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Renal damage: a possible role of diclofenac induced injury associated with altered acid phosphatase and collagen content in Balb/c mice

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ABSTRACT: Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most popular classes of drugs with anti-inflammatory, analgesic and antipyretic effects. NSAIDs interfere with certain metabolic pathways which are involved in formation of prostaglandins. The prostaglandins have been assigned many physiological roles including renal metabolism. Diclofenac, a NSAID, is prescribed for its anti-inflammatory and analgesic actions during different type of bone and muscle injuries. We hypothesized that other than treating different ailments, the drug can have some detrimental effects on kidney by possibly suppressing renal prostaglandins. To test this, Balb/c male mice were exposed to diclofenac sodium at dose rate of 10 mg/ kg/ body wt from 10-30 days. Collagen content of kidney was analyzed by estimating hydroxyproline content. Acid phosphatase was localized in renal tissue histochemically. Further, acid phosphatase was also assayed biochemically. A noteworthy hike in collagen concentration was noticed in diclofenac treated kidney throughout the study period ($p < 0.05$). Histochemical sections revealed initial decrease in acid phosphatase content with some localized areas of enzyme activity. Change in acid phosphatase content was accompanied by many pathological abnormalities. These findings were corroborated by biochemical results too.

Key words: Diclofenac, kidney, Acid phosphatase, Hydroxyproline

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INTRODUCTION

Non steroidal drugs are anti-inflammatory drugs along with being analgesics and antipyretics. The prime advantage of non steroidals is that they are non-narcotic. These drugs are prescribed for different type of muscle and bone problems. The term "nonsteroidal" is used to distinguish these drugs from steroids, which, amongst a broad range of other effects, have a similar eicosanoid-depressing, anti-inflammatory action. Eicosanoids are signaling molecules formed by oxidation of essential fatty acids (EFAs). These control a number of physiological functions, such as inflammatory processes and defense mechanism, and also act as messengers in the central nervous system. There are four families of eicosanoids: prostaglandins, prostacyclins, thromboxanes and leukotrienes. These are either derived from ω-3 or ω-6 essential fatty acids (EFAs). Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic to reduce pain in conditions as common as a tooth ache as well as arthritis and acute injury. Diclofenac, is an acetic acid derivative having molecular weight of 296.148 g/mol, while its IUPAC name is represented as 2-(2-(2,6-dichlorophenyl) amino) phenyl) acetic acid which gives this drug its common name, diclofenac. Diclofenac was first introduced in the UK in 1979[1].
Diclofenac inhibits isoenzymes, COX-1 and COX-2, by blocking arachidonate binding resulting in analgesic, antipyretic and anti-inflammatory effects[2]. The enzymes COX-1 and COX-2 catalyze the conversion of arachidonic acid to prostaglandin G2 (PGG2), the first step of the synthesis of prostaglandins (PG) and thrombaxanes that are involved in rapid physiological responses. Veterinary use of diclofenac in South Asia has resulted in the collapse of populations of three vulture species of the genus *Gyps* to the most severe category of global extinction risk[3]. Vultures exposed to diclofenac while scavenging on livestock treated with the drug shortly before death contained residues of diclofenac in their kidneys[4]. Concern about this issue led the Indian Government to announce a ban on the veterinary

use of diclofenac. Non selective as well as selective NSAIDs carry similar renal risks due to inhibition of COX-2 which is constitutively expressed in the kidney[5]. An important study has established that COX-2 enzymes are expressed at multiple nephron sites in the mammalian kidney including the cortical thick ascending limb, macula densa, medullary interstitial cells and the endothelium of arteries and veins as well as glomerular podocytes[6]. Thus, it is possible that inhibition of COX-2 enzymes may be associated with alterations in renal functions.

PGs (prostaglandins) have cytoprotective properties in the gastrointestinal tract and control renal functions. Most common renal toxicity, called reversible nephropathy is the result of inhibition of renal prostaglandins which is characterized by elevation of serum urea, creatinine, potassium and an increase in body weight due to fluid retention[7]. Furthermore, renal prostaglandins are produced in kidney in response to vasoconstriction caused by angiotensin II, norepinephrine and vasopressin. Vasodialatory renal prostaglandins especially prostaglandin E2 and prostacyclin act to maintain renal blood flow and glomerular filtration rate. Non-steroidal anti-inflammatory drugs exert their toxic effects on kidney through their inhibition of the renal prostaglandin (PG) synthesis, especially in cases where the systemic haemodynamic conditions are compromised. Kidneys being organs of excretion are affected by the toxic metabolites of the drugs. The measurement of different enzymes in tissues and body fluids serves as an index for disease diagnosis where acid phosphatase (ACPase) is a marker enzyme for lysosomal membrane.[8] Lysosomal acid phosphatase (3.1.3.2) is an orthophosphoric monoesterase of the endosomal/lysosomal compartment[9]. Besides ACPase, collagen also plays an important role in pathogenicity of kidney where renal collagen has been documented as a marker of progression of kidney disease[10]. The goals of this project were to evaluate toxicity of diclofenac on mice kidney in terms of histoarchitecture, acid phosphatase concentration and collagen profile.

MATERIAL AND METHODS

1. Animals and drug: Adult male swiss albino mice of Balb-c strain, weighing 22-24g were procured from the Central Research Institute, Kasauli (H.P.), India. All the experimental procedures were conducted strictly under the guidelines of the Institutional Animal Ethics Committee. The mice were maintained hygienically in the well ventilated room of animal house of the Department of Biosciences of H.P. University with 12 hrs day light and a temperature of 25 ± 2ºC. These were given commercial feed (Hindustan Lever Ltd.) and water *ad libitium.*

The drug, diclofenac sodium was purchased from Sigma Aldrich Co., USA and stock solution was made in distilled water. Further; dilutions were done according to the body weight records of the animals. The animals were divided into two broad groups: control group and experimental group. Each group was further divided into three subgroups: Day 10, Day 20 and Day 30 groups. Animals in the experimental group were administered diclofenac sodium intramuscularly at dose rate of 10mg /kg bwt. /day for 10, 20 and 30 days while that of the control group received normal saline. Mice were sacrificed by cervical dislocation. Kidneys were removed and cleared of fats and weighed.

2. Histochemical localization of Acid phosphatase activity: Acid phosphatase activity in kidney was accomplished by the method of Barka and Anderson (1962) in the fresh frozen hand cut sections[11]. Thin tissue slices were fixed for 24 hrs at 4°C in calcium formol. Further, fresh frozen sections were transferred onto the albuminized slides and were air dried for 2 hrs. These slides were incubated in the incubation medium (hexazonium pararosanilin solution containing pararosanilin hydrochloride and 4% sodium nitrite; and substrate solution having α-naphthyl phosphate in Michaelis veronal acetate buffer) for 30 minutes at room temperature. After incubation, rinsed in distilled water, dehydrated quickly in alcohol, cleared in xylene and mounted in DPX. Finally, the slides were photographed for observations.

3. Biochemical studies:

3.1 Acid phosphatase activity: A weighed amount of tissue was homogenized in ice cold distilled water which was centrifuged at 4000 rpm for 20 minutes at 4°C. The supernatant was used for the enzyme assay. Acid phosphatase activity in kidney was analyzed as per methods described earlier with some modifications[12,13]. The enzyme was assayed in supernatant in the incubation mixture containing sodium-

β-glcerophosphate, sodium barbitone and acetic acid having pH 4.9. Briefly, supernatant and incubation mixture were incubated in a test tube at 37°C for one hr. The reaction was stopped by adding 10% trichloroacetic acid (TCA). The resultant mixture was centrifuged for 10 minutes at 3000 rpm so as to get a clear supernatant. To estimate phosphate content in the supernatant, ferrous suphate ammonium molybdate reagent and ANSA and distilled water was added to supernatant. After incubating this mixture at 100°C for five minutes, the optical density was read at 650 nm in spectrophotometer. Total amount of phosphate released was calculated by plotting a standard curve of different known concentrations of KH_2PO_4 against optical density.

3.2 Protein estimation: Protein was estimated as per the method of Lowry et al. (1955)[14].

3.3 Collagen estimation: Total collagen concentration in kidney was determined by measuring hydroxyproline as per the method described elsewhere[15]. The tissue homogenate was prepared in 5 M HCl. The homogenate was incubated at 100°C for 4 hours followed by decolorizing by activated charcoal. Samples were gravity filtered and rinsed with 800 µM NaOH and distilled water. Tissue extract containing hydroxyproline was oxidized with oxidant solution containing chloramine-T for 20 minutes at room temperature which was followed by addition of Ehrlich's reagent containing p-dimethylamino-benzaldehyde. The reaction mixture was heated for 20 minutes at 60°C. The test tubes containing the digests were left as such at room temperature for 60-90 minutes. These test tubes were centrifuged at 3500 x g for 10 minutes and optical density was recorded at 550 nm. A standard calibration curve for 4-hydroxyproline (SRL Pvt., Bombay) was plotted and the concentration of 4-hydroxyproline in µg/ mg fresh tissue weight was determined. Assuming that collagen contained 14% of 4-hydroxyproline, a conversion factor of 7.14 was used to convert 4-hydroxyproline into collagen[16].

4. Statistical analysis: Data is represented as statistical mean ± SEM. Student's two tailed t-test was used to compare control and treated groups of each stage. The minimum level of significance was at p value equal to 0.05.

RESULTS AND DISCUSSION

Tissue sections of mice kidney used for acid phosphatase activity from control animals (Figure1a) revealed intense enzyme activity in the glomerulus as well as around the tubules, however, activity declined after 10 days of diclofenac treatment group. There was overall decrease in the color intensity but the intact epithelial lining of the glomerulus as well as glomerulus itself was strongly enzyme positive along with certain noticeable histopathological alterations such as increased urinary space, shrunken glomerulus etc. (Figure1b). The ACPase activity decreased in mice kidney as compared to the control after 20 days; intense colour reaction was observed in the glomerulus which seemed to have been swollen almost occupying the whole of urinary space. Few of the glomeruli having high enzyme activity in their epithelial lining were shrunken with increased urinary space (Figure 1 c, d). Amongst other pathological abnormalities, edema was prevalent in enzyme poor tubules at day 10 and day 20 stages in comparison to control (Figure 1 b, d). After one month of diclofenac treatment, acid phosphatase content increased as compared to the control group along with further deterioration of the structural organization of the mice kidney (Figure 1 e, f). The degenerated epithelial lining of the glomerulus exhibited poor distribution of ACPase activity while the intact epithelial boundary and the intact tubules were extremely rich in red precipitate distribution. The degenerating tubules in the immediate vicinity of the glomerulus appeared almost enzyme free in comparison to the control sections (Figure 1 f).

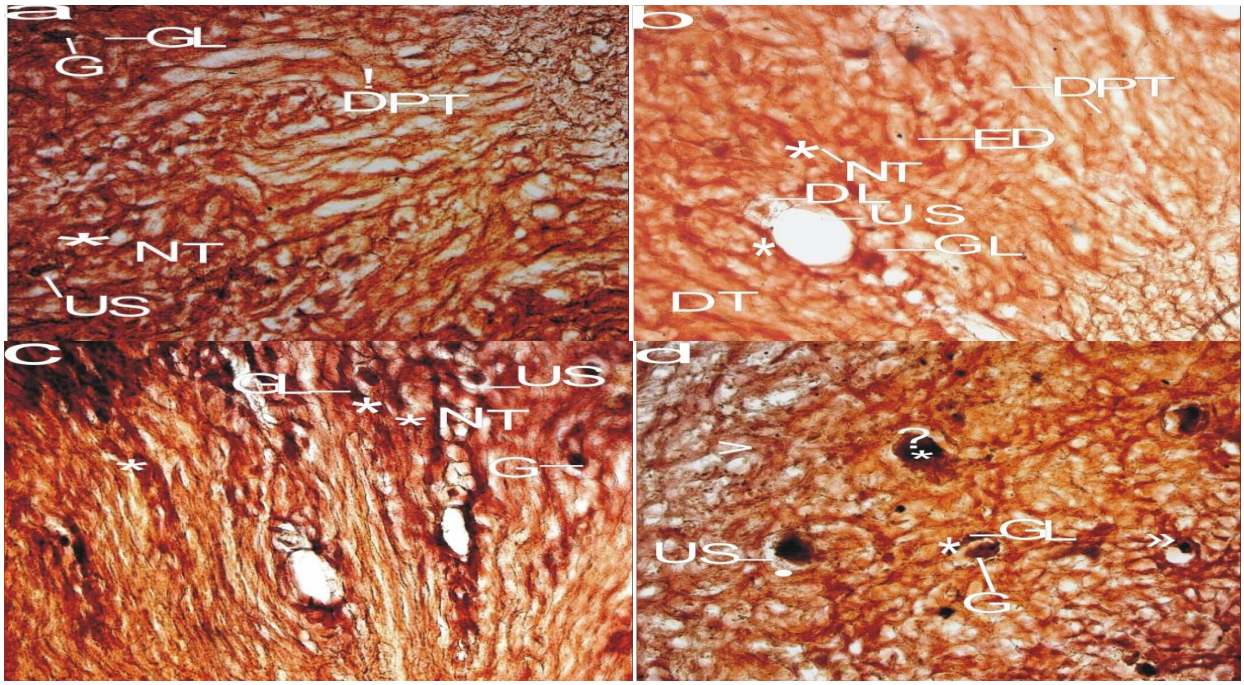

Figure 1 (a): Normal mice kidney at 10 days stage with glomerular lining (GL), glomerulus (G) with normal urinary space (US) and nephric tubules (NT) exhibiting intense acid phosphatase activity (*). Distal tubules (DT) poor in enzyme activity X100.
(b): Diclofenac treated kidney at 10 days showing overall decrease in ACPase activity. Intense enzyme positivity (*) in intact epithelial lining (GL) and intact nephric tubules (NT) is noticed. Degenerating lining (DL), degenerating tubules (DPT), edematous (ED) tubules with moderate and distal tubules (DPT) with negligible ACPase are seen X100.
(c): T.S. of normal mice kidney demonstrating enzyme positive areas (*) around normal glomerulus (G) with normal urinary space (US) lined by glomerular lining (GL) and nephric tubules (NT) X100.
(d): T.S. of mice kidney after 20 days of Diclofenac treatment exhibiting decreased ACPase activity in degenerating glomerular epithelium (o). Intense enzyme positivity (*) in glomerulus (G) and intact glomerular epithelium (GL); edema (>); shrunken glomerulus (>>) with increased urinary space (US) is noticeable X100.

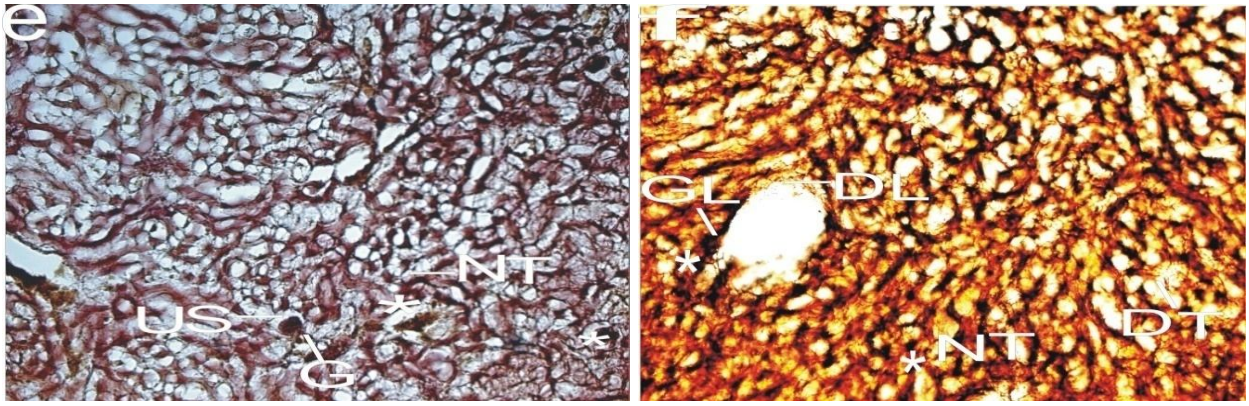

Figure (e): Normal kidney section at day 30 group demonstrating ACPase positive (*) glomerulus (G) with normal urinary space (US), and nephric tubules (NT) X100.
(f): DS treated mice kidney at 30 days depicting overall increased activity of ACPase in intact glomerular lining (GL) and normal tubules (NT). Widened urinary space (US) surrounded by partially necrosed epithelial lining (DL) with lesser APase activity, degenerated tubules (DT) with decreased ACPase activity are also visualized X100.

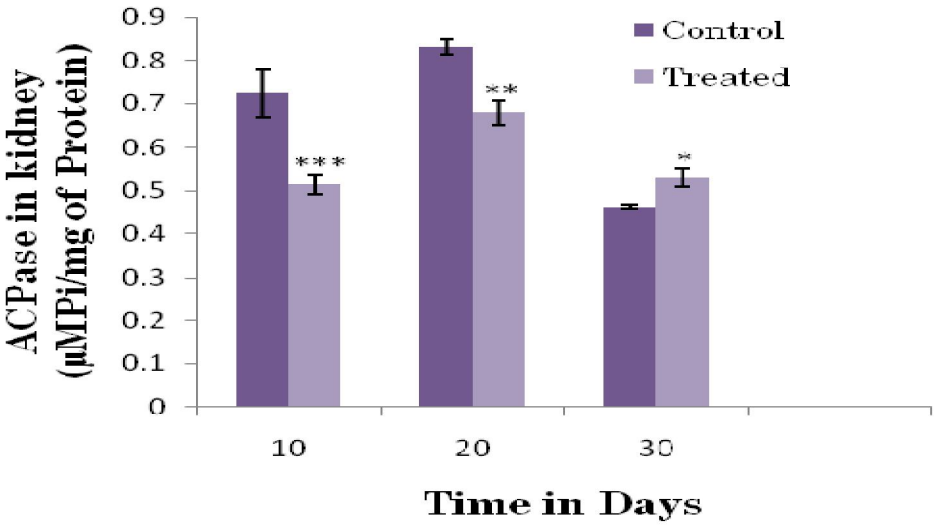

Figure 2: Acid phosphatase activity in control and diclofenac administered mice kidney from 10-30 days period. Values are mean ± SEM (*P<0.01, **P<0.02, *P<0.05, n= 6).**

Biochemical analysis for ACPase activity in kidney documented a diverse trend after diclofenac administration. Enzyme level diminished notably in diclofenac administered animals after 10 days to 0.513 ± 0.022 µM Pi/mg of kidney proteins in comparison to control group (0.723 ± 0.055 µM Pi/mg of kidney proteins; P<0.01). Similar decline in enzyme activity was noticed in animals after 20 days where diclofenac treated animals had 0.678 ± 0.028 µM Pi/mg as compared to the control animals (0.831 ± 0.017 µM Pi/mg) which was significant at P<0.02. Surprisingly, reversal of the previous results was noticed after 30 days treatment with diclofenac where a noteworthy increase (P<0.05) in the ACPase activity was recorded in treated animals (0.528 ± 0.020 µM Pi/mg of proteins) as that of the normal (0.463 ± 0.004 µM Pi/mg of proteins); Figure 2.

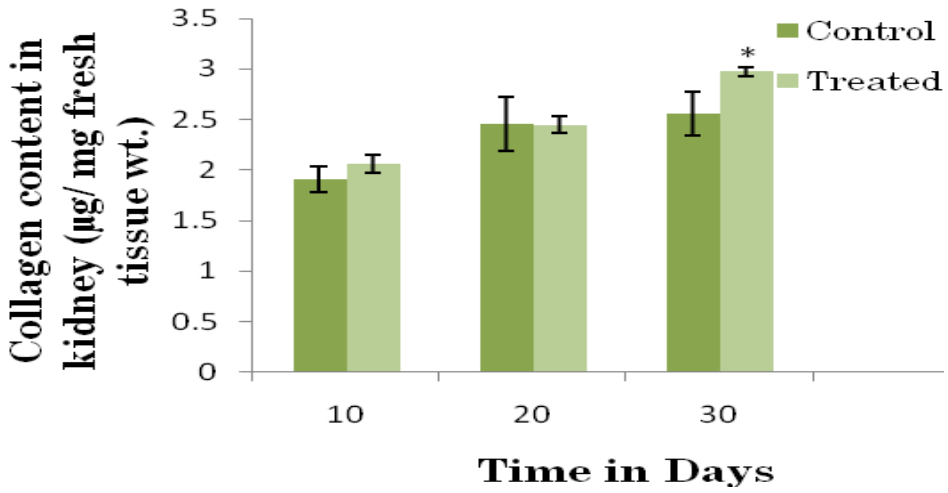

Figure 3: Change in collagen content (µg/ mg of fresh tissue weight) of diclofenac administered kidney of mice in comparison to control. Values are mean ± SEM; *P<0.05, n =6.

Hydroxyproline assay was performed to estimate collagen concentration. Diclofenac administration resulted in an increase in collagen concentration in mice kidney initially after 10 days as well as after one month of drug therapy. The collagen concentration was reported to show marginal rise (P>0.05) in the diclofenac treated animals for 10 days (2.056 ± 0.09 µg / mg of fresh tissue wt) in comparison to control

(1.909 ± 0.13 µg / mg of fresh tissue wt). After 20 days of drug treatment, there was no change in the collagen concentration of treated animals (2.449 ± 0.08 µg / mg of fresh tissue wt) as compared to the normal (2.458 ± 0.27 µg / mg of fresh tissue wt). However, after 30 days of diclofenac therapy, noteworthy hike in the collagen content of kidney was documented in the treated group (2.974 ± 0.044 µg / mg of fresh tissue wt) as compared to control group (2.557 ± 0.22 µg / mg of fresh tissue wt), $P<0.05$; Figure3.

NSAIDs are one of the most popular classes of drugs with anti-inflammatory, analgesic, and antipyretic effects. All these effects are mediated by inhibition of the biosynthesis of prostaglandins. Further, diclofenac is a common NSAID which is traditionally used as an anti-inflammatory agent and pain reliever for the pains associated with arthritis, osteoarthritis and ankolysing spondylitis. NSAIDs are amongst the most commonly used drugs for acute and chronic pain in clinical dentistry. Recently, the use of NSAIDs has been linked as chemopreventive measure against colorectal cancer and to lesser extent breast and lung cancer[17,18]. Diclofenac sodium is reported to exert hepatotoxic, nephrotoxic, and visceral gout inducing potentials in White Leghorn birds, especially at higher dose[19]. The effect of PGs on the renal vasculature is primarily vasodilatory. Owing to the inhibition of cyclooxygenase enzyme in PG synthesis, the synthesis of vasodilatory PG is diminished and severe renal vasoconstriction may develop resulting in acute renal failure[20]. The toxicity of diclofenac has become of ample interest due to its clinical use and for the study of the mechanisms of nephrotoxicity, renal dysfunction, hematotoxicity and hypersensitivity reactions. The drug in question has been a prime suspect in causing cell injury due to its ability to covalently bind to macromolecules in situations where intracellular levels of NADH, NADPH, GSH, and other reducing agents are very low[21]. Diclofenac is eliminated following conjugation by sulfate and glucuronic acid. Excretion and accumulation of conjugates have been correlated to renal function and end-stage renal disease. Usually after detoxification pathways are impaired, and if metabolites are formed in excessive amounts, they can cause cell injury leading to cell death.

Veterinary use of diclofenac has been reported to be a major cause of the catastrophic collapse of *Gyps* vulture populations in the Indian subcontinent[3]. Diclofenac is a widely available drug in the Indian subcontinent, where it is used for the symptomatic treatment and management of inflammation, fever, and/or pain associated with disease or injury in domestic livestock. It has been reported by workers that vultures got exposed to the drug when they consumed carcasses of cattle which were treated with diclofenac shortly before death[22]. Furthermore, following experimental exposure to diclofenac or diclofenac-contaminated tissues, *Gyps* vultures died within days from kidney failure with clinical signs of extensive visceral gout. Diclofenac-associated renal failure has been also reported in humans with prolonged exposure or with pre-existing renal disease although these reports do not describe renal pathology[23].

Our results document that chronic administration of diclofenac sodium brings about a number of structural modifications in kidney. Inhibition of prostaglandin synthesis has been reported to be the initiating factor for the pathological changes in kidney tissue[24]. During the present study, a number of histopathological changes in kidney due to diclofenac administration such as glomerular epithelial cell necrosis, tubular cell degeneration, edema of the tubules and infiltration of inflammatory cells were observed. Earlier, diclofenac-treated birds were reported to show histopathological lesions in liver and kidney[25]. Two different forms of acute renal failure have been shown to develop as a result of NSAID administration viz. haemodynamically-mediated ischaemic nephropathy and acute interstitial nephritis[26]. Edema of the nephric tubules in the diclofenac treated kidneys observed during this investigation is proposed to be a consequence of water retention due to hindrance in filtration process. Earlier, workers have reported that reduced production of PGs also decreases the glomerular filtration leading to water retention, hypertension and, in some cases, to renal failure; especially in patients with reduced renal function[27]. A possible explanation for the preferential necrosis of the proximal convoluted tubules caused by diclofenac may be related to the high metabolic activity of these cells which would make them more sensitive to hypoxia than cells in the distal or collecting tubules that are less metabolically active[28].

Diclofenac is a weak acidic drug having a tendency of binding to cell membrane as well as to plasma proteins *in vivo*[29]. As plasma proteins are not filtered through glomerulus, high concentration of

radiolabelled diclofenac was detected in kidney[30]. Free diclofenac and its metabolites might be taken up by the kidney through organic anion transporter (OAT) on membrane of the renal proximal tubular cells[31]. It was evident that diclofenac decreased the transport of uric acid by interfering with the organic anion transporter in both chicken and vulture cells[32]. Furthermore, general toxicity resulted from the loss of an intracellular anti-oxidant (uric acid), the additional production of ROS (reactive oxygen species) from the long-term exposure to diclofenac enhanced the overall oxidative stress experience by the RTE (renal tubular epithelial) cells i.e. they lost an anti-oxidant while being exposed to greater ROS production. Local acid microenvironment caused by inflammation might favour uptake of the neutral chemical form of diclofenac into the cell membrane and into the cytoplasm of cell, where binding to COX-2 enzymes may occur [33].

In histochemical studies, a decrease after 10 and 20 days but elevated acid phosphatase activity after 30 days of diclofenac treatment was documented. Biochemical analysis of acid phosphatase in kidney showed similar trend from 10 to 20 days of drug therapy which declined from 29.04% to 21.94% respectively. Our results are in agreement to earlier works on rats treated with diclofenac where acid phosphatase activity declined in liver and kidney but increased in testes[21]. Lysosomal enzymes association to pathological changes in different tissues like liver, kidney and spleen in adjuvant induced arthritic rats has been documented earlier[34]. It is hypothesized that diclofenac tends to stabilize lysosomal membrane thus inhbits the activity of ACPase. Later on due to breakdown of lysosomal membrane, concentration of ACPase hiked accompanied by pathological changes. Furthermore, the increased acid phosphatase activity has been correlated to tissue damage by radiation due to rupture of lysosomes[35]. The elevated levels of ACPase during last stage of the investigation could be related to increased functional activity of kidney itself to compromise oxidative stress induced by diclofenac. The hyperactivity of ACPase would consume phosphate esters of the tissue which could threaten cell life leading to pathophysiological changes. Previous studies showed that the activity pattern of rat liver and kidney enzymes change following administration of xenobiotics[36].

Collagen content in kidney after diclofenac administration augmented after 10 days (7.70%) as well as 30 days (16.31%), whereas no significant change was reported after 20 days. The hydroxyproline concentration in the early postoperative period following experimental colonic anastomosis was documented to increase after piroxicam, ibuprofen and flurbiprofen treatment[37]. Dissimilar results among various studies show that different NSAIDs can affect collagen production and consumption in different ways. The high mean hydroxyproline level at the early postoperative period on metamizole treatment was recorded by researchers[38]. Previously workers reported that endocytic uptake of proteins disturbs collagen homeostasis in proximal tubular cells leading to interstitial fibrosis, an important cause of renal insufficiency development[39]. Furthermore, albuminuria is linked to enhanced renal TGF- β1 expression in rats[40]. Transforming growth factor (TGF-β1) plays a key role in initiating the fibrosis cascade[41]. The regeneration of injured muscle due to fibrosis was delayed by up-regulating transforming growth factor (TGF-β1) in mice gastrocnemius by NS-398, a specific COX-2 inhibitor[42]. Diclofenac possibly up regulates TGF- β1, hence proliferating fibroblasts and increasing collagen concentration in kidney.

CONCLUSION

Present study shows evidence of a possible deleterious effect of chronic administration of diclofenac on kidney in terms of changed ACPase activity which is one of the marker enzyme for renal function. Further, increased collagen concentration was indicative of fibrosis which could be a factor for kidney malfunctioning. These physiological changes were accompanied by noticeable pathological evidences. Therefore, the drug should be carefully indicated with cautions as a pain-killer or as an anti-inflammatory entity keeping in view previous renal history and that too for a short duration in the patients particularly with some or the other kidney malfunction history for short term therapy.

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