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Can spesific biomarkers be used to enlighten the major mechanisms of acute high dose diclofenac sodium-related nephrotoxicity?

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Aim: The aim of this study was to examine the basic mechanisms that play a role in the acute nephrotoxicity caused by diclofenac sodium.
Materials and Methods: Only water was given to the control group; however, the diclofenac sodium group was group intoxicated by giving water-soluble, 240 mg/kg, oral single dose diclofenac sodium. After 24 hours, all animals were sacrificed and histopathological analyzes were performed. The levels of spesific biomarkers (Vascular endothelial growth factor [VEGF], Nuclear Factor-Kappa B [NF-kB], Matrix Metalloproteinase-9 [MMP-9], Metalloproteinase Tissue Inhibitor-1 [TIMP-1] and Carcinoembryonic antigen [CEA]) that may be related to the nephrotoxicity mechanism were evaluated.
Results: As a result of biochemical analysis we found that VEGF, TIMP-1, NF-kB and CEA levels were significantly higher and MMP-9 levels were significantly lower in diclofenac sodium group compared to control group. Nephrotoxicity related histopathological changes were observed in the sections of diclofenac sodium group.
Conclusion: This study has shown that the biomarkers we evaluated in the diclofenac sodium-induced acute high-dose intoxication model we created can help us to identify the nephrotoxicity and to explain the nephrotoxicity mechanism with the 3 main steps (the hemodynamic-related pathway, the inflammation-related pathway, and the oxidative stress-related pathway). With a simple version of this panel adapted to emergency departments, we may be able to diagnose diclofenac sodium-related nephrotoxicity.

Keywords: Diclofenac Sodium, Intoxication, Nephrotoxicity
Short Title in English: Mechanisms of acute diclofenac sodium-related nephrotoxicity

Abstract

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently preferred drugs with their analgesic and antipyretic properties. They are also widely used in acute and chronic inflammation [1]. Diclofenac sodium as a NSAID inhibits cyclooxygenase (COX) also known as prostaglandin endoperoxide synthase, reversible. Thus, it shows analgesic and anti-inflammatory activity by reducing the production of prostaglandin and thromboxane A2 [2]. In addition to being a non-selective (both COX-1 and COX-2 enzymes) inhibitor of COX, diclofenac sodium causes a decrease in the level of leukocyte intracellular free arachidonic acid. This situation is associated with the decrease in fatty acid levels released from the cell [3].

Similar to all NSAIDs on the market, diclofenac sodium is commonly available over the counter, which may be the main reason for the increase in overdose-related organ damage cases [4]. People with acute overdose due to diclofenac sodium may have serious clinical manifestations such as convulsions, metabolic acidosis, coma, acute renal failure and acute liver failure [5]. Although some progress has been made in understanding diclofenac sodium-related mechanisms of organ toxicity, the pathways in the process of renal toxicity have not been fully elucidated [6]. It is possible to classify the mechanisms that are thought to be associated with acute damage mainly under the following 3 headings;

hemodynamics associated pathway (renal perfusion disorder), immune-mediated inflammation of the interstitium (Acute interstitial nephritis or Glomerulopathy) and oxidative stress (OS) [6,7,8].

Vascular endothelial growth factor (VEGF) is a powerful endothelial cell mythogen that supports angiogenesis, increases vascular permeability and is chemotactic for monocytes. VEGF embryogenesis plays an important role in placenta growth, tumor growth, diabetes, wound healing, inflammatory response and tissue regeneration. VEGF, a glycoprotein of 45 kDa, is synthesized in podocytes and tubular epithelium. Antiprostaglandin activity and vasoconstriction due to non-selective COX enzyme inhibition cause hypoxia that increases VEGF. Although it is known that hypoxia is the main stimulation that increases VEGF synthesis; factors such as prostaglandins, mechanical stress, hyperglycemia and reactive oxygen species (ROS) are thought to cause an effect in the same direction [9]. Conversely, VEGF levels were found to decrease in antineoplastic-related nephrotoxicity with endothelial and glomerular damage [10].

Nuclear Factor-Kappa B (NF-kB) is a nuclear transcription factor that acts as an inflammatory regulator. NF-kB induces the release of inflammatory mediators that play an important role in the OS-related damage mechanism, especially TNF-α [11]. With this activation of NF-kB, it is thought that it plays a role in the formation of nephrotoxicity by leading the cell to apoptosis and death [12]. Likewise, in the diclofenac-induced intoxication model, it was observed that NF-kB activation and NF-kB-dependent proinflammatory cytokine production were induced in mice. This process led to apoptosis of kidney cells [13, 14]

Matrix Metalloproteinases (MMP) is a family of enzymes synthesized in the kidney from glomerular cells, mesenchymal cells and tubular epithelium [15]. The MMP enzyme family consists of 4 different groups: collagenases (MMP-1, -8, -13, -18), stromelins (MMP-3, -7, -10, -11, - 12), gelatinases (MMP-2, -9) and membrane type MMPs (MT-MMP-14, -15, -16, -17) [16]. Among these, MMP-9 has been shown to increase in experimentally created nephrotoxicity and renal fibrosis [17]. The main reason for this increase is that MMP-9 is induced by increased TNF-α in case of OS. Metalloproteinase Tissue Inhibitor-1 (TIMP-1) has been defined as the main inhibitor of MMP-9. MMP-9 and TIMP-1 are cytokines that play an important role in the formation of inflammation in the parenchymal organs. It is also known that the balance between these two biomarkers is disturbed in the disease settings accompanied by renal scarring, which is associated with drugs and diabetes, especially immunosuppressives [18]. Carcinoembryonic antigen (CEA) is a glycoprotein that plays a role in cell adhesion. CEA is normally produced in gastrointestinal tissue during fetal development, but production stops before birth. Today, it is used as a tumor marker for the diagnosis and treatment of adenocarcinoma and colorectal cancers [19]. It is usually found at very low levels in the blood of healthy adults. In contrast, CEA serum was found to be abnormally high in cases such as hepatic cirrhosis/inflammation, cardiometabolic diseases, pulmonary emphysema, rectal polyps, colon inflammation and chronic kidney

diseases [20-22]. The increase in CEA in these non-neoplastic settings is thought to be associated with inflammation [23].

In our study, we aimed to create a setting of oral, high dose diclofenac sodium intoxication for suicidal purposes that we often encounter in the emergency departments. In the literature, nephrotoxicity patients due to chronic and therapeutic diclofenac sodium intake were examined but the mechanism of nephrotoxicity was not clearly explained [24,25]. Our main goal in our study was to evaluate the roles of specific biomarkers in the diagnosis of diclofenac sodium-induced nephrotoxicity and to define the main steps of the nephrotoxicity mechanism using these biomarkers. For this purpose, we evaluated the levels of biomarkers (VEGF, NF-kB, MMP-9, TIMP-1, and CEA) within 24 hours that may be related to the nephrotoxicity mechanism.

Methods

The study was started with the permission obtained from Kafkas University Local Ethics Committee for Animal Experiments (KAU-HADYEK/2020-025). A total of 14, 4-6-month-old female wistar albino breed rats were used in the study. All animals were fed as ad libitum. The rats were housed in an environment with an automatically adjusted light with a 12/12 hours cycle of light/dark. Before starting work, the rats were kept for seven days for the adaptation process. After the adaptation process, the rats were divided into two groups, with 7 rats in each group. We referred to the study of Dass et al. for toxication model [26]. Diclofenac sodium tablet (Dicloron®, Deva 50mg tablet) was used in this study.

Groups;

Control Group: Only water was given orally.

Diclofenac sodium Group: The group intoxicated by giving water-soluble, 240 mg/kg, oral single dose diclofenac sodium.

24 hours after the application of diclofenac sodium, kidney tissue samples required for analysis were collected after animals were sacrificed through the use of cervical dislocation under anesthesia [ketamine hydrochloride (75 mg/kg,) (Ketalar®, Pfizer), and xylazine (15 mg/kg) (Rompun®, Bayer) intramuscular] in accordance with ethical rules. After the kidney tissues were homogenized, they were centrifuged at 3000 rpm and their homogenate was separated. The separated homogenates were placed in Ependorf tubes and stored at -20 C° until the time of analysis.

Biochemical analysis

Tissue samples were homogenized with phosphate buffer (pH 7.4) and then, centrifuged at 3000 RPM for five minutes. The homogenates obtained were stored at−20 °C until the time of analysis. Total protein analysis and gram protein calculation was performed according to the method developed by Lowry et

al. from the homogenates of kidney tissue [27]. VEGF, NF-kB, MMP-9, TIMP-1, and CEA analyses were done according to the kit procedure using the commercial ELISA kit (ELISA- YL Biotech Company, Shanghai). The data taken after the ELISA measurement were calculated with the previously determined protein analysis data and the data were given in grams of protein. Serum creatinine values were measured at the end of 24 hours to evaluate renal functions. To explain the nephrotoxicity mechanisms associated with diclofenac sodium, biomarkers were associated with 3 basic steps: hemodynamics-related pathway, inflammation-related pathway, and OS-related pathway.

Histopathological analysis

Renal tissue samples collected from the experimental rats were fixed in neutral buffered 10% formalin. Following routine tissue follow-up procedures, sections with a thickness of 4 μm were taken from the prepared paraffin blocks. Haematoxylin and eosin (H&E) stain was applied to the sections to identify the histopathological changes.

Statistical Analyses

Unpaired t test analysis was conducted for all the biochemical parameters to test whether there is a difference between the two groups. An experiment-wise p-value of ≤ 0.05 was deemed to be statistically significant throughout the study. All the analyses were conducted using GraphPad 8.1 (San Diego, CA, USA).

Results

Biochemical results

VEGF, NF-kB, MMP-9, TIMP-1, and CEA parameters determined as a result of the analyses were given in Figure 1-A, B, C, D, E respectively. VEGF levels, which played an important role in renal perfusion, were higher in the diclofenac sodium group compared to the control group. We found that this difference between the two groups was statistically significant ($p=0.001$). When MMP-9 and its inhibitor TIMP-1, which play a role in the OS-related damage mechanism, were examined; we found that the mean MMP-9 levels were lower in the diclofenac sodium group compared to the control group. This difference between two groups was statistically significant ($p=0.003$). TIMP-1 levels were statistically significantly higher in the diclofenac sodium group than in the control group ($p=0.007$). We found that average levels of NF-kB, another OS-related biomarker, were also higher in the diclofenac sodium group. This difference was statistically significant ($p=0.006$). We found that CEA levels, which are biomarkers associated with inflammation, were statistically significantly higher in the diclofenac sodium group than in the control group ($p=0.002$). We also found that the creatinine values measured at the end of the experiment in the diclofenac sodium group were significantly higher than the control group. (the mean difference: 0.48, 95% CI: 0.39-0.57; $p<0.001$).

Histopathological results

In histopathological examinations, no pathological finding was observed in the sections belonging to the control group, except mild congestion and erythrocyte extravasation, which were thought to be related with resection. Additional to mild congestion, interstitial edema and mixed type inflammatory infiltrate containing interstitial neutrophils and lymphocytes were observed in kidney histopathology of rats induced with diclofenac sodium. Focal tubular damage and degenerative changes were also detected in this histopathological sections of diclofenac sodium group (Figure 2).

Discussion

The primary outcome of this study was to explain the basic mechanisms that play a role in the nephrotoxicity model due to acute high dose diclofenac sodium intake that we have created in rats. For this purpose, we evaluated three main mechanisms which are the most associated with NSAID nephrotoxicity; the hemodynamic-related pathway (renal perfusion impairment), the inflammation-related pathway, and the OS-related pathway. In order to evaluate the role of these classified mechanisms in diclofenac sodium nephrotoxicity, we investigated the biomarkers associated with these pathways. We examined the level changes of these biomarkers in a short period of 24 hours, which provide easy use in emergency services because they are produced as a kit.

Diclofenac sodium, as a NSAID, is a highly used drug that is sold as analgesic and as antipyretic, both as prescription drugs and over the counter purchases [3]. This is also the main reason why we often encounter diclofenac sodium-related toxicity cases in emergency departments. Although the potential to cause multiorgan toxicity of diclofenac sodium is known, its mechanisms remain unknown [8]. In studies in the literature, it was agreed that the main cause of the diclofenac sodium-related acute kidney injury setting may be perfusion disorder developed as a result of vasoconstriction [4, 28]. This condition was associated with antiprostaglandin activity caused by non-selective COX enzyme inhibition, which is the most fundamental feature of diclofenac sodium [29]. In this mechanism, defined as 'hemodynamics-related pathway', hypoxia is the main stimulant, and the role of VEGF released in response to hypoxia has often been studied in the literature [30, 31]. Various growth factors and cytokines, such as epidermal growth factor, are active in the expression of VEGF in transformative growth factor β (TGF-β), platelet-induced growth factor (PDGF), insulin-like growth factor I (IGF-I), angiotensin II, interleukin-1 (IL-1) and IL-6. VEGF can also be induced by prostaglandins, mechanical stress, hyperglycemia, protein kinase C (PKC) and ROS [32]. In renal tissue with diclofenac sodium-induced nephrotoxicity, the main cause of the increase in VEGF has been associated with hypoxia [33]. There are studies in the literature showing that there may be an increase in VEGF levels in drug-related nephrotoxicity models [34-37]. However, there is no NSAID-related acute high dose toxicity data in the literature. Similar to our study, Kaur and his colleagues applied diclofenac sodium at a dose of 8mg/kg per oral (18 weeks) [38]. At this dose of diclofenac sodium intake, VEGF levels were found to be low,

similar to the control group. In our study, we found that VEGF levels were significantly higher in acute diclofenac sodium toxicity group. We believe that this increase in VEGF levels in our study may be a guide for understanding the main "hemodynamic and circulatory-related" toxicity mechanism in diclofenac sodium-related nephrotoxicity.

Cytochrome P450 mediated metabolization of diclofenac sodium is an inducer for ROS production [25]. Increased ROS triggers the formation of OS reactions, thus damaging cellular macromolecules and leading to cell death [39]. Oxidative stress is one of the main pathways in the mechanism of kidney damage. There are studies indicating that there is up-regulation in the activity of NF-kB in many cells due to damage caused by the OS-related ROS response [40, 41]. In our study, we found that NF-kB increased significantly in the group developing nephrotoxicity due to acute diclofenac sodium. In the literature, we did not encounter a study examining NF-kB levels in acute high-dose diclofenac sodium-induced nephrotoxicity model similar to our study. However, a study of different nephrotoxicity models linked to cadmium and chemotherapeutics has shown that levels of NF-kB increase in toxicity groups [42].

Other biomarkers we examined in our study due to their activities in the OS-related nephrotoxicity mechanism were MMP -9 and its inhibitor TIMP-1. It has been stated in the literature that TNF-α, one of the cytokines known as the OS marker and found to have increased in the diclofenac-induced renal toxicity, induced the release of MMP-9 [24]. It can be observed that MMP-9 levels vary in different ways as the clinical situation of acute kidney injury changes. In their study, Caron et al. found that MMP-9 levels increased in AKI due to ischemia perfusion damage, while TIMP-1 levels decreased [43]. However, this picture can be reversed in chronic kidney diseases associated with glomerulosclerosis or tubulointerstitial fibrosis. Sharma et al. found that MMP-9 levels decreased and TIMP-1 levels increased in the unilateral ureteral obstruction model they created in their study [44]. Unlike the literature, we found that MMP-9 levels were low and TIMP-1 levels were significantly higher in the nephrotoxicity-related AKI model we created in our study.

Inflammation is known to be one of the main mechanisms involved in the development of acute kidney injury process. While nephrotoxic drugs generally cause inflammation in the glomerulus, proximal tubules and surrounding cellular matrix, it is known that in NSAID-induced nephrotoxicity acute interstitial nephritis also play a role in this process [45]. However, this process, which is associated with drug-related inflammation in the literature, has often been associated with chronic NSAID usage [46]. CEA, which we examined in our study, is widely used as a tumor marker. However, there have been recent studies that CEA levels are also high in low-grade chronic inflammation-related conditions such as atherosclerosis, type 2 diabetes and metabolic syndrome [47, 48]. It is thought that this low-grade chronic inflammation process, which is also seen in chronic kidney diseases, may explain why CEA shows high blood levels [49]. In our study, we believe that high CEA levels are associated with

'inflammation-related pathways' in diclofenac sodium-related nephrotoxicity. However, the increase in CEA levels we found occurred in a short period of 24 hours, and it occurred in a much shorter time compared to the chronic inflammation process mentioned in the literature.

Conclusion

This study has shown that the biomarkers we evaluated in the diclofenac sodium-induced acute high-dose intoxication model we created can help us to identify the nephrotoxicity and to explain the nephrotoxicity mechanism with the 3 main steps. Of these biomarkers, a statistically significant decrease has been observed in the MMP-9 levels in the diclofenac sodium group compared to the control group whereas there has been a significant increase in the VEGF, TIMP-1, NF-kB and CEA levels in the diclofenac sodium group compared to the control group. With a simple version of this panel adapted to emergency departments, we may be able to diagnose diclofenac sodium-related nephrotoxicity and refer patients to appropriate centers. In addition, in the light of the parameters we examine, we think that understanding the basic mechanisms that play a role in the diclofenac sodium-related kidney injury can be a guide in the research for specific treatments.

Declaration of Conflicting Interest: The Authors declare that there is/are not conflict(s) of interest.

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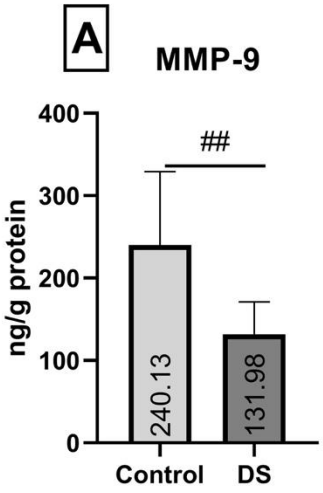

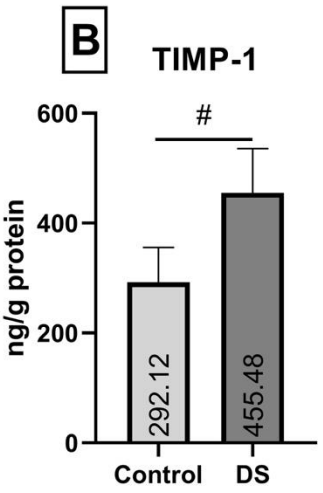

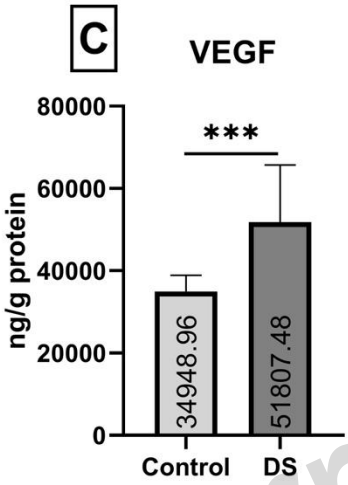

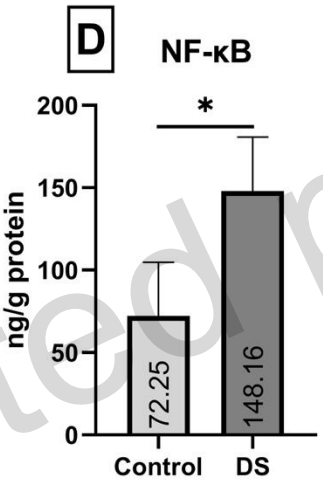

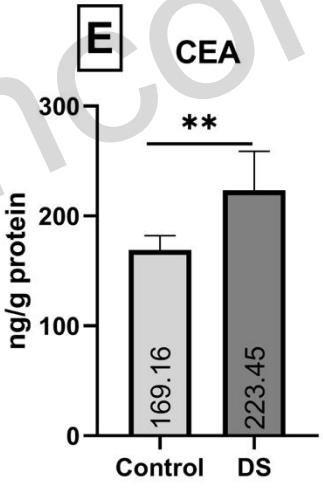

Figure 1. Means and Standarts Errors of the two groups for biochemical parameters (A, B, C, D, E). *p=0.006, **p=0.002, ***p=0.001, #p=0.007, ##p=0.003.

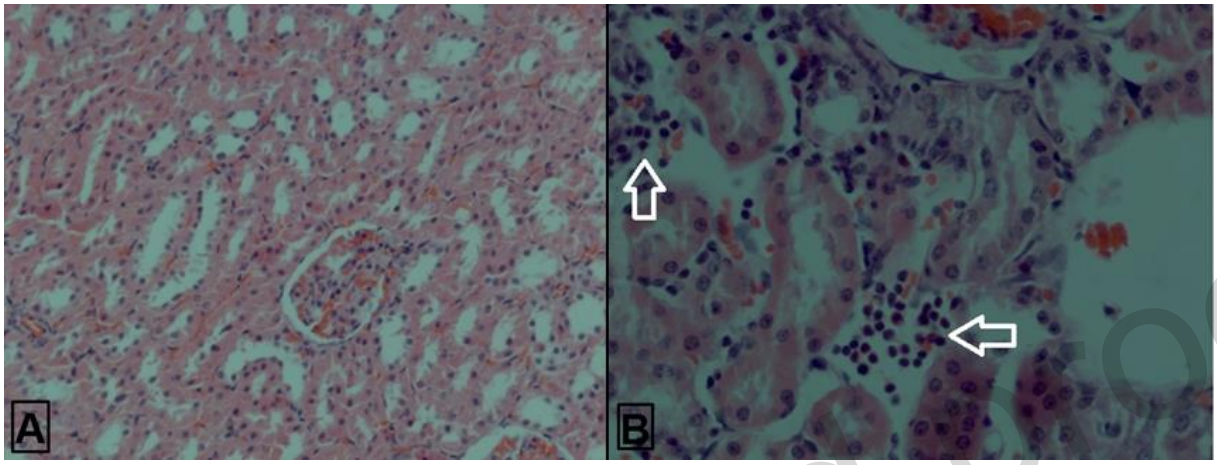

Figure 2. Kidney histopathology: A) Control group shows normal kidney morphology with minimal congestion and erythrocyte extravasation (H & E staining, x200). B) Diclofenac Sodium induced nephrotoxicity group shows interstitial edema and mixed type inflammatory infiltrate containing interstitial neutrophils and lymphocytes (H & E staining, x400). Focal tubular damage and degenerative changes were also detected in this histopathological sections (white arrows).