney in response to the administration of indomethacin. These effects include oxidative damage and impairment of structure and function of mitochondria and appear to be mediated through the production of free radicals[34]. We determined myeloperoxidase activities and TBARS levels in renal tissue, in order to evaluate the role of oxidative stress on the damage of renal cells; however there was no change in these parameters. Since Hickey et al showed a dose-dependent increase in MDA levels and SOD activity we suggest that the dose of diclofenac sodium used in this study was not enough to increase in TBARS levels[35].

Histopathological examination of kidney sections revealed numerous apoptotic nuclei across proximal and distal tubular cell linings. Collectively, these data for the first time suggest that diclofenac induced nephrotoxicity may involve production of reactive oxygen species leading to oxidative stress and massive genomic DNA fragmentation, and these two free radical mediated events may ultimately translate into apoptotic cell death of kidney cells in vivo, and reveal a DNA-active role for diclofenac. Hickey et al used nephrotoxic doses of diclofenac (100, 200, 300 mg/kg, po) for one day. In present study we used the usual therapeutic dose, so its clear that although theraputic doses NSAIDs mostly used post-operatively for analgesia, may cause unfavorable effects on kidneys by oxidative stress[35].

In the current investigation, in histological assessment degeneration was present only in NSAID group. Although it wasn't reach the statistical significance inflammation was lower than the control group. Cyclooxygenase-2 expression was disappeared in NSAID group immunohistochemically. Thyroidisation was not seen in any group indicating those effects were acute rather than chronic. Both alterations suggest a cellular degeneration[36-37]. In another study the administration of celecoxib resulted in increase in oxidative stress parameters and also moderate tubulointerstitial nephritis in rats[38]. For this, oxidative stress seems to be one contributor to NSAID-induced acute nephrotoxicity.

In conclusion, therapeutic dose of NSAID led to both damage in renal cells proven by histopathologically and an increase in oxidative stress in erythrocytes resulted to increase in lipid peroxidation and inhibition of eSOD activity. These data suggest that oxidative stress may play a role in NSAID induced renal damage althought clinically it seems as if NSAIDs are innocent.

Conflict of interest: Authors have not any conflict of interest.

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