Turk J Vet Anim Sci
27 (2003) 1131-1140

Research Article

Histopathologic Changes in Liver and Renal Tissues Induced by Different Doses of Diclofenac Sodium in Rats

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Received: 22.05.2002

Abstract: Non-steroidal anti-inflammatory drugs (NSAIDs) are in common use worldwide. These drugs may sometimes be used in high or toxic doses by mistake. In this study we investigated the effects of different doses of diclofenac sodium on liver and renal tissues. Forty albino adult male Wistar rats weighing 200 to 220 g were divided equally into four groups. The rats in the control group (n = 10) were each intramuscularly injected with 1 cc of physiologic saline. The other three groups were given diclofenac sodium doses. The rats in the first (n = 10), second (n = 10) and third (n = 10) groups were intramuscularly injected with diclofenac sodium at a low, medium and high dose of 50, 100 and 150 mg/kg live weight/day, respectively, every day for 5 days. At the end of the experimental period (5 days), after the animals were sacrificed, they were autopsied and liver and kidney tissue samples were prepared for histopathologic assessment.

No significant ($P > 0.05$) changes were observed in the histopathology of the liver or kidney tissues of the control rats. The diclofenac sodium treatment significantly ($P < 0.001$) affected the histopathology of both the liver and kidney. Histopathologic changes in the liver sections stained with hematoxylin and eosin in all diclofenac groups included claudy swelling and hydropic degeneration of the liver cells, focal sinusoidal and vena centralis dilatation, proliferation of the bile duct in portal areas, enlargement of the periportal area with mononuclear cell infiltration, hyperemia and dose-dependent fibrous tissues proliferation and focal necrosis. Cloudy swelling and hydropic degeneration were seen in the tubular epithelial cells of the kidney tissue of all diclofenac sodium treated groups. Necrosis, peritubular lymphocyte infiltration, stromal fibrous tissue proliferation and hyperemia were observed in the second and third groups. In the liver and kidney tissue of the third group, which was given a high dose of diclofenac sodium, necrosis, cloudy swelling and hydropic degeneration and inflammation were rather widespread and intensive, as compared to the group given a low dose. The increase in fibrous tissue in the kidney and liver that caused irregularities in the periportal areas was only seen in the group given a high dose. These results suggest that a high dose of diclofenac sodium causes meaningful changes in liver and kidney tissue.

Key Words: Diclofenac sodium, liver, kidney, histopathologic changes

Farklı Dozlarda Diclofenac Sodium'un Ratların Karaciğer ve Böbrek Dokusunda Oluşturduğu Histopatolojik Değişiklikler

Özet: Non-steroidal anti-inflamatuar ilaçlar (NSAIDs) dünyada yaygın olarak kullanılırlar. Bu ilaçlar bazen yalnışlıkla yüksek ya da toksik dozda kullanılabilirler. Bu çalışmada, farklı dozlarda diclofenac sodium'un karaciğer ve böbrek dokuları üzerine etkilerini araştırmayı amaçladık. Wistar Strain cinsi ağırlıkları 200-220 gram olan 40 adet olgun Albino ratlar, her biri 10 rattan oluşan 4 gruba ayrıldı. Kontrol grubu olan ratlara (n = 10) intramüsküler olarak 1 cc serum fizyolojik enjekte edildi. Diğer üç gruba diclofenac sodium'un farklı dozları uygulandı. Birinci (n = 10), ikinci (n = 10) ve üçüncü (n = 10) grup ratlara 5 gün süre ile hergün 50, 100, 150 mg/kg, düşük, orta ve yüksek dozlarda diclofenac sodium intramüsküler olarak enjekte edildi. Beş günlük deney süresi sonunda hayvanlar ötenazi edildikten sonra nekropsileri yapılarak, karaciğer ve böbrek doku örnekleri histopatolojik inceleme için hazırlandı. Kontrol grubu ratların karaciğer ve böbrek dokularının histopatolojik incelenmesinde önemli değişiklikler gözlenmedi ($P > 0,05$). Diclofenac sodium tedavisi, karaciğer ve böbrek dokularını önemli derecede etkiledi ($P < 0,001$). Diclofenac tedavili bütün grupların, hematoksilen-eosin ile boyalı karaciğer kesitlerindeki histopatolojik değişiklikler: Karaciğer hücrelerinde bulanık şişkinlik ve hidropik dejenerasyon, fokal sinüsoidal ve santral ven genişlemesi, portal alanlarda safra kanal proliferasyonu, mononükleer hücre

infiltrasyonu ile birlikte periportal mesafe genişlemesi, hiperemi, fokal nekrozlar ve doza bağlı fibröz doku artışıydı.

Diclofenac sodium tedavili bütün grupların böbrek dokularının tübüler epitel hücrelerinde bulanık şişkinlik ve hidropik dejenerasyon görüldü. Fakat, nekroz, peritübüler lenfosit infiltrasyonu, interstisiyumda fibröz doku artışı, hiperemi ikinci ve üçüncü gruplarda gözlendi. Yüksek doz diclofenac sodium verilen üçüncü grubun karaciğer ve böbrek dokularında görülen nekroz, hidropik dejenerasyon ve inflamasyon, düşük dozla karşılaştırıldığında, daha yaygın ve yoğun idi. Böbrek dokusunda ve karaciğerin periportal alanlarında düzensizliğe neden olan bağ dokusu artışı sadece yüksek doz alan grupta görüldü. Bu bulgular, yüksek doz diclofenac sodium'un karaciğer ve böbrek dokularında anlamlı değişikliklere neden olduğunu göstermektedir.

Anahtar Sözcükler: Diclofenac sodium, karaciğer, böbrek, histopatolojik değişiklikler

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), which are often used for the relief of non-specific fever (1), continue to be important for the palliation of pain (2). They are the most frequently used medications for the treatment of a variety of common chronic and acute inflammatory conditions (3), and continue to be important for the palliation of pain and in decreasing inflammation and fever (4-7). Diclofenac sodium is a non-steroidal anti-inflammatory agent (8). Diclofenac belongs to a chemical subgroup of NSAIDs that are an arylalkanoic group of phenylacetic acid (9) and advocated for use in painful and inflammatory rheumatic and non-rheumatic conditions (4-6). It may be used for the treatment of fetal developmental problems and the prevention of premature birth (5).

Diclofenac sodium has antipyretic, analgesic and anti-inflammatory effects, is an inhibitor of cycloxigenase enzyme and is decreased in leukocyte intracellular free arachidonate level (10). The exact mechanism is not known but it is probably related to the decrease in the fatty acid entering the cell or released from the cell (10). The literature has continued to broaden our understanding of the potential toxic events associated with the use of these agents (4-6).

Several studies have indicated that NSAIDs can generally prevent prostaglandin synthesis from arachidonic acid by inhibiting the activity of the prostaglandin synthesizing enzyme, cyclooxygenase. Prostaglandins are formed from dietary essential fatty acids (principally arachidonic acid) esterified to phospholipids and in some instances to trigliycerides. These products have some potent biological activities affecting cell function in every organ. The high levels of NSAIDs also inhibit the activities of various enzymes, the proteoglycan synthesis from chondrocytes, the ionic exchange rate and the processes depending on prostaglandins (4-6).

Diclofenac is extremely metabolized in the liver (11). Therefore, the present study aimed to investigate only the hepatotoxic and nephrotoxic effects of increasing doses of diclofenac on liver and kidney tissue in rats.

Materials and Methods

Forty albino adult male Wistar rats weighing 200 to 220 g were used in this study. They were divided into four groups: a control group and three diclofenac groups, each with 10 rats. The rats in the control group (n = 10) were intramuscularly injected with physiologic saline (1 cc per rat). The rats in the first (n = 10), second (n = 10) and third diclofenac groups (n = 10) were intramuscularly injected with 50, 100 and 150 mg/kg live weight/day diclofenac sodium, respectively. The experiment lasted 5 days. Twenty-four hours after the last injections at the end of 5 days, all the rats were cut under ether anesthesia and tissue samples were taken from the liver and kidney. Tissue samples were then fixed in a buffered formalin, processed through graded alcohols and xylene, and embedded in paraffin blocks. Tissue sections of 4-6 µ were made at multiple levels. Sections were routinely stained with hematoxylin and eosin and reticulum and Masson's trichrome. Mounted slides were examined and photographed under a light microscope.

Changes in the experimental histopathologic parameters for liver and kidney tissues were graded as follows: (-) showing no changes, and (+) (++) and (+++) indicating minimum, moderate and maximum changes, respectively. These values were considered to be non-parametric, and therefore the data was statistically analyzed by a Kruskal-Wallis test in order to determine

the effects of all groups on each of the experimental parameters, and a Mann-Whitney U test was used to compare the means of each parameter between the groups. A P value of < 0.05 was considered significant.

Findings

Histologic examination of the liver and kidney tissues in the control group revealed no significant deviation from the normal histological structures (Figs. 1 and 2). The changes in the histologic structure of the liver and kidney were significantly ($P < 0.001$) affected by the first, second and third diclofenac sodium treatments (Tables 1 and 2).

In the first diclofenac group although there were mononuclear cell infiltrations, bile duct proliferation in the periportal areas and minimal enlargement in the periportal areas, sinusoidal and central vein dilatation

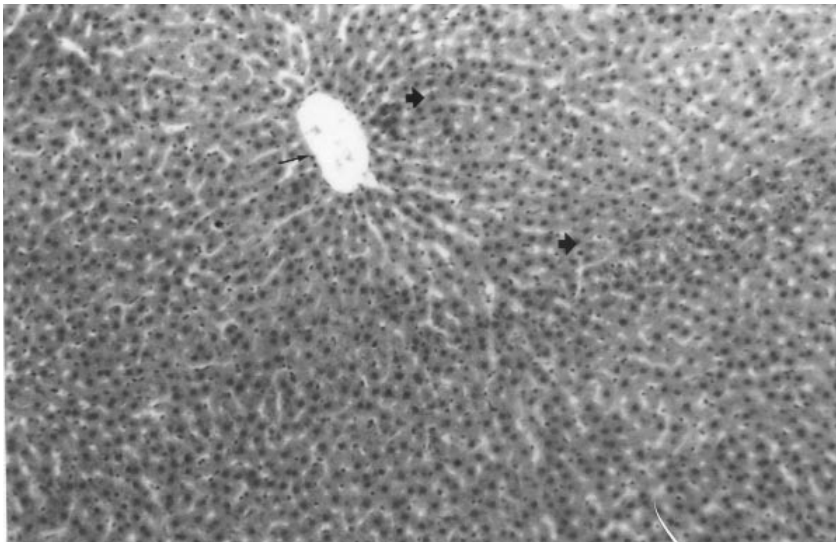

Figure 1. Histologic appearance of rat liver from the control group. Normal lobular structure is seen. Central vein (thin arrow) and hepatocytes (thick arrows) (Hematoxylin-eosin, x 48).

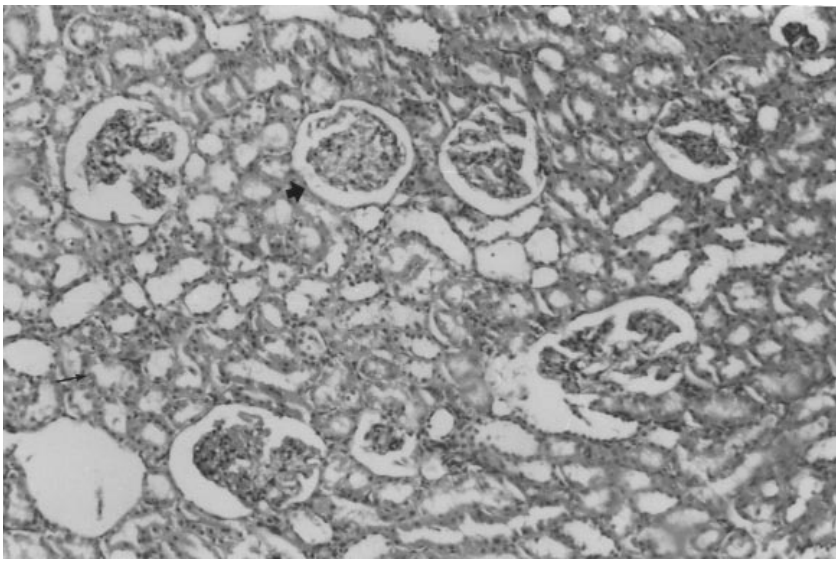

Figure 2. Histologic section of rat kidney from the control group. Normal tubular (thin arrow) and glomerular (thick arrow) structures are seen in the cortex (Hematoxylin-eosin, x 120).

Table 1. Histopathologic changes induced by different doses of diclofenac sodium in liver tissue.*

	Control group n = 10	First group n = 10	Second group n = 10	Third group n = 10	P[1]
Hepatocyte degeneration	+	+	++	+++	< 0.001
Sinusoidal dilatation	+	+	++	+++	< 0.001
Central vein dilatation**	+	2	3	4-5	< 0.001
Enlargement of periportal area**	-	2	3	4	< 0.001
Bile duct proliferation**	-	2	3-4	4-5	< 0.001
Mononuclear cell infiltration	-	+	++	+++	< 0.001
Pleomorphism of the hepatocyte	-	-	+	+++	< 0.001
Conspicuous nucleolus	-	-	+	++	< 0.001
Hepatocyte with eosinophilic cytoplasma and pyknotic nucleus	-	-	+	++	< 0.001
Parenchymal necrosis	-	-	+	++	< 0.001
Limiting plate irregularities	-	-	-	++	< 0.001
Increased fibrous tissue	-	-	-	+	< 0.001

[1] The data was statistically analyzed by a Kruskal-Wallis test in order to determine the effects of all groups on each of the experimental parameters.
* Histopathologic assessments of the experimental parameters were graded as follows: (-) showing no changes and (+), (++) and (+++) indicating mild, moderate and severe changes, respectively.
** The number of specified parameters was counted under the light microscope of 10 high power field.
n, number of observations.

Table 2. Histopathologic changes induced by different doses of diclofenac sodium in kidney tissue.*

	Control group n = 10	First group n = 10	Second group n = 10	Third group n = 10	P[1]
Tubular epithelial cell degeneration	+	+	++	+++	< 0.001
Tubular epithelial cell necrosis	-	-	+	+++	< 0.001
Atrophic glomerulus and tubulus	-	-	-	++	< 0.001
Eosinophilic secretion in the tubulus lumen	-	-	+	++	< 0.001
Interstitial mononuclear cell infiltration	-	-	-	++	< 0.001
Increased fibrous tissue	-	-	+	++	< 0.001
Hyperemic vessels in the interstitium	+	+	++	+++	< 0.001

[1] The data was statistically analyzed by a Kruskal-Wallis test in order to determine the effects of all groups on each of the experimental parameters.
* Histopathologic assessments of the experimental parameters were graded as follows: (-) showing no change and (+), (++) and (+++) indicating mild, moderate and severe changes, respectively.
n, number of observations.

occurred in the liver tissues (Table 1); however, these changes were not significantly ($P > 0.05$) different from those observed in the control group (Table 3). In the second diclofenac group we observed dilatation of the central vein, sinusoidal dilatation around the central vein, bile duct proliferation, enlargement of some portal areas and cloudy swelling, parenchymal cell necrosis, mild pleomorphism and a conspicuous nucleolus in the hepatocyte. Some hepatocytes have eosinophilic cytoplasma and a pyknotic nucleus. Furthermore, there was mononuclear cell infiltration in the periportal areas (Table 1). Of the above changes, only bile duct proliferation ($P < 0.001$), mononuclear cell infiltration ($P < 0.001$) and parenchymal cell necrosis ($P < 0.001$) were

Table 3. Statistical comparison for the differences between the experimental groups for liver parameters[1].

Liver tissue	Control with First group	Control with Second group	Control with Third group	First group with Second group	First group with Third group	Second group with Third group
Hepatocyte degeneration	> 0.05 (ns)	> 0.05 (ns)	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Sinusoidal dilatation	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Central vein dilatation	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Enlargement of periportal area	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Bile duct proliferation	> 0.05 (ns)	< 0.001	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Mononuclear cell infiltration	> 0.05 (ns)	< 0.001	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Pleomorphism of the hepatocyte	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Conspicuous nucleolus	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Hepatocyte with eosinophilic cytoplasma and pyknotic nucleus	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	> 0.05 (ns)
Parenchymal necrosis	> 0.05 (ns)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Limiting plate irregularities	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	< 0.001
Increased fibrous tissue	> 0.05 (ns)	> 0.05 (ns)	> 0.05	> 0.05 (ns)	> 0.05 (ns)	> 0.05 (ns)

[1] Mann-Whitney U test was used to compare the means of each parameter between the groups.
ns: not significant

significantly different from those in the control group (Table 3).

In the third diclofenac group the liver tissue samples showed large widespread changes. The insignificant histopathologic changes seen in the second diclofenac group together with other parameters were significantly ($P < 0.001$) different in the third diclofenac group campared with the control group (Table 3). Additionally, there were patchy parenchymal necrosis ($P < 0.001$), limiting plate irregularities ($P > 0.05$), diffuse sinusoidal dilatation ($P < 0.001$) and slightly increased fibrous tissue ($P > 0.05$) as compared with the changes in the control group (Figs. 3 and 4) (Table 3).

In the first diclofenac group kidney tissue samples showed degenerative changes in the tubular epithelium and hyperemic vessels in the cortical and medullar interstitial areas (Table 2). However, these changes were not significantly ($P > 0.05$) different from those in the control group (Table 4). In the second diclofenac group the changes observed compared with those in the first diclofenac group were very common. In addition to focal tubular epithelial degeneration and destruction ($P < 0.001$) there were mild fibrosis areas ($P < 0.001$), which were stained with Masson's trichrome in the interstitium as compared with the control group (Tables 2 and 4).

In the third diclofenac group there were cortical and medullar tubular epithelial degeneration ($P < 0.001$), focal tubular epithelial necrosis ($P < 0.001$), some atrophic glomerulus and tubulus ($P < 0.001$), intratubular eosinophilic secretion ($P < 0.001$) and increased fibrous tissue ($P < 0.001$) and mononuclear cell infiltration in the interstitium ($P < 0.001$) as compared with the control group (Tables 2 and 4) (Figs. 5 and 6).

Discussion

In the present study, dose-dependent changes were seen in the liver tissue samples. In the first diclofenac group, these changes were very mild and reversible. Similar changes were also seen in the second diclofenac group. In the third diclofenac group the changes seen compared with those in the first diclofenac group were aggressive and common: there were hepatocyte necrosis, mild fibrous tissue proliferation and interstitial and periportal inflammation, which indicate acute hepatitis. These findings suggest that a high dose of diclofenac sodium causes irreversible cell death, as well as cell damage, fibrous tissue proliferation and acute hepatitis (12,13). The association of NSAIDs with liver disease is poorly documented. Reports on hepatic injury have ranged from insignificant and transient liver enzyme

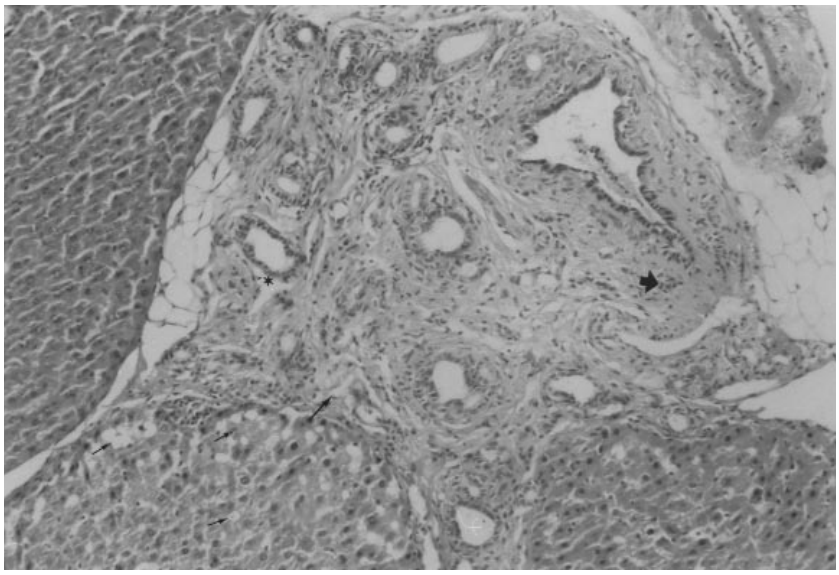

Figure 3. High dose diclofenac sodium administered group. Bile duct proliferation (star) in portal area, enlargement in periportal area (thin arrow) with mononuclear cell infiltration (thick arrow) and parenchymal cell degeneration (short arrows) are seen (Hematoxylin-eosin, x 120).

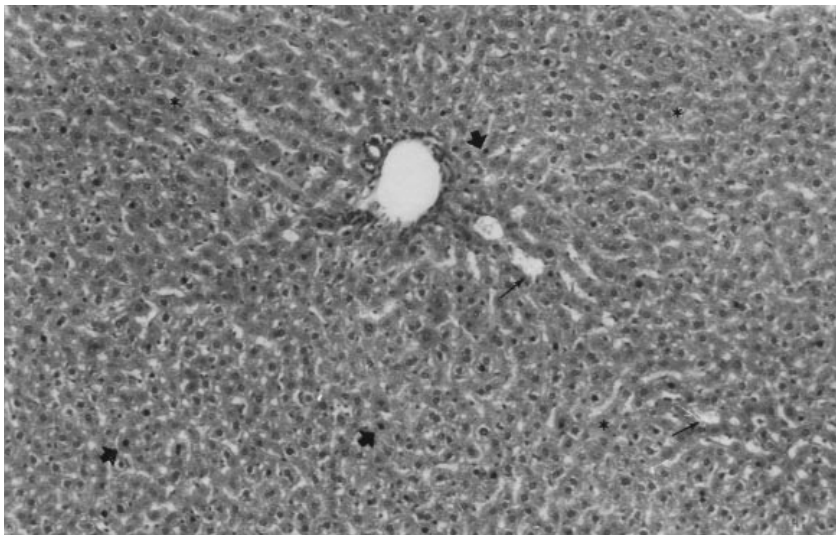

Figure 4. Higher magnification of liver lobule in high dose diclofenac sodium treated group. Parenchymal cell necrosis (stars), sinusoidal dilatations (thin arrows), hepatocytes with pyknotic nucleus and eosinophilic cytoplasma (thick arrows) are seen (Hematoxylin-eosin, x 120).

elevation to severe and fulminant hepatitis (3). Diclofenac sodium causes a rise in liver function, and has also been reported to cause hepatitis (14-16). The hepatotoxic and nephrotoxic effects of diclofenac sodium in both humans and experimental animals have been reported (16-19). Nevertheless, the exact mechanism of the toxic effects induced by diclofenac sodium has not yet been determined (6). Diclofenac sodium is metabolized in liver tissue and so is toxic to liver cells (11). The toxic effects of diclofenac could be acute or reversible (6). Several

Table 4. Statistical comparison for the differences between the experimental groups for kidney parameters[1].

Kidney tissue	Control with First group	Control with Second group	Control with Third group	First group with Second group	First group with Third group	Second group with Third group
Tubular epithelial cell degeneration	> 0.05 (ns)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Tubular epithelial cell necrosis	> 0.05 (ns)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Atrophic glomerulus and tubulus	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	< 0.001
Eosinophilic secretion in the tubulus lumen	> 0.05 (ns)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interstitial mononuclear cell infiltration	> 0.05 (ns)	> 0.05 (ns)	< 0.001	> 0.05 (ns)	< 0.001	< 0.001
Increased fibrous tissue	> 0.05 (ns)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Hyperemic vessels in the interstitium	> 0.05 (ns)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[1] Mann-Whitney U test was used to compare the means of each parameter between the groups.
ns: not significant

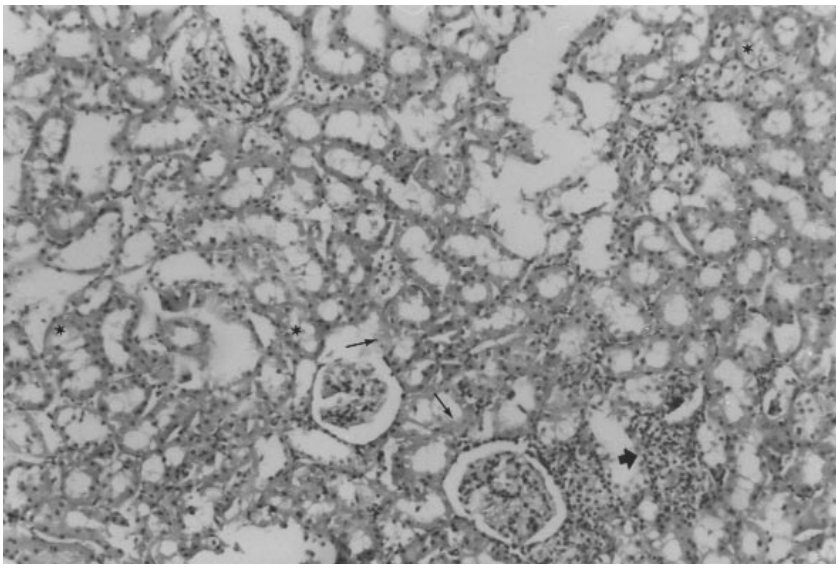

Figure 5. Histologic section of rat kidney from the high dose administered group showing degeneration (stars) and epithelial cell necrosis (thin arrows), in the epithelial lining some of the tubules and mononuclear cell infiltration (thick arrow) in the interstitium (Hematoxylin-eosin, x 120).

investigators have attempted to clarify the mechanism of diclofenac-sodium-induced hepatotoxicity. It has been reported that the damage in hepatocytes induced by diclofenac sodium was associated with an idiosyncratic reaction (14).

The effects of the anti-inflammatory drug diclofenac on mitochondrial respiration, ATP synthesis and membrane potential are known. These drugs stimulate ATP synthesis and collapsed membrane potential in mitochondria. Diclofenac blocks the activity of the adenine nucleotide translocase and inhibits mitochondrial ATPase activity (20). Diclofenac is more cytotoxic to drug metabolizing cells than to non-metabolizing cell lines. Despite the fact that diclofenac itself was effective in impairing ATP synthesis by mitochondria, there was evidence that toxicity was also related to the drug metabolism and was reduced by the addition of cytochrome p-450 inhibitors to the culture medium (21).

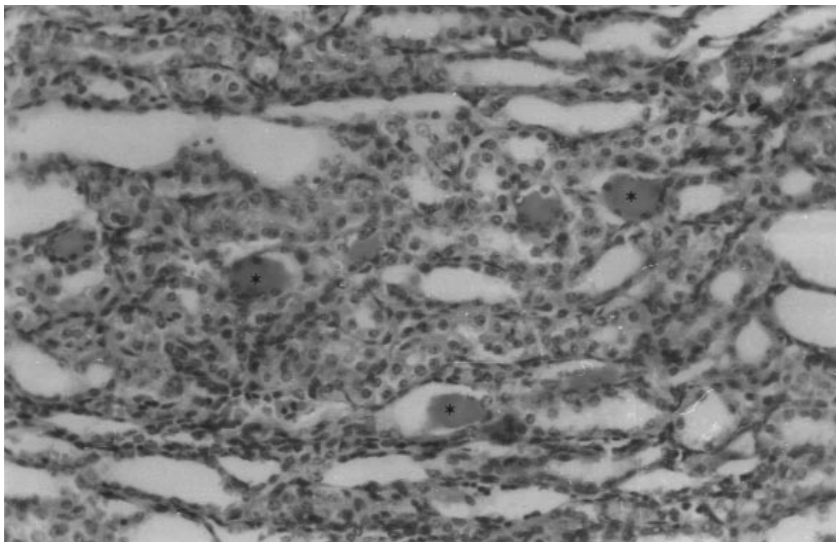

Figure 6. Histologic examination of rat kidney from high dose treated group showing eosinophilic secretion in the tubulus lumen (stars) (Hematoxylin-eosin, x 120).

The non-steroidal anti-inflammatory drug diclofenac causes a rare but potentially fatal hepatotoxicity that may be associated with the formation of reactive metabolites (22,23). These products presumably were formed via the hepatic cytochrome P-450 (CYP)-catalyzed oxidation of diclofenac to reactive benzoquinone imines that are trapped by GSH (glutathione) conjugation. It is, therefore, possible that reactive benzoquinone imines may be formed and contribute to diclofenac-mediated hepatic injury (24). Three metabolites of diclofenac sodium are reported to be responsible for diclofenac sodium toxicity in the liver, namely 4' hydroxy 3 diclofenac, 5' hydroxy 4 diclofenac and 5' hydroxy 6 diclofenac (22,24). Some experimental results suggest that the toxic effect of diclofenac on hepatocytes may be caused by drug-induced mitochondrial impairment together with a futile consumption of nicotinamide adenine dinucleotide phosphate (NADPH) (21). Mitochondrial damage and NADPH deficiency are thought to be responsible for diclofenac sodium hepatotoxicity (25).

We tested the effects of different doses of diclofenac treatment in order to investigate histopathologic differences between low and high doses. The present histopathologic finding in the liver was in agreement with diclofenac-sodium-induced hepatotoxicity that may appear in acute or chronic liver disease. The hepatic changes we obtained were dose-dependent because we observed meaningful histopathologic liver changes accompanying with acute hepatitis and irreversible cell changes with the use of high dose diclofenac compared to low dose treatment.

The effects of NSAIDs in therapeutic doses on oxidative stress have been established (23), but there is little knowledge on the effects of high doses of these drugs (26). The kidney is another organ affected by the toxic effects of NSAIDs (27-29). It is known to be an important target organ for the untoward effects of NSAID, which can produce acute, reversible or permanent effects (22,23,30-36). The toxic effects of diclofenac can be acute or reversible (19). The NSAIDs adversely change the kidney functions (10) and may play a role in the induction of membranous nephropathy (37). During diclofenac sodium metabolism the number of reactive oxygen species can be increased. These products induced prooxidative damage in renal tissue. The increase in SOD levels (endogenous antioxidant enzymes) and MDA activity (indicating lipid peroxidase) in renal tissue may indicate peroxidative damage. In the renal tissue, structural changes confirm oxidative changes including reductions in GSH-Px and GR activities in the kidney and increased MDA levels in serum. The decrease in GSH-Px activity possibly destroys the process of H_2O_2 convertion into H_2O (12,13).

Thus, an increased level of H_2O_2 can also cause cell damage in kidney tissue (12). Diclofenac sodium causing nephrotoxicity could be associated with the potent inducer of the membrane permeability transition (MMPT) in renal cortex mitochondria (19). MMPT increases calcium uptake in mitochondria in the presence of reactive oxygen species. The mitochondrial degeneration thus occurs. This is associated with an increased level of intra-mitochondrial calcium in the renal tissue (38-42).

In the present study the high dose of diclofenac caused tubular epithelial cell degeneration, and necrosis that may be reversible or irreversible. With the low dose of diclofenac sodium histopathologic changes in the kidney tissue were reversible, but with the high dose diclofenac these changes were common and irreversible and there was also increased fibrous tissue in the interstitial tissue of the kidney.

In a previous study, nephrotic syndrome (NS) was said to be associated with NSAID use if the patient developed NS while taking NSAID and if other causes of membranous nephropathy were excluded, and a rapid remission of the NS followed withdrawal of the drug (37). It can be concluded that NS due to membranous nephropathy should be recognized as an idiosyncratic drug reaction to many NSAIDs (37).

In this experimental study there were significant differences between the different doses of diclofenac. Dose-dependent administration of diclofenac significantly altered the histopathologic structure. The major histopathologic events in the course of diclofenac cytotoxicity are hepatocytes and renal tubule epithelial cell death, acute hepatitis and moderate histopathologic changes that are seen as in the case of chronic pyelonephritis.

In conclusion, pathologically liver injury associated with NSAIDs is not prevalent but we think it is interesting to report these results for the recognition of the histopathologic events and disease in the course of diclofenac cytotoxicity.

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