Stereological and biochemical evaluation of diclofenac-induced acute nephrotoxicity in rats

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SUMMARY

The aim of this study was to evaluate the effects of diclofenac-induced acute nephrotoxicity using stereological and biochemical parameters in rats. For that, 20 male Wistar rats allotted in 4 equal groups were intraperitoneally injected by 0, 10, 50 and 100 mg/kg diclofenac, respectively and 8 hours after injection, blood serum samples were collected for assessment of urea, creatinine, fibrinogen, antithrombin III, malondialdehde (MDA), nitric oxide, vitamin C and β-carotene concentrations and adenosine deaminase (ADA) and superoxide dismutase (SOD) activities. After slaughtering, left kidneys were stereologically evaluated. Despite slight but more often not significant variations especially observed in rats treated with 50 mg/kg diclofenac, stereological renal parameters, oxidative stress markers and coagulation markers were considered to be within usual values in rats. By contrast, significant and dose related increases in uraemia were evidenced in intoxicated animals. It was concluded that diclofenac-induced kidney damage cannot be evaluated by kidney stereological and serum oxidative stress parameters, however, more severe kidney damage may cause observable alterations in these parameters.

Keywords: Diclofenac, nephrotoxicity, rat, acute toxicity, stereology, oxidative stress.

Introduction

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID) which belongs to the acetic acid group [11, 30], is frequently prescribed in human and veterinary medicine as an anti-inflammatory, antipyretic and analgesic agent. Diclofenac acts by inhibiting cycloxygenase-1 (COX-1) and cycloxygenase-2 (COX-2) enzymes, thus preventing prostaglandin synthesis from arachidonic acid [11]. COX-1 and COX-2 are continuously expressed in the kidney and its vessels [11, 17, 22]. Diclofenac may cause stomach-related side effects and nephrotoxicity, similar to other NSAIDs, by blocking prostaglandin synthesis [30]. Liver synthesized fibrinogen which is converted into fibrin and participates in coagulation, while antithrombin acts as an anti-coagulant. NSAIDs inhibit thromboxane production and platelet aggregation, thus expressing anti-coagulation activity [12, 20]. In addition, NSAID administration depresses the excessive production of adenosine deaminase by immune system cells during infection [2, 36].

Stereology is a group of methods which have generated significant interest during recent years and are used to obtain quantitative data from biological tissues. Stereology has been used to evaluate the three-dimensional properties of two-dimensional, systemic, tissue and organ cross-sections, by utilizing mathematical and statistical methods [25]. The total volume of organs can be qualified by Archimedes' method [1], while stereological methods can be utilized to determine the volume of their subcomponents [5]. Stereological methods have been successfully applied to calculate the volume of subcomponents of the kidney such as the cortex, medulla and pelvis [5, 25, 26]. Some serum parameters are used to evaluate organ damage. Increased serum urea and creatinine concentrations are accepted as indicators of kidney damage. It has been shown that diclofenac may cause nephrotoxicity in rodents [3, 33] and increased serum urea and creatinine concentrations are accepted as markers of infection- and chemical agent-induced nephrotoxicity in rats [9, 10, 31].

Reactive oxygen species (singlet oxygen, superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide,
peroxynitrite, etc.) are continuously produced in the body [21, 34] and are deactivated by enzymatic (superoxide dismutase, glutathione peroxidase, catalase, etc.) or non-enzymatic (glutathione, vitamin A, vitamin C, etc.) substances [21, 34]. Normally, oxidants and antioxidants are in balance in the body. However, oxidative stress occurs when reactive oxygen radicals are produced excessively and/or antioxidants are insufficient. As a result, oxidative stress causes lipid peroxidation. Malondialdehyde (MDA) is the world-wide accepted biological marker of lipid peroxidation [4, 21, 34]. NSAIDs can affect oxidative balance in the body, and some of them have antioxidant activity while others exhibit oxidant activity [18, 30].

Similar to other NSAIDs, diclofenac inhibits prostaglandin thus causing kidney ischemia and affects the coagulation mechanism; hence it has been hypothesized that it may have dose-dependent effects on kidney-linked stereological parameters such as renal damage, oxidative stress, plasma coagulation parameters and adenosine deaminase activity. The aim of this study was to determine the effects of diclofenac-induced nephrotoxicity, at different doses, on stereological parameters of the kidney (total renal volume, cortex volume, medulla volume, pelvis volume and fractions), serum markers of kidney damage (urea and creatinine concentrations), oxidative stress (malondialdehyde, nitric oxide, vitamin-C, β-carotene concentrations and superoxide dismutase activity), plasma coagulation (fibrinogen and antithrombin III concentrations) and serum adenosine deaminase activity.

Material and Methods

ANIMALS AND EXPERIMENTAL DESIGN

Twenty male Wistar rats (50-60 days old, 170-190 g, Kobay Deney Hayvanlari AS, Ankara, Turkey) were used in this research. The research protocol was approved by the Ethical Committee of Kobay Laboratory Animal Organization.

The rats were divided into four equal groups, three groups received intraperitoneally a single dose of diclofenac sodium (Diclomec Amp., Mecom Saglik Urnerleri A.S., Istanbul) at 10 mg/kg, 50 mg/kg and 100 mg/kg, respectively, and the remaining group served as the control. Blood samples were collected from the heart under thiopental sodium anaesthesia (70 mg/kg, intraperitoneal route, Pental sodium® 1 g inj., I.E. Ulagay Ilac Sanayi, Istanbul, Turkey) 8 hours after the administration of diclofenac.

STEREOLOGICAL ANALYSIS

The rats were then euthanized by cervical dislocation under anaesthesia, and their left kidneys were immediately collected for subsequent stereological methods. The collected kidneys were kept in 10% neutral formaldehyde solution for 15 days. Following routine histological processing, paraffin kidney blocks were prepared and sectioned in accordance with systematic random sampling [15]. Using a rotary microtome (Leica, RM2125RT, Germany), 11-13 pieces of transverse cross-section samples 10 μm thick per 1000 μm distance were obtained from all kidneys. All samples were stained with haematoxylin-eosin and covered by Entellan®. Volume and fraction calculations were performed using Cavalieri estimator command of Stereo Investigator software (ver. 10, MicroBrightField Inc., VT, USA). A point grid with 100 μm distance between two points was overlaid with images of the cross-sections. Hitting points on the cortex, medulla and renal pelvis were counted using the above-mentioned software (figure 1). The volumes of each subcomponent of the kidneys were calculated using the formula: \( V = t \times a(p) \times \Sigma P \), where \( V \) refers to the volume of the subcomponent of interest, \( t \) is the cross-section thickness, \( a(p) \) is the area of one point (1000 \( \mu m^2 \)) within the point counting grid and \( \Sigma P \) is the total number of points in the subcomponent of interest. Volume fractions of the cortex, medulla and pelvis were expressed as percentages.

BIOCHEMICAL ANALYSIS

Serum urea and creatinine concentrations were measured using an auto-analyzer (ILab-300 Biomerieux Diagnostic, Milano, Italy), and citrated plasma fibrinogen and antithrombin III concentrations were determined by a coagulometer (Siemens Sysmex CA 1500 Model, Japan). Serum MDA [7], nitric oxide [24], vitamin-C [19] and β-carotene [28] concentrations as well as the superoxide dismutase [27] and adenosine deaminase [14] activities were determined by ELISA reader (MWGt Lambda Scan 200, BioTek Instruments, USA) with previously reported methods.

STATISTICAL ANALYSIS

All values are expressed as mean ± standard error (SE). One way analysis of variance (ANOVA) and Tukey tests were used to evaluate the results. Differences were considered as significant when \( p < 0.05 \).
Results

The effects of diclofenac on kidney stereological parameters are shown in Table I. Although not significantly, the total renal and the medulla volumes tended to decrease whereas the pelvic volume tended to increase in diclofenac treated rats, particularly in the group treated by 50 mg/kg. In parallel, the medulla fraction was lowered and the pelvic fraction elevated in this group compared to the others but differences were not statistically significant.

As shown in Table II, serum urea concentrations were markedly higher in diclofenac treated rats than in the negative controls (p < 0.05) and the increase in uraemia was gradual accordingly the injected dose (100 mg/kg vs. 10 mg/kg: p < 0.05). It was observed that creatinine concentrations also increased in intoxicated animals, particularly in those treated with 50 mg/kg but differences compared to the controls were not statistically significant. As far as the coagulation system was concerned, some modifications were recorded in animals receiving the lowest dose (10 mg/kg) of the NSAID: in this group, the mean fibrinogen concentration was significantly enhanced compared to the others (p < 0.05) and the antithrombin concentration also tended to increase but not significantly. The serum adenosine deaminase activity remained similar in all groups.

The effects of diclofenac on serum oxidative stress parameters are shown in Table III. Although the oxidant MDA and NO concentrations did not significantly change in treated animals, some slight alterations in antioxidant systems were noticed. The β-carotene concentrations slightly increased in intoxicated animals, particularly in those treated with 50 mg/kg but differences compared to the controls were not statistically significant. As far as the coagulation system was concerned, some modifications were recorded in animals receiving the lowest dose (10 mg/kg) of the NSAID: in this group, the mean fibrinogen concentration was significantly enhanced compared to the others (p < 0.05) and the antithrombin concentration also tended to increase but not significantly. The serum adenosine deaminase activity remained similar in all groups.

### Table I: Effects of different doses (0, 10, 50 and 100 mg/kg) of diclofenac intraperitoneal administration on kidney stereological parameters in rats. Results are expressed as mean ± standard error (SE).

<table>
<thead>
<tr>
<th>Diclofenac doses</th>
<th>0 mg/kg</th>
<th>10 mg/kg</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total renal volume (mm³)</td>
<td>302.0 ± 11.5</td>
<td>284.0 ± 5.7</td>
<td>280.0 ± 9.5</td>
<td>289.0 ± 13.2</td>
</tr>
<tr>
<td>Cortex volume (mm³)</td>
<td>167.0 ± 8.1</td>
<td>152.0 ± 4.9</td>
<td>155.0 ± 6.2</td>
<td>155.0 ± 5.6</td>
</tr>
<tr>
<td>Medulla volume (mm³)</td>
<td>130.0 ± 5.2</td>
<td>126.0 ± 5.3</td>
<td>118.0 ± 5.7</td>
<td>127.0 ± 8.5</td>
</tr>
<tr>
<td>Pelvic volume (mm³)</td>
<td>4.21 ± 0.52</td>
<td>6.10 ± 0.77</td>
<td>7.21 ± 1.29</td>
<td>7.09 ± 1.50</td>
</tr>
<tr>
<td>CV/TRV (%)</td>
<td>55.4 ± 1.3</td>
<td>53.1 ± 1.4</td>
<td>55.3 ± 1.5</td>
<td>53.7 ± 1.5</td>
</tr>
<tr>
<td>MV/TRV (%)</td>
<td>43.2 ± 1.2</td>
<td>44.4 ± 1.6</td>
<td>42.1 ± 1.4</td>
<td>43.8 ± 1.2</td>
</tr>
<tr>
<td>PV/TRV (%)</td>
<td>1.40 ± 0.01</td>
<td>2.16 ± 0.01</td>
<td>2.54 ± 0.01</td>
<td>2.44 ± 0.01</td>
</tr>
</tbody>
</table>

CV: Cortex volume; MV: Medulla volume; PV: Pelvic volume; TRV: total renal volume.

### Table II: Effects of different doses (0, 10, 50 and 100 mg/kg) of diclofenac intraperitoneal administration on serum biochemical parameters in rats. Results are expressed as mean ± standard error (SE).

<table>
<thead>
<tr>
<th>Diclofenac doses</th>
<th>0 mg/kg</th>
<th>10 mg/kg</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (µmol/L)</td>
<td>63.65 ± 3.54</td>
<td>69.84 ± 1.77</td>
<td>76.91 ± 4.42</td>
<td>65.42 ± 2.65</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>16.12 ± 0.62</td>
<td>26.31 ± 1.46</td>
<td>32.63 ± 2.58</td>
<td>36.13 ± 1.95</td>
</tr>
<tr>
<td>Fibrinogen (mg/L)</td>
<td>964 ± 23</td>
<td>1070 ± 22</td>
<td>1000 ± 5</td>
<td>1000 ± 18</td>
</tr>
<tr>
<td>Antithrombin III (%)</td>
<td>103.0 ± 0.2</td>
<td>117.0 ± 7.6</td>
<td>102.0 ± 0.7</td>
<td>103.0 ± 0.4</td>
</tr>
<tr>
<td>ADA (U/L)</td>
<td>23.5 ± 1.3</td>
<td>23.4 ± 3.8</td>
<td>21.2 ± 1.9</td>
<td>25.5 ± 1.3</td>
</tr>
</tbody>
</table>

ADA: adenosine deaminase.
Different superscripts a,b,c in the same row indicate significant difference between groups (p < 0.05).

### Table III: Effects of different doses (0, 10, 50 and 100 mg/kg) of diclofenac intraperitoneal administration on oxidative stress parameters in rats. Results are expressed as mean ± standard error (SE).

<table>
<thead>
<tr>
<th>Diclofenac doses</th>
<th>0 mg/kg</th>
<th>10 mg/kg</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (mmol/L)</td>
<td>6.24 ± 0.54</td>
<td>5.74 ± 1.17</td>
<td>4.22 ± 0.50</td>
<td>5.11 ± 0.28</td>
</tr>
<tr>
<td>Nitric oxide (µmol/L)</td>
<td>11.1 ± 0.5</td>
<td>14.1 ± 1.4</td>
<td>10.1 ± 1.1</td>
<td>12.6 ± 1.7</td>
</tr>
<tr>
<td>Vitamin C (µg/L)</td>
<td>10.0 ± 1.4</td>
<td>10.8 ± 1.5</td>
<td>24.8 ± 2.7</td>
<td>12.8 ± 2.5</td>
</tr>
<tr>
<td>β-carotene (µg/L)</td>
<td>5.6 ± 0.7</td>
<td>6.7 ± 0.6</td>
<td>6.5 ± 0.5</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>SOD (U/L)</td>
<td>37.6 ± 6.3</td>
<td>36.2 ± 1.6</td>
<td>51.2 ± 4.1</td>
<td>52.8 ± 3.2</td>
</tr>
</tbody>
</table>

MDA: malondialdehyde; SOD: superoxide dismutase.
Different superscripts a,b in the same row indicate significant difference between groups (p < 0.05).
increased but not significantly in all treated groups, the serum SOD activity tended to increase when rats received 50 and 100 mg/kg compared to the not treated controls and the vitamin C ascorbate was significantly and markedly increased in the group treated with 50 mg/kg diclofenac (p < 0.05).

Discussion

Diclofenac may induce some adverse effects similar to any other anti-inflammatory drugs [6, 32]. In this study, diclofenac did not significantly affect the stereological parameters of the kidney (p > 0.05), and the volumes of rat kidneys varied between 280 and 302 mm³. It was previously reported that the volumes of rat kidneys vary between 160 and 675 mm³ [23], and it is well known that the volumes of organs differ between species and with age. The cortex, medullar and pelvic fractions were 53-55%, 42-44% and 1.4-2.5%, respectively, in this study. It has been reported that the cortex, medullar and pelvic fractions of the kidney in different species were between 60 and 70%, 27 and 37% and 2.1 and 3.8%, respectively [5, 13, 23, 26]. In this research, the calculated mean coefficient of error (CE) of the cortex, medulla and pelvis were 1.35%, 1.57% and 14.1%, respectively. However, the CE is preferred to be lower than 5% [1, 29]. A high CE value in extremely small structures may be corrected by increasing the number of cross-sections or the point frequency in the point grid [5, 26, 29]. However, in the present study, slight renal stereological changes (decrease in medulla volume and increase in pelvic volume) were suspected in rats treated with 50 mg/kg compared to the controls of the same age.

In this study, uraemia, as an indicator of kidney damage, was significantly increased depending on dose. Diclofenac may cause kidney damage depending on dose and this effect may also be observed in rodents [3, 16, 33]. NSAIDs-induced nephrotoxicity may be due to the inhibitory effect of these drugs on prostaglandin synthesis, thus causing kidney ischemia [32].

In the current study, diclofenac at 10 mg/kg significantly increased the plasma fibrinogen concentration. The observed variance in this parameter may be within the reference range, as fibrinogen concentrations can raise to 1760 mg/L in healthy rats [36].

Diclofenac has not altered serum MDA concentrations in this study. However, diclofenac has been shown to increase MDA contents in liver and kidney tissue [30] while another NSAID, flunixin, decreased them in heart, kidney and spleen tissues [18]. The reason for the unchanged serum MDA concentrations after diclofenac administration in this study may be due to kidney damage which would be not enough severe to affect serum parameters and to lead to significant accumulation of labile compounds such as oxidant species. Nevertheless, a statistically significant increase in the vitamin C concentration was observed with 50 mg/kg diclofenac treatment, whereas the highest dose (100 mg/kg) has not affected this parameter. This result suggests that in fact, vitamin C concentrations would be within usual values in all groups. In addition, it has also been reported that NSAID administration did not altered vitamin C concentrations during infection [8, 35].

It is concluded that depending on the dose of diclofenac administered which increased urea concentrations, an indicator of kidney damage in serum, kidney damage cannot be evaluated using the stereological method or by serum oxidative stress parameters despite slight variations of them mainly observed with 50 mg/kg diclofenac. More severe damage in organs and/or tissues is necessary in order to evaluate damage using the above-mentioned stereological method or oxidative stress parameters.

Acknowledgments

The authors thank to Departments of Pharmacology (SU Veterinary Faculty, Konya) and Physiology (AKU, Veterinary Faculty, Afyon) for analyzing the blood parameters. A part of this study was performed in the Scientific and Technological Research Laboratories of Kirikkale University (KUBTAL).

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