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Original article

AN INVESTIGATION OF TOXICITY POTENTIAL OF NIMESULIDE IN JUVENILE RATS

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Abstract

Nimesulide has been widely used in pediatry for treatment of inflammation associated to respiratory tract infections, fever, several chronic inflammatory conditions, and pain in many countries. However, very few but serious cases of adverse effects, particularly hepatic dysfunction and liver injury have been reported. Reactive oxygen species (ROS) has been implicated in nimesulide-induced adverse effects, including hepatotoxicity. However, several reports demonstrated the reducing effect of nimesulide on oxidative damage and its direct free radical scavenging activity. This study was performed to investigate the effects of nimesulide on oxidative stress and antioxidant enzymes in juvenile rats as well as its tissue damage potential. Four weeks-old Wistar albino, female rats were used. Nimesulide was given by gavage at two doses for 14 days. Blood and tissue samples were taken under pentobarbital anesthesia. Nimesulide treatment caused increase in plasma malondialdehyde (MDA) levels and decrease in catalase (CAT) and glutathione peroxidase (GPx) activities; superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G-6P-DH) activities were not changed. Tissue damage and changes in some serum parameters were also observed.Our results, indicating the possibility of tissue damage and alterations of oxidant/antioxidant status by nimesulide, might provide important contribution to the literature about the cautions for nimesulide use in juveniles.

Key words: *Nimesulide, Antioxidant enzymes, Oxidative stress, Juvenile.*

Juvenil Sıçanlarda Nimesulid'in Toksisite Potansiyelinin Araştırılması

Nimesulid, birçok ülkede, çocuklarda solunum yolu enfeksiyonlarına eşlik eden inflamasyonlarda, ateş, ağrı ve çeşitli kronik inflamasyonlu olgularda yaygın olarak kullanılmaktadır. Ancak az sayıda fakat ciddi advers etkiler, özellikle hepatik disfonksiyon ve karaciğer hasarı vakaları bildirilmiştir. Reaktif oksijen türleri (ROS) hepatotoksisiteyi de içeren nimesulide bağlı advers etkilerle ilişkilendirilmektedir. Ancak, çeşitli raporlar nimesulidin oksidatif hasarı azaltıcı etkisini ve direkt serbest radikal süpürücü aktivitesini belirtmektedir. Bu çalışma genç sıçanlarda nimesulidin oksidatif stres ve antioksidan enzimler üzerindeki etkileri ile doku hasarı oluşturma potansiyelini araştırmak amacıyla yapılmıştır. Dört haftalık dişi Wistar albino sıçanlar kullanılmıştır. Nimesulid gavajla 2 dozda ve 14 gün süreyle uygulanmıştır. Kan ve doku örnekleri pentobarbital anestezisi altında alınmıştır. Nimesulid uygulaması, plazma malondialdehid (MDA) düzeylerinde artışa ve katalaz (CAT) ve glutatyon peroksidaz (GPx) aktivitelerinde azalmaya neden olmuştur; süperoksit dismutaz (SOD) ve glukoz-6-fosfat dehidrojenaz (G-6P-DH) aktiviteleri değişmemiştir. Doku hasarı ve bazı serum parametrelerinde de değişiklikler gözlenmiştir. Nimesulidin doku hasarı oluşturabileceği ve oksidan/antioksidan durumda değişikliğe neden olduğunu ortaya koyan sonuçlarımız, juvenillerde nimesulid kullanımında dikkatli olunmasına yönelik olarak literatüre önemli katkı sağlayacaktır.

Anahtar kelimeler: *Nimesulid, Antioksidan enzimler, Oksidatif stres, Juvenil.*

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INTRODUCTION

Nimesulide (N-(4-nitro-2-phenoxy-phenyl)-methanesulfonamide) is probably the first sulfonanilide to be developed which has emerged as a clinically successful cyclooxygense-2 (COX-2) selective non-steroidal anti-inflammatory drug (1-3). It has been widely used in pediatry for treatment of inflammation associated to respiratory tract infections, fever, rheumatic disease and other chronic inflammatory conditions, peri/post-operative and cancer pain (4,5). It was reported that nimesulide can cause fatal skin reactions and neonatal kidney insufficiency (6,7). However, the most of reported cases were related to hepatotoxicity of the drug, particularly among women and children (8-11). After its launch, very few but serious cases of hepatic dysfunction and liver injury were reported (11-13). These fatal cases caused some countries to re-examine the safety profile of the drug, especially in children. Pediatric formulations were withdrawn/prohibited in most of the countries worldwide, due to those fatal cases as well as the conflicting data about safety of nimesulide in children (12). In Turkey, pediatric dosage form of nimesulide is not available since 2003. The drug is commercially available as tablets (100 mg) and gels (1%); the patient information leaflet informs that the use of nimesulide in children under the age of 12 is contraindicated.

Oxidative stress has been implicated in nimesulide-induced adverse effects, including hepatotoxicity (14,15). It was claimed that bio-activation related to the reduction of aromatic nitro group may cause oxidative stress in molecular level and reactive mid-products will induce covalent engagement to the proteins (16). However, it was also suggested that the development of nimesulide was predicated on the search for antioxidant compounds (17). Several reports demonstrated the reducing effect of nimesulide on oxidative damage and its direct free radical scavenging activity (18-20). There is limited data regarding the effects of nimesulide in children. Juvenile toxicology studies in animals may provide useful information for the assessment of adverse reactions in children especially on growth and development (21). Therefore, this study was performed to investigate the effects of nimesulide on oxidative stress and antioxidant enzyme activities in juvenile rats as well as tissue damage potential of the drug.

EXPERIMENTAL

Materials

Nimesulide kindly donated by Helsinn Chemicals SA (Switzerland). Diagnostic kits for biochemical measurements were purchased from Med-Tec (Germany) and Randox (UK). All other chemicals and reagents were of analytical grade.

Experimental procedure

Four weeks-old female Wistar albino rats were obtained from Experimental Surgical and Research Centre, Faculty of Medicine, Ege University. Animals were housed in individual cages, under controlled temperature (22±2 °C) and 12-h on-off light schedule. They were fed with standard laboratory pellet and given free access to tap water. After acclimatization to laboratory conditions for one week, animals were randomly divided into three groups (n=22/each). Nimesulide was suspended in 0.5 % w/v carboxymethyl cellulose (CMC) and given by gavage, twice daily for 14 days. The total daily doses administered were 5 mg/kg/day (Group N-5) or 10 mg/kg/day (Group N-10). Rats in control group received only CMC (Group C).

All rats were observed daily for any changes in their general conditions. Body weights were determined daily. On 15th day, rats were anesthetized with sodium pentobarbital (50 mg/kg; ip). Blood samples for biochemical analysis were collected via abdominal aorta. Liver and kidneys were excised, weighed and fixed in buffered formalin immediately. The study was approved by

the Animal Ethic Committee, Faculty of Pharmacy, Ege University. All animal experiments were conducted in accordance with the internationally accepted laboratory animal use and care, and guidelines (guiding principles in the use of animals in toxicology, adopted by the Society of Toxicology in 1999).

For the evaluation of oxidative stress and antioxidant status plasma MDA levels as an indicator of lipid peroxidation and erythrocyte antioxidant enzyme activities were determined. Tissue damage was estimated by histopathological examination of organ sections.

Plasma MDA levels were measured by modification of the method of Satoh and Yagi (22,23). The principle of the method was based on measurement of the absorbance of the pink color produced by the interaction of thiobarbituric acid (TBA) with MDA at 533 nm.

CAT activity was measured spectrophotometrically by the method of Aebi (24). Briefly, hydrogen peroxide (H_2O_2) was used as a substrate and the decrease in H_2O_2 concentration by CAT was recorded at 240 nm. One unit of CAT activity is defined as the amount of enzyme that degrades 1 μmol H_2O_2 per min.

SOD activity was measured by the method of Misra and Fridovich (25). The method was based on the inhibition of autooxidation of epinephrine at pH 10.2, which can be measured at 480 nm. One unit of SOD activity was considered as the amount of enzyme that causes a 50% decrease in the rate of epinephrine autooxidation to adrenochrome.

GPx activity was determined by the method of Pleban *et al.* (26). The principle of the method was based on the decrease in NADPH absorbance at 340 nm by GPx. One unit of GPx activity was defined as the amount of enzyme required to cause the oxidation of 1 nmol of NADPH per min.

For determination of G-6P-DH activity, Randox diagnostic kits were used. The enzyme activity was determined by measurement of the rate of absorbance change at 340 nm, due to the reduction of $NADP^+$.

Enzyme activities were expressed in U/g Hb of hemolysate. Hemoglobin content was determined by cyanmethemoglobin method (27).

Serum alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities, alanin aminotransferase (ALT), aspartat aminotransferase (AST), total bilirubin, urea, uric acid, triglyceride (TG), total cholesterol, high-density cholesterol (HDL), and low-density cholesterol (LDL) levels were measured by commercially available kits (Med-Tec).

Histopathology

Tissue damage was determined by histopathological examination of organ sections. Organ samples fixed in formalin solution were embedded in paraffin, after processing in ethanol and xylol. The blocks were cut in 5-μm sections and stained with hematoxylin, eosin and periodic acid-Schiff base for light microscopy studies. Slides were examined by an experienced pathologist unaware of the treatment.

Statistical analyses

Results were analyzed by Student's t and Mann-Whitney U tests, using Statview II statistical package program for Macintosh. Data were presented as mean ± standard deviation. Differences were considered significant when $p<0.05$.

RESULTS

In the present study, no significant changes had been observed in body weights and physical characteristics of the treated groups as compared with controls (Table 1). Results of all measurements are shown in Table 2 and Table 3.

Drug treatment increased plasma MDA levels significantly at both doses used, reflecting the increase in lipid peroxidation. GPx and CAT activities were decreased significantly, while SOD

and G-6P-DH activities remained unaltered by treatments. Nimesulide caused prominent increases in serum LDH and ALP (by 10 mg/kg) activities and AST, TG and uric acid levels. HDL levels decreased significantly.

Table 1. Body weight gain and the weights of the liver and kidneys relative to body weights.

	BODY WEIGHTS (BW) (g)			**ORGAN WEIGHTS RELATIVE TO BODY WEIGHTS (g/100 g BW)**	
	Before treatment	15^{th} day	% Increase	Liver	Kidney
Group- C	41.77 ± 8.4	63.41 ± 12.3	67.16 ± 22.1	4.69 ± 0.79	1.04 ± 0.14
Group N-5	38.86 ± 5.4	59.22 ± 10.8	57.64 ± 26.1	5.02 ± 0.60	1.16 ± 0.23*
Group N-10	39.99 ± 7.29	63.29 ± 9.05	60.21 ± 19.12	5.15 ± 0.90	0.95 ± 0.20

Values are expressed as the mean ± standard deviation.
* Statistically significant at $p<0.05$, compare with controls.

Table 2. MDA levels and antioxidant enzyme activities of groups.

	GROUP-C	**GROUP N-5**	**GROUP N-10**
GPx (U/gHb)	94.76 ± 40.3	64.84 ± 24.3*	68.06 ± 27.5*
CAT (k/gHb)	50.25 ± 17.5	34.07 ± 11.0**	32.0 ± 8.33•
SOD (U/gHb)	2987.19 ± 914.9	2962.44 ± 791.6	2906.44±1328
G-6P-DH (mU/gHb)	10620.11 ± 2853.41	11783.10 ± 2286.23	9511.51±2412
MDA (nmol/ml)	1.45 ± 0.3	2.11 ± 0.6 ••	2.37±0.7••

Values are expressed as the mean ± standard deviation.
Statistically significant at * $p<0.05$, ** $p<0.005$, • $p<0.01$, •• $p<0.0005$, compare with controls.

Light-microscopic examinations of all tissue sections from untreated rats showed normal histology. In both nimesulide-treated groups, histopathological examination of liver tissues showed Kupffer cell proliferation, congestion and hydrotropic degeneration. Intrahepatic cholestasis and focal necrosis in liver were observed only in those of rats from Group N-10 (Figure 1). Histopathological examination of kidney tissues of treated rats revealed congestion (Figure 2).

Table 3. Serum parameters of groups.

	GROUP-C	**GROUP N-5**	**GROUP N-10**
AST (U/L)	51.35 ± 26.8	82.92 ± 18.2•	85.64 ± 19.3•
ALT (U/L)	25.28 ± 4.1	28.58 ± 5.9	22.87 ± 7.0
LDH (U/L)	215.44 ± 123.7	347.02 ± 31.8***	540.85 ± 165.02 ***
ALP (U/L)	250.94 ± 98.4	222.00 ± 70.1	489.63 ± 115.9 ***
TG (mg/dl)	46.91 ± 12.0	62.86 ± 17.8 **	58.86 ± 17.5 *
Total Cholesterol (mg/dl)	95.89 ± 29.7	88.50 ± 12.3	92.81 ± 13.0
HDL (mg/dl)	52.45 ± 18.6	31.23 ± 19.7 ***	36.21 ± 12.3*
LDL (mg/dl)	41.17 ± 16.3	43.49 ± 16.0	48.36 ± 19.8
Urea (mg/dl)	39.07 ± 8.6	44.91 ± 5.9 *	42.17 ± 7.05
Uric Acid (mg/dl)	1.11 ± 0.5	2.89 ± 1.1 •	2.01 ± 0.4**
Total Bilirubin (mg/dl)	0.94 ± 0.1	1.18 ± 0.3**	0.92 ± 0.2
Creatinine (mg/dl)	0.87 ± 0.1	1.23 ± 0.4**	0.94 ± 0.06

Values are expressed as mean ± standard deviation.
Statistically significant at *p<0.05, **p<0.01, ***p<0.005, • p<0.0005 compare with controls.

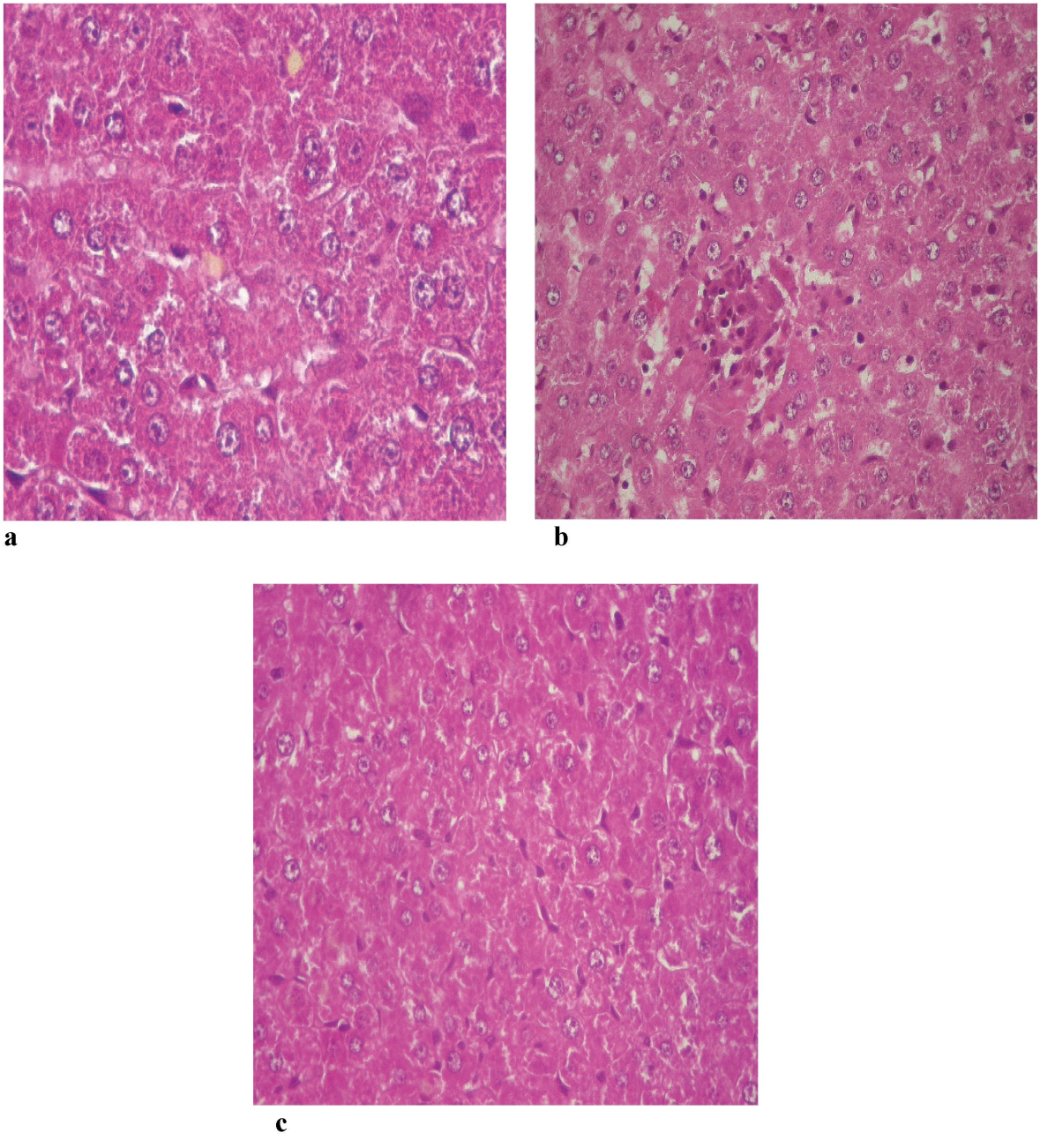

Figure 1. (a) Intrahepatic cholestasis (H.E. X400), (b) Focal necrosis (H.E. X200) and (c) Kupffer cell proliferation (H.E. X100) in liver from nimesulide-treated rats.

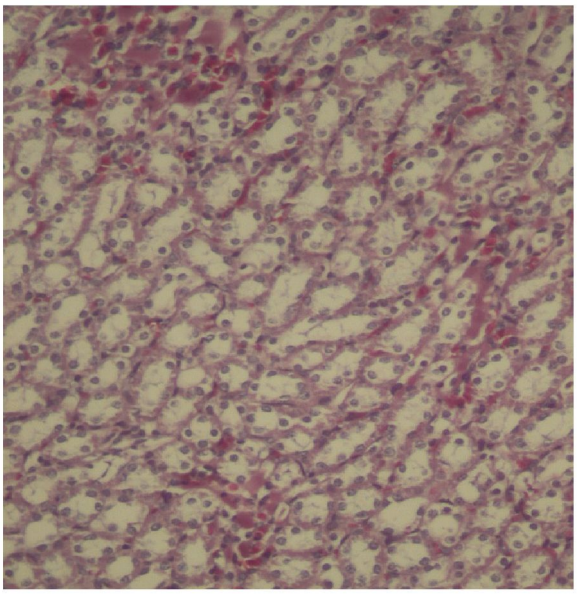

Figure 2. Congestion in kidney (100XHE) from nimesulide-treated rats.

DISCUSSION

In the present study, we administered nimesulide at two doses as a therapeutic dose (5 mg/kg/day) and a higher dose (10 mg/kg/day). In literature, 5 and 2.8 mg/kg/daily doses of nimesulide have been demonstrated as therapeutic doses in children (28-31). There are limited studies regarding pharmacokinetics of nimesulide in children and animals. Bernareggi demonstrated that the pharmacokinetic profile of nimesulide in children and the elderly did not differ from that of healthy young individuals (32). Wallace *et al.* suggested that the COX-2 inhibitors (including nimesulide) needed to be given at doses which precluded their selectivity in order to achieve a desirable anti-inflammatory effect in rats (33). In a study performed in dogs, it was suggested that some COX-1 inhibition is necessary to achieve a clinically useful anti-inflammatory effect (34). The doses used in this study were determined considering all these references and other nimesulide studies performed in rats (35-37).

Increased MDA levels, reflecting the increase in lipid peroxidation, confirm the induction of oxidative stress by nimesulide treatment in the present study. This finding is in agreement with previous studies (15,38,39). In our earlier study performed in adult rats, there was almost 2-fold increase in MDA levels by nimesulide treatment (40). Nimesulide has been shown to cause significant increase in formation of ROS, confirming enhanced oxidative stress in rat hepatocytes *in vitro* (15). It is well known that lipid peroxidation, leading enhanced oxidative stress, may play important role in tissue damage (20,41). In the present study, intrahepatic cholestasis and focal necrosis were observed in liver tissues of rats from Group N-10, which was associated with significant increases in serum AST, ALP and LDH as compared with

controls. We didn't observe any damage except mild congestion in kidneys by nimesulide treatments. Suleimani *et al.* has also reported that nimesulide treatment (20 mg/kg/day for 5 days) did not cause any significant effect on renal function and also any damage on kidneys of Wistar Kyoto rats (42). However, Prevot *et al.* reported that long-term administration of nimesulide (12 months) had a deleterious effect on renal function of newborn rabbits (43). In the present study serum uric acid levels were increased by nimesulide treatments. Hyperuricemia has been considered as an important risk factor for gout and may be associated with oxidative stress conditions (44). Several pharmacologic agents modulate urate excretion, including salicylates. Salicylates have been shown to produce urate retention or uricosuria under different experimental conditions (45). Hyperuricemia secondary to drugs results from their interference with excretion as well as their stimulation of production of uric acid. Because of the increases in urea and creatinine levels in our study, we can speculate that nimesulide-induced hyperuricemia results from its effect on kidney function in juvenile rats.

There are conflicting results about the effects of nimesulide on oxidative stress and antioxidant enzymes. In the present study, MDA levels were increased, CAT and GPx activities were decreased; SOD and G-6P-DH activities were not changed. Kowalczyk *et al.* observed similar effects on adult rats by nimesulide treatment at the doses of 2,5 and 12,5 mg/kg bw (46). These observations may support the suggestions about the effect of nimesulide on antioxidant mechanisms. The decrease in enzyme activities could be attributed to a feedback inhibition or oxidative inactivation of enzyme proteins caused by ROS generation (47). Nimesulide was suggested to cause oxidative stress as a result of increased formation of ROS during their nitro-reductive metabolism (48, 49). Superoxide is one of the main reactive oxygen species in the cells and nimesulide decreases the production of the superoxide anion and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Sohi and Khanduja have demonstrated that SOD activity was suppressed in rats by nimesulide treatment (50). The unaltered SOD activity in the present study might be a consequence of the effects of nimesulide on superoxide anions and SOD activity. In an *in vitro* study of Orhan *et al.*, nimesulide treatment did not cause any changes in SOD and CAT activities, but a significant decrease in GPx activity. Authors suggested that the effect was related to the direct interaction of nimesulide with the enzyme (51).

Interestingly, nimesulide treatments caused significant decreases in serum HDL levels in our study. HDL is a known antioxidant. It was reported that COX-2 levels and prostacyclin production were increased by HDL and this mechanism was described as the basis of cardioprotective effect of HDL (52). Our finding needs to be considered carefully in juveniles.

We did not find any data in literature about nimesulide usage in juvenile rats regarding the parameters we evaluated. Our results, indicating the possibility of liver damage at high dose and alterations of oxidant/antioxidant status by nimesulide treatment, might provide important contribution to the literature about the cautions for nimesulide use in juveniles.

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