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Decreased Expression of Alpha Smooth Muscle Actin and Desmin Contributes to the Protection of Vitamin D3 against Diclofenac Induced Nephrotoxicity in Rats

Sahar Youssef *[1,2], Marwa Salah[3]

Abstract

Background: Diclofenac is widely prescribed for its analgesic and anti-inflammatory actions but it also has some harmful effects on the kidney. The current study was conducted to elucidate the possible mechanism of action of diclofenac sodium on kidney, and if it is affected by the addition of vitamin D.

Methods: Rats were divided into 4 equal groups. G1 was the control group that received no treatment; G2 was treated with intramuscular injection of vitamin D (1,000 IU/kg, 3days/week); G3 was treated with intramuscular injection of diclofenac sodium (3.6 mg/kg, 3 days/week) and G4 treated simultaneously with both diclofenac (3.6 mg/kg, 3 days/week) and vitamin D (1,000 IU/kg, 3days/week) intramuscularly for four weeks. Kidneys sections were stained with H&E, Masson's trichrome and immunohistochemical staining against α –SMA and desmin followed by the morphometric and statistical analysis.

Results: Kidney sections from diclofenac sodium treated group showed degeneration and necrosis, small or atrophic glomeruli with dilated Bowman's space and some of the renal tubular lining cells appeared vacuolated with small pyknotic nuclei. Renal fibrosis was confirmed by significant increase in collagen fibers, α –SMA and podocytes injury by significant increase of desmin. However, in diclofenac- vitamin D treated group significantly the expression of α –SMA and desmin were decreased.

Conclusion: The current data suggested that vitamin D might play a protectant role against diclofenac induced kidney injury in rats through the preservation of the histological architecture of renal corpuscles, renal cortical tubules and down regulation of collagen, α - SMA and desmin.

Introduction

Kidney is one of the most important organs in the human body. Kidney involved in removing of drugs or toxic substances, regulates blood pressure, acid base balance, electrolytes and maintaining the production of prostaglandins via cyclooxygenase (COX). Kidney injuries often arise due to their function involvement in storage, detoxification and excretion. Certain prescriptions such as non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to control pain and fever, however, nephrotoxicity are recognized on their repeated and high dosage. Diclofenac sodium is a phenyl acetic acid NSAIDs broadly used as an antipyretic, analgesic, anti-rheumatic and anti-inflammatory [1]. It is prescribed for treatment of osteoarthritis, myalgia and rheumatic disorders by suppressing the synthesis of prostaglandin by inhibiting COX enzymes activity [2]. Although normal therapeutic usage of diclofenac exhibited beneficial effects but adversarial side possessions has been reported such as liver injury, gastrointestinal diseases, hematotoxicity and acute kidney injury [3]. The exact mechanism of nephrotoxicity by diclofenac administration is not fully known, there is evidence suggested that diclofenac can cause inhibition of prostaglandin synthesis leads to irregular renal function and the deterioration in glomerular hydraulic pressure. Diclofenac can cause mitochondrial injury by stimulated oxidative stress and disturbing kidney mitochondrial complex I, leading to a decline of ATP production [4]. Also, diclofenac effects were shown to be associated with increased collagen content in mice [5]. So, the potential antioxidant therapeutic drugs that might prevent oxidative stress mediated cellular injury can be the protectant approach against harms affecting renal glomerular and tubular function.

Antioxidants play a vital role in protecting the body from toxic substances. Vitamin D is a fat soluble vitamin produced in the skin, kidney, liver, and can be absorbed from food. It is known to have wide range functions such as regulation of calcium and phosphorus metabolism, regulation of renal, cardiovascular functions and modulation of immune reactions [6]. Vitamin D has an antioxidant and anti- apoptotic action [7]. It has been linked with regulation of fibrosis or has been recommended for treating of fibrosis in several organs. For instance, vitamin D prevents bleomycin induced pulmonary fibrosis in mouse model [8]. Also, supplementation of vitamin D decreased hepatic fibrosis associated with hepatitis C [9]. However, the roles of vitamin D in the treatment and pathogenesis of kidney linked to NSAIDs are largely unknown.

The aim of current study was to examine the effect of diclofenac administration on the kidney of male albino rat and if these results were affected by the concomitant administration of vitamin D using histological and immunohistochemical analyses of α-SMA and desmin.

Methods

Drugs

Diclofenac sodium (Voltaren 75 mg/ 3 ml) ampoules produced by (Novartis Pharma Co. Cairo, Egypt). Vitamin D3 was in the form of ampoules; Devarol-S 200,000 I.U.; (Cholecalciferol) manufactured by (Memphis Co. for Pharm. & Chem. Ind., Cairo, Egypt).

Animals

Forty adult male Wistar rats weighing 180-200g were controlled and carried out according to the Animal Ethical Committee of Faculty of Medicine, Assiut University, Egypt. Rats remained acclimatized for one week before the beginning of the experiment and housed in special cages under standard laboratory circumstances at an appropriate humidity room and temperature (23±1°) with 12-h light/dark cycles. Ordinary rat pellet and water *ad libitum* were supplied.

Experimental Design

In the current study, male rats were used instead of female because females might have a greater variability due to the alteration of hormonal changing in the oestrous cycles affecting the physiological and pharmacological characteristics. Rats were divided into four equal groups (n=10 per group) including the control group (G1) in which rats kept without treatment, vitamin D treated group (G2) in which rats were intramuscular injected with vitamin D3 diluted with saline at dose (1000 IU/Kg/day; 3 days/week) [10], for four weeks. Diclofenac treated group (G3) in which rats received intramuscular injection of diclofenac sodium at a dose of 3.6 mg/kg**,** [11], 3days/week for four weeks and diclofenac - vitamin D treated group (G4) in which rats were given diclofenac sodium (3.6 mg/kg) and vitamin D (1000 IU/kg/day) simultaneously 3 days/week for four weeks.

Histological Analysis

At the end of experiment, rats from different studied groups were dissected. Kidney tissue samples from each rat were cut longitudinally and renal specimens were collected and fixed in 10 % neutral buffered formalin solution. Dehydration was performed in ascending grades of the ethanol and embedding in paraffin and processed for sections of 5 µm thickness. These sections were stained with hematoxylin and eosin (H&E) to study general histological structure and Masson's trichrome to detect collagen fibers [12].

Immunohistochemical Analysis

Immunohistochemical staining for α–SMA and Desmin, was carried out using the technique according to the previously literature methods [13, 14]. Sections were cut into 3µm thickness, after deparaffinization and rehydration; methanol treatment including 0.03 % hydrogen peroxide for 20 min was used. Kidney sections were incubated with normal serum for 20 min to block non-specific antibody, then incubated with anti-mouse monoclonal primary antibody against- *α*-SMA (1:100, DAKO, Denmark) [8], followed by the secondary antibody anti-mouse IgG (1:500, Sigma-Aldrich). Other sets of sections were incubated with anti-mouse monoclonal primary antibody against desmin (1:100, DAKO, Denmark) [15]. The secondary antibody, peroxidase anti-mouse IgG (1:100, DAKO, Denmark)

was performed. Reaction was visualized by using the chromogen Diaminobenzidine, DAB (Dako, Glostrup, Denmark). Slides were counterstained with Mayer's hematoxylin and finally dehydrated, rendered transparent with xylene and cover slipped. The negative control was performed by neglecting the primary antibody. Slides were analyzed under a light microscope to identify areas with brownish color which considered as sign of a positive reaction. Photography was conducted at the Mycology and Biotechnology Unit, Al-Azhar University, Cairo, Egypt.

Morphometric Analysis

The mean area percentage of Masson's trichrome histochemical stain, desmin and α-SMA immunoreactivity using (Leica Qwin 500 image system, Cambridge, England) was carried out for image analysis. The measurements were conducted in 10 non-overlapping fields in stained slides chosen from each animal in all groups at a magnification of×400.

Statistical Analysis

The results were analyzed using one way analysis of variance (ANOVA), followed by the Tukey's post-hoc test. All values are presented as the mean (M) ± standard deviation (SD). Differences between the groups were considered significant when the probability of chance (p) is less than 0.05 ($p < 0.05$). All the data collected from the experiment was calculated and analyzed using SPSS software version 16 (SPSS, Chicago, USA).

Results

Histological Results

H&E Results

The renal cortex of the control group (G1) stained with H&E showed normal architecture of renal corpuscles. The renal corpuscle consists of glomerular capillaries and Bowman's capsule and the two layers of Bowman's capsules could be observed. The outer parietal layer consists of simple squamous epithelium, the inner visceral layer formed of podocytes and the two layers were separated by a urinary space (Fig.1a). Numerous proximal convoluted tubules with narrow lumina were lined with simple cubical cells with basal spherical nuclei and deeply acidophilic cytoplasm. The distal convoluted tubules with wide lumina were lined with simple cubical cells, apical spherical or central nuclei and pale acidophilic cytoplasm (Fig.1a).

The histological architecture of the renal cortex of the vitamin D treated group (G2) was similar to that of control rats (Fig.1b). The renal cortex of the diclofenac group (G3) showed some small glomeruli or atrophic glomeruli with a widening of Bowman's space. Some of the renal tubular lining cells appeared with vacuolated cytoplasm and small pyknotic nuclei (Fig.1 c). Sections from Diclofenac-vitamin D treated group (G4) revealed apparently normal glomeruli that were surrounded with Bowman's space. Most of the renal tubules appeared nearly normal however few tubules appeared dilated (Fig.1 d).

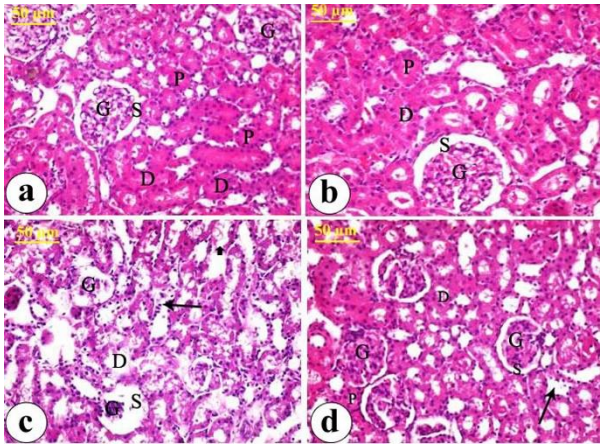

Figure 1: Photomicrographs of renal cortical sections of rat from different studied groups **(a); (b)** Control group (G1) and vitamin D group (G2) respectively showing Malpighian renal corpuscles containing glomeruli and surrounded with Bowman's space, proximal convoluted tubules with narrow lumina and distal convoluted tubules with wider lumina. **(c)** Diclofenac sodium (G3) showing partial destruction of some of renal corpuscles containing glomerular tufts and atrophy of some glomeruli with widening of Bowman's space. Dilated (D) and degenerated tubules can be seen. Vacuolated cytoplasm appears in the tubular lining cells (short thick arrow) and pyknotic nuclei (thin arrow) **(d)** Diclofenac and vitamin D group (G4) showing nearly normal renal corpuscles with preservation of most of glomeruli, almost all tubular cells have vesicular nuclei but widening of some tubules (arrow). Glomeruli (**G**), Bowman's space (**S**), proximal convoluted tubules (**P**), distal convoluted tubules (**D**); Scale bar 50µm; (H&E, x400).

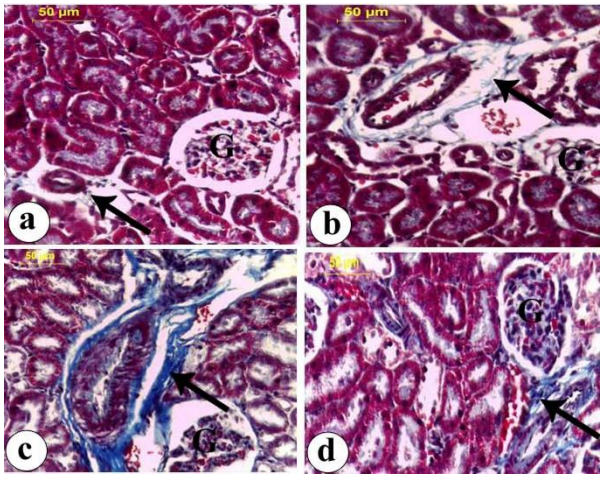

Figure 2: Photomicrographs of renal cortical sections from different studied groups stained with Masson's trichrome stain **(a); (b)** Control group (G1) and vitamin D group (G2) respectively, showing few collagen depositions in the glomeruli and around blood vessels (arrows). **(c)** Diclofenac group (G3), showing marked deposition of collagen fibers among glomeruli and around the dilated congested blood vessels (arrow). **(d)** Diclofenac-vitamin D group (G4) showing mild deposition of collagen fibers in glomeruli and around blood vessels (arrow). Glomeruli (G); Scale bar 50µm; (Masson trichome, x400).

Masson's Trichrome Results

Examination of the renal cortical sections of the control (G1) and vitamin D (G2) groups after Masson's trichrome stain, showed the presence of a minimal amount of collagen which appeared as fine fibrous tissue among glomerular capillaries and around blood vessels (Fig. 2 a; b). Renal cortical sections from diclofenac group (G3) revealed marked deposition of collagen fibers within the glomeruli in the renal corpuscles and around the dilated blood vessels (Fig.2 c). However, renal cortical sections from diclofenac - vitamin D group (G4) showed mild deposition of fibrous tissue in the renal corpuscles and around blood vessels (Fig.2 d).

Immunohistochemical Results

Expression of α-SMA

Examination of rat's cortical sections from the control (G1) and vitamin D (G2) groups showed positive α-SMA immunostaining localized to the wall of blood vessels and very negligible expression in the renal glomeruli can be seen (Fig.3 a, b). Immunoexpression in the cortical sections of the diclofenac treated group (G3) appeared with strong positive reaction of α -SMA in the glomeruli, blood vessels (Fig.3 c) and in some of renal tubules (Fig.3d) as compared to that of the control group. Sections from diclofenac- vitamin D (G4) treated rats displayed a moderate reaction of α -SMA localized to the wall of blood vessels and minimal expression in the glomeruli (Fig.3e) as compared to rats received diclofenac only.

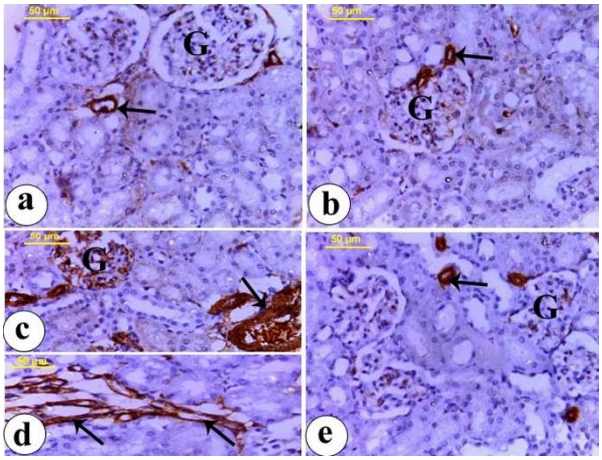

Figure 3: Photomicrographs of rat renal cortical sections from different studied groups **(a); (b)** control& vitamin D groups (G1&G2 respectively) showing positive immune reaction of α-SMA in the blood vessels (arrows) and minimal positive reactions in the glomeruli. **(c)** Diclofenac sodium group (G3) showing strong positive immune reaction of α- SMA in the wall of dilated blood vessels (arrow) and in the renal glomeruli. **(d)** Diclofenac sodium group (G3) showing strong positive immune reaction of α-SMA in the renal tubules (arrows). **(e)** Diclofenac - vitamin D (G4) showing positive immune reaction of α-SMA in the blood vessels (arrow) and minimal expression in the glomeruli. Glomeruli (G); Scale bar 50µm; (α- SMA X400 immunostaining).

Expression of Desmin

Renal cortical sections from the control (G1) and vitamin D (G2) groups were nearly similar and showed slight desmin positive immunoreactivity localized in the renal glomerular cells, however, negative desmin expression in the interstitium can be observed (Fig.4 a; b). Desmin immunostaining became abundant in sections of diclofenac treated group (G3) in the renal glomerular cells (Fig.4c). On the other hand; diclofenac and vitamin D group (G4) exhibited moderate desmin immunoreactivity in the renal glomerular cells (Fig.4d).

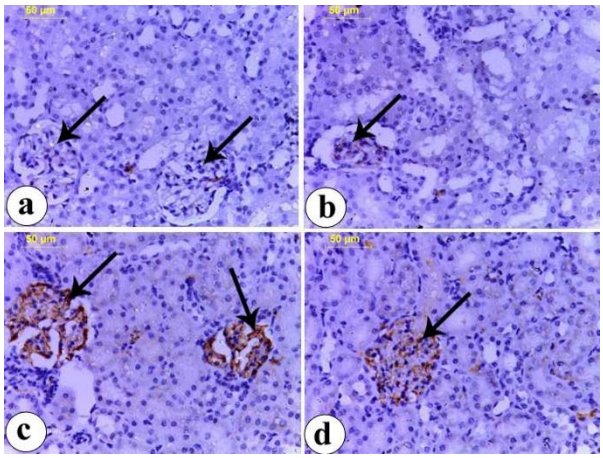

Figure 4: Photomicrographs of desmin immunostained cortical sections in the renal cortex showing **(a); (b)** Control & vitamin D group (G1, G2 respectively) showing minimal glomerular positive immunostaining in the glomerular cells (arrows). **(c)** Diclofenac group (G3) showing strong positive desmin immunoreaction in the glomeruli (arrows). **(d)** Diclofenac - vitamin D group (G4) showing apparent reduction in the positive desmin immuno-reaction in the glomerular cells (arrow). Scale bar 50µm; (Desmin x 400 immunoreactivity).

Morphometric Results

Regarding the morphometric data of the mean area percentage of collagen fibers, there was a significant increase of collagen fiber deposition ($P < 0.001$) in the diclofenac group (G3), (20.2±1.3) as compared to the control (G1); (6.3±1.2) and the vitamin D group (G2); (7.1±0.8). On the other hand, there was a significant ($p < 0.001$) decrease of collagen fiber accumulation in the diclofenac-vitamin D group (G4), (12.4±0.99); as compared to the diclofenac group (Fig.5).

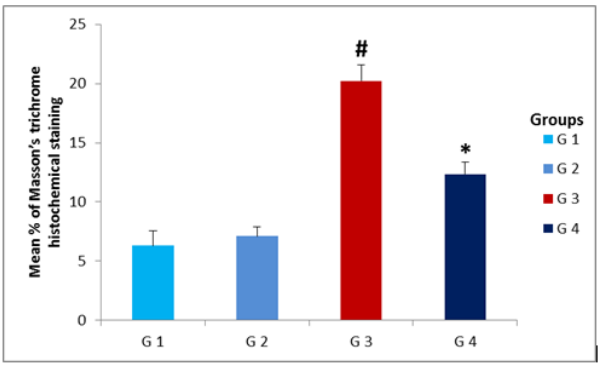

Figure 5: Representative bar graph of Masson's trichrome stain in the different experimental groups. Data are presented as means ± SD. # denotes $p < 0.001$ versus control group; * denotes $p < 0.001$ versus diclofenac group.

Morphometric results of the area percentage of α-SMA from diclofenac treated group (G3) showed a significant

increase (p <0.001) in both the glomeruli and the interstitium (21.22±1.4; 13.56. ±1.5 respectively) versus the control (G1) and vitamin D (G2) groups (5.64±0.7; 5.72±0.5); (4.45±1.5; 4.57±0.3 respectively). Co-administration of vitamin D with diclofenac (G4) revealed a significant (p <0.001) decrease in the area percentage of positive α-SMA immunostaining in the glomeruli and in the interstitium (7.23±0.9; 6.35±0.9 respectively) as compared to the diclofenac group (Fig.6).

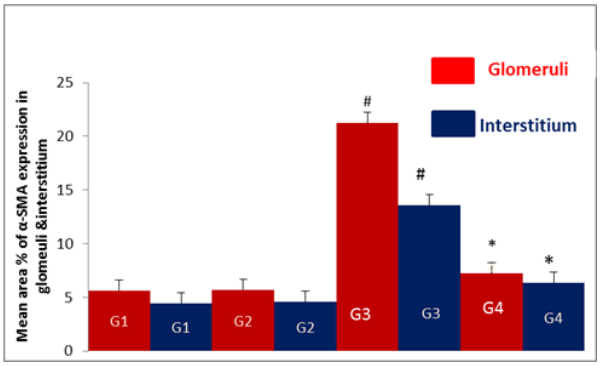

Figure 6: Representative bar graph of mean area % of α-SMA in glomeruli and interstitium of the different experimental groups. Data are presented as means ± SD. # denotes p< 0.001 versus control group; * denotes p < 0.001 versus diclofenac group.

Statistical analysis of the morphometric findings of desmin immunostaining in the renal cortex showed a significant (p <0.001) increase in the area percentage of desmin in the diclofenac treated group (G3); (22.3±1.1) as compared to the control (G1) (2.5±0.6); and the vitamin D groups (G2); (3.2±0.8). While, vitamin D - diclofenac group (G4) manifested a significant (p <0.001) decrease (8.3±0.5) in the area percentage of the desmin as compared to the diclofenac group (Fig.7).

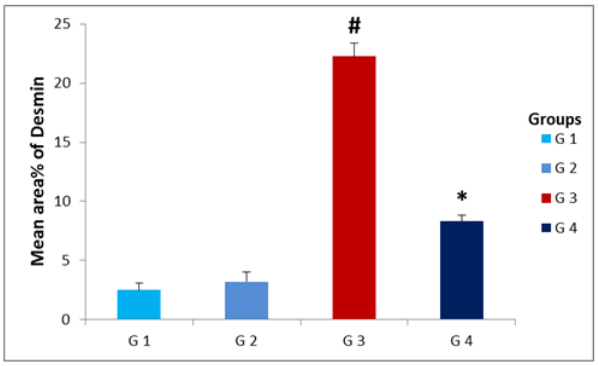

Figure 7: Representative bar graph of mean area% of desmin immunoreactivity in the different experimental groups. Data are presented as means ± SD. # denotes p < 0.001 versus control group; * denotes P < 0.001 versus diclofenac group.

Discussion

The present study adds new data regarding the possible defensive effect of vitamin D against diclofenac sodium induced renal injury in rats. Atrophic glomeruli with a widening of Bowman's space in the diclofenac group in the present study were clearly observed. Atrophy of the glomeruli was observed after diclofenac administration from other studies [16- 18].

The current study revealed tubular dilatation and vacuolated cytoplasm of the lining epithelium of the renal tubules in the diclofenac treated rats. This is in agreement with other investigations. In humans, patient presented with acute tubular damage used diclofenac treatment for knee arthropathy and chronic muscular pain [19]. Similar alterations have been reported by several authors in diclofenac [20], and from other drug such as atorvastatin [21]. The cytoplasmic vacuolation of the renal tubules observed in the current study could be due to increased fluid up take as a consequence of oxidative stress induced cell membrane injury [22]. Notably, pyknotic nuclei in the cells lining of the renal cortical tubules in the current study could be described as a pattern of nuclear alterations triggered by a non-specific interruption of DNA, leading to the irreversible condensation of chromatin into a solid basophilic mass in the cells undergoing necrosis and or apoptosis [23].

In the present study, marked deposition of collagen within the glomeruli and around the dilated blood vessels in the diclofenac group and supported by a morphometric analysis, where the mean area percentage of collagen fibers was significantly higher than the control. Crucially, fibroblasts initiate mainly inside the interstitial tissue through epithelial mesenchymal transition procedure. Then, tubular epithelial cells changed to a mesenchymal phenotype started by disruption in the equilibrium of the cytokine concentrations. Finally, fibroblasts increase their numbers and secreting large quantity of extracellular matrix and disrupt normal interstitial structure of the kidney due to chronic inflammation of renal parenchyma. Numerous studies hypothesized that in renal fibrosis, more than thirty percentage of all disease related in which fibroblastic proliferation resulted in tubulointerstitial nephritis and finally irreversible renal failure [24]. Other studies confirmed that an increase in the connective tissue in the rat kidneys of the diclofenac treated group [11, 25]. The injured tubule cells promote inflammation, lead to the elimination of all tubular remnants and eventually formation of area of fibrosis. Some authors reported that degenerating tubule surrounded by inflammatory process consists of the enormous accumulation of myofibroblasts that form the interstitial fibrosis by producing type I collagen [26, 27].

The present study have been shown a significant increase in α- SMA immunoreactivity in diclofenac sodium treated group and confirmed by morphometric study. Importantly, α-SMA actin expression is used as a key index of mesangial cell activation [28]. Mesangial cells play a significant role in glomerular injury and have a vital impact to the extracellular matrix production and glomerulosclerosis [29]. α- SMA is greatly expressed in kidneys and recognizes smooth muscle cells and myofibroblasts. Interestingly, the overproduction of α-SMA partly results from tubular epithelial-myofibroblast transdifferentiation, which act as an essential role in renal interstitial fibrosis [30].

Desmin is an intermediate filament protein and act as an indicator of podocyte injury. The podocytes are crucial

for the glomerular filtration barriers that are damaged at an early stage of glomerular injury. The current study supports the hypothesis of renal injury in the diclofenac sodium group came from the strong desmin positive immunoreactivity within the cytoplasm and the processes of the podocytes. Increased desmin immunoreactivity was further supported by the significant increase in its mean area percentage as compared to the control group. Indeed, desmin expression was increased in various glomerular diseases in which podocytes damage. This finding is in line with other studies [31,32] which mentioned that desmin glomerular expression is significantly upregulated in podocytes of diabetic nephropathy and in the nandrolone decanoate induced renal cortical damage [33].

Vitamin D Administration with diclofenac sodium ameliorated most of the histological changes in the current study. Diclofenac-vitamin D group have been shown nearly normal renal histological architectures and minimal changes. Masson's trichrome-stained sections showed mild deposition of collagen fibers in renal corpuscles and around blood vessels. Statistically, the mean area percentage of collagen fibers was significantly lower when compared to the diclofenac group. These findings were in parallel and supported by Cohort study in patients with severe liver fibrosis and cirrhosis in which vitamin D alleviated the risk of hepatic decompensation and inflammation by reducing numerous inflammatory cytokines [34]. Many experimental studies suggested that vitamin D protects against lead induced renal and testicular injuries in rats and act as an anti-oxidant and anti-inflammatory [35]. Other authors have been stated that vitamin D improved the hepatorenal damage triggered by monosodium glutamate by decreasing the levels of Malondialdehyde (MDA), increasing antioxidants and inhibiting cell apoptosis in rats [36].

Decreased α-SMA immune reaction in the current work in Diclofenac-vitamin D group was detected. This finding is in accordance with in vitro study which suggested that active vitamin D3 suppressed the expression of α-SMA and inhibited the upregulation of fibronectin and collagen [37].

In the present study, decreased desmin positive immunostaining with a significant decrease in its mean area percentage in diclofenac-vitamin D group was observed. These results were in agreement with the earlier results [38], which suggested that the antioxidant lycopene modified podocyte foot process changes and had a protecting influence on podocytes and decrease desmin positive immunoreactivity in nandrolone–lycopene group. .

The histological, and immunohistochemical findings obtained in the present study support the view that diclofenac sodium has damaging effects and causes a variety of the histological alterations in the renal cortex. Crucially, vitamin D protects against renal fibrosis by decreasing the collagen, α-SMA expression and podocyte injury by decreasing expression of desmin. Though, additional studies are needed to detect further molecular mechanisms.

Author Contributions

Sahar Youssef designed the paper and both Sahar Youssef and Marwa Salah contributed to methodology, investigation, formal analysis, software, original draft preparation, writing, review, editing, final proofreading corrections and funding. This research article received no external funding.

Competing Interest

All authors declare no conflicts of interest in this paper.

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