

Full Length Research Paper

Oxidative stress, decreased activities of anti-oxidant enzymes, and neutrophil infiltration contribute to Tenofovir disoproxil fumarate induced renal damage in rats

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The mechanism by which tenofovir (TDF) causes renal damage is not clear. To examine the role of oxidative stress in TDF induced renal damage. Rats were administered by gavage 600mg /kg body weight Tenofovir disoproxil fumarate for 35 days. The kidneys were used for light microscopy and lectron microscopy as well as for the assay of markers of oxidative stress and activities of antioxidant enzymes and myeloperoxidase activity, a marker of neutrophil infiltration. TDF administration to the rats resulted in glomerular and tubular damage. Electron microscopically, mitochondrial swelling, disruption of cristae and accumulation of amorphous deposits in the matrix were observed. Significant increase in protein carbonyl content, decrease in reduced glutathione and protein thiol, decrease in the activities of the antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione S transferase and glutathione reductase and a massive increase in myeloperoxidase activity was observed in the kidneys of TDF treated rats. Oxidative stress contributes to TDF induced renal damage in rats. The source of reactive oxygen species may be the damaged mitochondria and or activated neutrophils.

Keywords: Tenofovir; renal damage; mitochondria; oxidative stress; neutrophil infiltration; rat animal models; antioxidants; peroxidation; proximal tubular cells, reactive oxygen species.

INTRODUCTION

The widespread introduction of highly active antiretroviral therapy (HAART) in the mid-1990s dramatically altered the course of human immunodeficiency virus (HIV) infection, with improvements in survival and reductions in the incidence of AIDS-defining illnesses. (Hull et al., 2011; Krambovitis et al., 2005). Although, antiretroviral therapy has been shown to reduce the incidence of both AIDS-defining and non-AIDS conditions, long-term exposure to HAART may also be associated with significant toxicity. Tenofovir and related nucleotide analogs have primarily been associated with proximal tubular dysfunction and acute kidney injury (Prie et al.,

2004; Perazella et al., 2004). Glomerular abnormalities have been observed less frequently (Quinn, 2010). Kidney damage related to antiretroviral therapy is typically reversible with early recognition and timely discontinuation of the offending agent, and nephrologists should be familiar with the potential toxicity of these agents to avoid delays in diagnosis. The mechanism by which tenofovir causes renal damage is not well characterized. Recent studies have implicated that mitochondrial damage may play an important role in TDF induced renal tubular damage in both humans and animal models (Babiak et al., 1998; Biesecker et al., 2003; Birkus et al 2002; Breton et al 2004; Buss et al., 2000).

Although mitochondrial damage has been suggested to be the cause of TDF induced renal tubular dysfunction, the mechanism by which mitochondrial damage results in renal tubular dysfunction is not

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known to the best of our knowledge. It is well known that the mitochondria are the primary intracellular source of reactive oxygen species (ROS), as they generate huge numbers of oxidative-reduction reactions and use massive amounts of oxygen (Circu et al., 2010; Cuzzocrea et al., 1997; Dalakas et al., 1990; Dalle-Donne et al., 2006). ROS that is released by the damaged mitochondria are superoxide anion, hydrogen peroxide, and hydroxyl radical. Significant increase in ROS results in oxidative stress. High levels of ROS exert its toxic effect on biomolecules such as DNA, proteins, and lipids, thus leading to the accumulation of oxidative damaged products within the cell leading to the deregulation of redox-sensitive metabolic and signaling pathways, and to cell death. To overcome ROS the ROS generation, cells are equipped with antioxidant defense systems that minimize the susceptibility to ROS. These defense mechanisms include antioxidant glutathione (Marí et al., 2009; Circu, 2010) and antioxidant enzymes such as superoxide dismutases, catalase, glutathione peroxidase, glutathione reductase and glutathione S transferase (Güngör et al., 2007; Haafte et al., 2001; Habeeb, 1972). The decrease in the antioxidant system in the cell can increase susceptibility of the cells to the toxicity of ROS resulting in oxidative stress. Thus, oxidative stress can result from overproduction of ROS and/ decrease in the antioxidant system in the cell.

In general, besides their direct damaging effects on the tissues, reactive oxygen species trigger the accumulation of leukocytes in the tissues involved, and thus aggravate tissue injury indirectly through activated neutrophils. The activated neutrophils secrete myeloperoxidase (MPO) and other proteases (Zimmerman et al., 1992). In turn, MPO plays important role in oxidant production by neutrophils. MPO activity is considered to be marker for neutrophil infiltration (Queiroz-Junior et al., 2009).

We hypothesize that ROS generated by the damaged mitochondria and activated neutrophils may play an important role in TDF induced renal damage. Therefore in the present study we investigated whether oxidative stress plays a role in TDF induced renal damage by the assay of parameters of oxidative stress such as malondialdehyde, protein carbonyl content, reduced glutathione and alteration in cellular antioxidant defense mechanism using a rat model.

We suggest that elucidation of the mechanism of renotoxicity of tenofovir will help minimise its renal side effects and improve the therapeutic efficacy of the drug.

MATERIALS AND METHODS

Animals and treatments

Adult male Wistar rats (200–250 g) were used for the experiments. The dose, route and duration of

administration of TDF was based on that described by Biesecke et al (2003) with modifications.

Experimental design

The rats were divided into 2 groups and were treated as follow.

Group I (control)

The rats in this group (n = 6) received sterile water.

Group II

The rats (n = 8) in this group received 600 mg/ kg body weight Tenofovir disoproxil fumarate by gavage for 35 days.

All the rats were sacrificed 24 hours after the final dose of TDF / sterile water, after withdrawal of blood under light haloethane anesthesia. The kidneys were removed and used for histological and biochemical studies.

Histology

Light microscopy

For light microscopic studies tissue was fixed in 10% buffered formaldehyde and paraffin embedded. Four-micron serial sections were cut and stained with haematoxylin and eosin.

Scanning electron microscopy of kidney

The kidney tissues were fixed in 3% glutaraldehyde and washed in buffer, post fixed by 1% osmium tetroxide and washed in buffer, and, dehydrated in increasing concentrations of alcohol. The tissues were washed with propylene oxide and embedded in epoxy-resin embedding medium. Semi-thin sections approximately 1 micron thicknesses were cut with a glass knife on Leica ultra-cut (UCT) ultramicrotome. Sections were stained with toluidine blue. Ultrathin (below 100 nm) sections were collected on copper grids, stained with uranyl acetate and Reynold's solution (sodium citrate and lead nitrate), and examined with transmission electron microscope (Philips 201C by Netherland) and photographed. Mitochondria were examined in the tubules. Parameters included the presence of structurally abnormal mitochondria, increased numbers of mitochondrial profiles per field, intra-mitochondrial lamellar bodies, abnormal cristae density, cristae reduplication, mitochondrial swelling, and intra-mitochondrial

paracrystals (Dalakas et al., 1990).

(A) Immunohistochemical localization of nitrotyrosine in kidney

Nitrotyrosine was detected immunohistochemically as described by Cuzzocrea et al (1997). The kidney tissue was fixed in 10% formalin, 4 μ thick sections obtained from paraffin-embedded tissues.

After deparaffinization, the sections were permeabilized with 0.1% Triton X-100 in Tris buffered saline for 15 min. The primary monoclonal anti-nitrotyrosine antibody, designated 39B6, raised against 3-(4-hydroxy-3-nitrophenylacetamido) propionic acid-bovine serum albumin conjugate was obtained from Santa Cruz and the Super Sensitive Polymer/HRP/DAB kit was obtained from BioGenex were used. Endogenous hydrogen peroxidase was quenched by 3% hydrogen peroxidase. After the buffer wash, the universal protein blocking agent was applied over the sections. Then the primary antibody was applied over the sections and incubated overnight. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate. After that the slides were counterstained with Harris hematoxylin and mounted.

(B) Immunohistochemical localization of PARP in the kidney by Kupper JH et al. (1996)

The kidneys were fixed in 10% formalin and 4 micron thick sections were obtained from paraffin-embedded tissues. After deparaffinization, the sections were permeabilized with 0.1% Triton X-100 in Tris buffered saline for 15 min. The primary polyclonal anti-PARP [poly (ADPribose) polymerase] antibody obtained from Sigma and the Super Sensitive Polymer/HRP/DAB kit obtained from BioGenex were used. Endogenous hydrogen peroxidase was quenched by 3% hydrogen peroxidase. After the buffer wash, the universal protein blocking agent was applied over the sections. The sections were then incubated overnight with 1:500 dilution of primary antibody. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymerase polymer and DAB substrate. After that the slides were counterstained with Harris hematoxylin and mounted.

Biochemical studies

Biochemical studies were carried out on 10% (w/v) of kidney homogenates prepared in ice-cold 1.5% KCL.

Myeloperoxidase (Wallace et al, 1989, Diaz-Granados et al, 2000)

Myeloperoxidase activity was measured with O-dianisidine-H₂O₂ assay. The rate of decomposition of H₂O₂ by myeloperoxidase was determined by measuring the rate of colour development at 460nm. To 10 μ l of sample, 11 μ l of H₂O₂, 17 μ l of O- dianisidine and 962 μ l of phosphate buffer were added and the color read at 460nm at an interval of 30 seconds for 4 minutes and the rate of change/minute was determined. Extinction coefficient of 1.13 X 10⁴ cm⁻¹ was used for the calculation. One unit is the amount of enzyme decomposing 1 μ mole of peroxide per minute.

Malondialdehyde

Malonaldehyde content was measured as describe by Ohkawa et al (1979). The mixture consisted of 0.8 ml of sample (1mg), 0.2ml of 8.1 % SDS, 1.5 ml of 20 % glacial acetic acid adjusted to pH 3.5, and 1.5 ml of 0.8 % aqueous solution of TBA. The mixture was made up to 4ml with distilled water and heated at 95oC for 60 min using a glass ball as condenser. After cooling with tap water, 1ml distilled water and 5ml n-butanol and pyridine mixture (15:1) were added and the solution was shaken vigorously. After centrifugation at 2000g for 10 minutes the absorbance of the organic layer was measured at 532nm. Amount of thiobarbituric reacting substances formed is calculated from standard curve prepared using 1, 1', 3, 3' tetramethoxy propane and the values expressed as nmoles per mg protein.

Protein carbonyl content

Protein carbonyl content was measured using DNPH as described by Sohal et al (1993). To 0.5 ml of sample (1-2mg), an equal volume of 10 mM DNPH in 2 N HCl was added and incubated for 1 hr shaking intermittently at room temperature. Corresponding blank was carried out by adding only 2N HCL to the sample. After incubation, the mixture was precipitated with 10 % TCA (final concentration) and centrifuged. The precipitate was

washed twice with ethanol: ethylacetate (1:1) and finally dissolved in 1 ml of 6 M guanidine HCl, centrifuged at low speed and the supernatant was read at 366nm. The difference in absorbance between the DNPH treated and HCl treated sample is determined and expressed as nmoles of carbonyl groups per mg of protein, using extinction coefficient of $22 \text{ mM}^{-1}\text{cm}^{-1}$.

Assay of anti-oxidant enzyme activities

Superoxide dismutase

Superoxide dismutase was measured as described by Ohkuma et al (1982). The assay mixture consisted of 100 μ l of phosphate buffer, 10 μ l of BSC, 50 μ l of Triton X-100, 5 μ l of EDTA, 5 μ l of xanthine oxidase, 50 μ l of xanthine is added. To this finally 150 μ l MTT and sample (50-150 μ g protein) were added and, the volume is made up to 1 ml with water. The mixture was incubated for 5 minutes at room temperature (30°C) and the reaction was terminated with the addition of 1ml of stop buffer. This was read at 540nm. Amount of superoxide formed is calculated using the molar extinction coefficient of MTT formazan E540 of $17,000 \text{ M}^{-1}\text{cm}^{-1}$ at pH 7.4 to 10.5. The percentage of inhibition by the presence of SOD is calculated from the reduction of the MTT colour formation as compared to the MTT formazan formed in the absence of SOD, which is taken as 100 %. One unit of SOD is defined as the amount of protein required to inhibit MTT reduction by 50%.

Catalase

Catalase activity is estimated by measuring the change absorption at 240 nm using H_2O_2 as substrate (Aebi, 1984). To 1ml of 30 mM buffered H_2O_2 , the enzyme (sample) was added to start the reaction. The final volume was made up to 2 ml with 0.05M phosphate buffer pH 7.0. Change in OD was observed for 2 min at 240 nm. One unit is the activity that disproportionates H_2O_2 at the rate of 10^{-3} absorbance/sec.

Glutathione-S-transferase (GSTase)

The activity of GSTase is measured spectrophotometrically using the substrate 1-chloro-2,4 dinitrobenzene (CDNB) (Awasthi et al., 1980). To 0.1 ml of 1M potassium phosphate buffer pH 6.5, following reagents were added: 0.1 ml of 10 mM GSH, 0.05 ml 20mM CDNB and water and made up the volume to 1 ml. The reaction was started by adding the enzyme and change in OD at 340 nm is measured for 1-2 min. One unit of enzyme is the amount required to conjugate 1 μ mole of substrate with glutathione in one minute.

Glutathione reductase

In the presence of enzyme, hydrogen is transferred from NADPH to GSSG and the reaction can be measured at 340 nm (Racker, 1955). To the reaction mixture containing 0.05 ml of 1 M phosphate buffer pH 7.6, 0.15 ml of 10 mM EDTA, 0.1 ml of 1mM NADPH, and 0.1 ml 10 mM GSSG, the enzyme was added. The volume was made up to 1 ml and the decrease in OD at 340nm was measured for 2-3 min. One unit is the amount of enzyme needed to oxidise 1 μ mole of NADPH/min.

Glutathione peroxidase

Total peroxidase is determined by following the oxidation of NADPH at 340 nm using hydrogen peroxide (Nakamura and Hosada, 1974). To 0.25 ml of 0.4 M phosphate buffer, 0.2 ml of 4 mM EDTA, 0.2 ml of 10 mM GSH, 0.2 ml of NaN₃, 0.2 ml of 1.6 mM NADPH, 0.03 ml glutathione reductase (one unit) and the enzyme (sample) was added. Total volume was made up to 2 ml with water. Reaction was started by adding 0.2 ml of H_2O_2 and change in OD at 340 nm was followed. Extinction coefficient of 6.1 mm^{-1} was used for the calculation. One unit is the amount needed to oxidize 1 nmole of NADPH/min.

Nonprotein thiol (glutathione) (Sedlak and Lindsay, 1968)

Nonprotein thiol was determined by the method described by Sedlak and Lindsay (1986). Briefly, proteins were removed by the addition of 21 μ l 50% trichloroacetic acid (TCA) to 200 μ l of sample. Then, samples were centrifuged at 12000 rpm for 10minutes. Then, 50 μ l obtained TCA extract and 100 μ l 6mmol/l dithionitrobenzene (DTNB) (Ellman's reagent) were added successively to 850 μ l 0.2mmol/l phosphate buffer, pH 8.2, and after 1 hour the absorbance was measured at 412nm. The results were read from a standard curve prepared from 1mmol/l solution of reduced glutathione.

Protein thiol groups

Thiol groups were measured as described by Habeeb (1972). To 1 ml of the sample suspension (1 mg protein /ml), 1 ml of 10 % TCA containing 1 mM EDTA was added. The protein precipitate was separated by high speed centrifugation for 10 min. For total thiol estimation the sample was taken directly without precipitation. To this, 1 ml of solution I and 0.5 % SDS were added followed by 2 ml of solution II and 30 μ l of

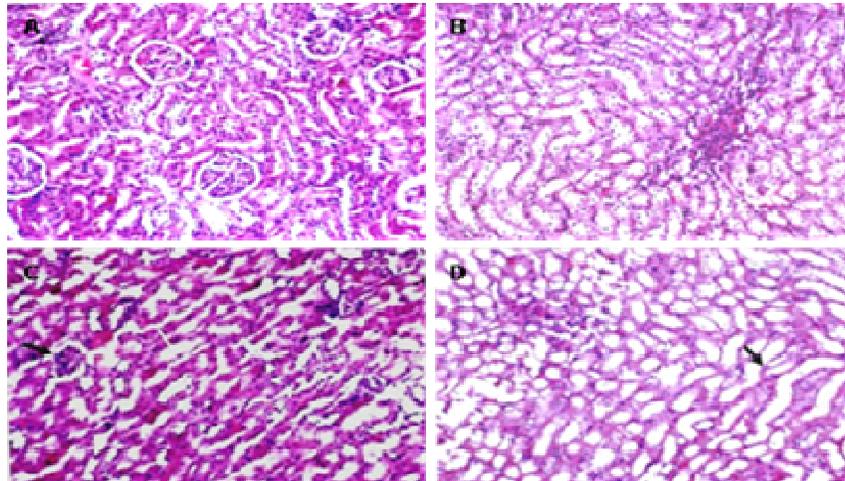


Figure 1. (A) Renal cortex of a control rat shows normal architecture [Hematoxylin and Eosin X 200] (B) Renal medulla of a control rat. Shows normal architecture [Hematoxylin and Eosin X 200] (C) Renal cortex of a TDF treated rat shows destruction of the glomeruli and some glomeruli were shrunken (black arrow). The convoluted tubules were distorted and their lining epithelium was destroyed (white arrow). Hematoxylin and Eosin X 200. (D) Renal medulla of a TDF treated rat– There was destruction of the lining epithelium of the loops of Henle and the convoluted tubules (black arrow) Hematoxylin and Eosin, X 200)

DTNB. The tubes were mixed well and kept in the dark for 15 min at room temperature. The intense yellow colour of the nitromerupto benzoate anion formed from the DTNB reaction with the thiol was read at 412 nm which has a molar absorption of $13,600 \text{ m}^{-1}\text{cm}^{-1}$.

The protein content of the homogenate/ supernatant was determined as described by Lowry et al (1951).

Statistical analysis

The results are expressed as mean \pm SD. Data were analysed with Mannwhitney 'U' test. Student's 't' test with Bonferroni correction was used to compare individual means in the case of a significant F.

RESULTS

Histological Analysis of Kidney Tissues

Kidneys were histologically assessed to determine whether treatment with TDF yielded microscopic changes in renal tubules or glomeruli. TDF induced renal damage involved the cortex and the medulla. In the cortex, there was destruction of the glomeruli and some glomeruli were shrunken. The convoluted tubules were distorted and their lining epithelium was destroyed. In the medulla there was destruction of the lining epithelium of the loops of Henle and the collecting duct. Figure 1

EM Features of Mitochondria in Tubular Epithelium

To investigate the organelle-specific effect of tenofovir on renal proximal tubules, ultra structural changes in renal tubular epithelial mitochondria were defined parametrically. Renal tubular epithelia of TDF-treated rats showed damage to the mitochondria. The mitochondria were swollen, cristae were disrupted, and amorphous deposits were observed in the matrix. Besides, increase in number of mitochondria was observed in the cytoplasm of basal part of tubule cell. Figure 2

Immunohistochemistry

Immunohistochemical staining of Nitrotyrosine and PARP shows a positively stained glomerulus and tubules of 35 days treated with TDF compared to Control group. (Figure 3A and 3B)

Biochemical Paramaters

The biochemical parameters are shown in below graph. A 2 fold increase in protein carbonyl content was observed in the kidneys of TDF treated rats as compared with the control. Malondialdehyde, an indicator of lipid peroxidation was increased in the kidneys of TDF treated rats as compared with the control, but the increase was not statistically significant.

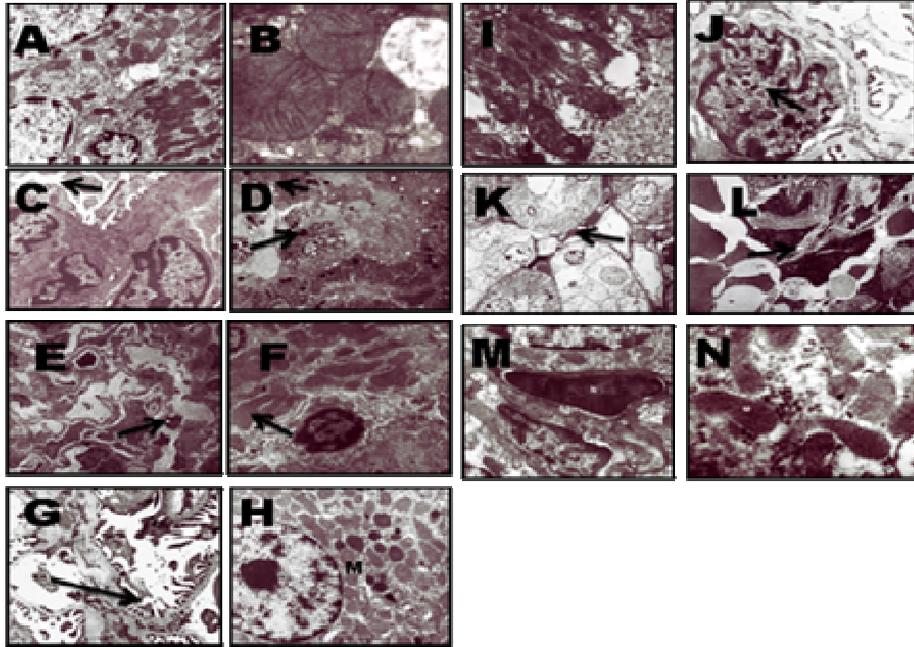


Figure 2. (A). Normal Kidney tubules (original magnification $\times 22000$) (B) Normal mitochondrial structure in the renal tubules of control rats (original magnification $\times 22000$) (C) Arrow indicates fusion of foot processes (original magnification $\times 22000$) (D) Vacuoles seen in the cytoplasm of the kidney tubule (black arrow) Less number of lysosomes (black arrowhead) (original magnification $\times 22000$) (E) Nucleus shrunken. Chromatin deposits are less in the nucleus of the endothelial cell (arrowhead) (original magnification $\times 22000$) (F) Disruption of mitochondrial cristae (black arrow), mitochondrial swelling (black arrow) in the renal tubules of TDF treated rats (original magnification $\times 22000$) (G) Fusion of foot processes (arrow) (original magnification $\times 22000$) (H) Increased number of mitochondria [M] in tubule cytoplasm (original magnification $\times 22000$) (I) Mitochondria swollen (arrow). Cristae destroyed. Amorphous deposits in matrix of mitochondria (arrowhead) (original magnification $\times 22000$) (J) Increased chromatin deposits in mesangial cell nucleus (original magnification $\times 22000$) (K) Increased mitochondria in basal part of tubule cell (arrow) (original magnification $\times 22000$) (L) Podocytes destroyed (arrow) (original magnification $\times 22000$) (M) Nucleus of mesangial cell [N] distorted (original magnification $\times 22000$) (N) Swollen mitochondria [M] (original magnification $\times 22000$).

Reduced glutathione, an important intracellular antioxidant was decreased by 61 % and protein thiol by 33 % in the kidneys of TDF treated rats as compared with the control.

A nine fold increase in myeloperoxidase activity, a marker of neutrophil infiltration was observed in the kidneys of TDF treated rats.

The activities of all the antioxidant enzymes estimated were significantly decreased in the kidneys of TDF treated rats as compared with the control. The activity of superoxide dismutase (SOD) was decreased by 61%, glutathione S transferase by 47 %, and glutathione reductase by 43% as compared with control. A 4 fold decrease in the activity of glutathione peroxidase was observed in the TDF treated rats. The activity of catalase in the kidneys of TDF treated rats was not significantly altered as compared with the control. Increase in nitrate level with a pvalue <0.008

was observed in the kidneys of TDF treated rats for 35 days.

No significant alteration in plasma urea and creatinine were observed in the TDF treated rats as compared with control.

DISCUSSION

Mild tubular dysfunction is recognized in a substantial proportion of TFV-treated individuals and tends to increase with cumulative exposure, (Karras et al., 2003) In a recent study, Rodríguez-Nóvoa et al (2010) examined the relationship between TFV exposure and kidney tubular dysfunction (KTD) prospectively in 92 HIV-infected individuals. Median TFV plasma trough concentration was higher in patients with KTD than in the rest. These authors have suggested that the dose-

(A) NITROTYROSINE

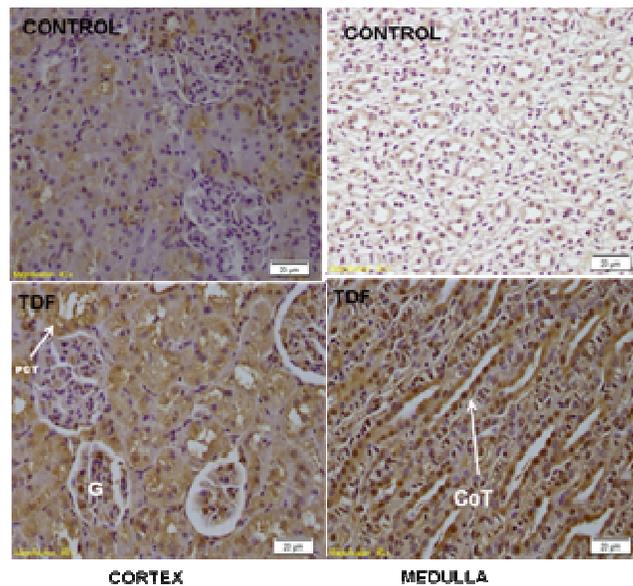


Figure 3A. Nitrotyrosine staining in the kidneys of control rat is minimal. In TDF treated the cortex, both PCT and DCT stained for NT. Glomerulus (G) showed mild staining for NT. In the medulla, loop of henle and collecting tubules (CoT) stained strongly for NT. (X40)

(B) PARP

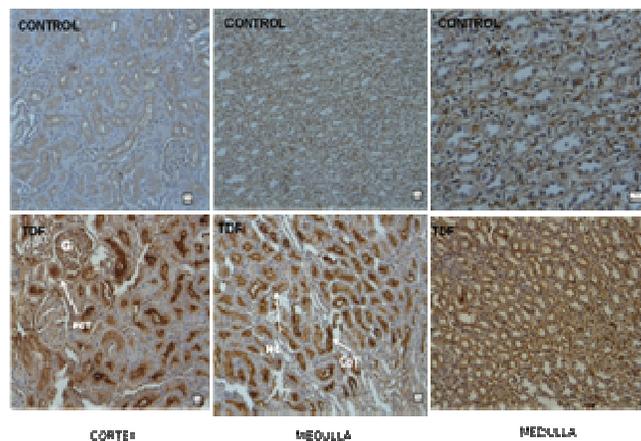


Figure 3B. Immunohistochemical appearance of the kidney-control (G) Glomerulus shows negligible staining for PARP. In TDF treated the cortex, the glomerulus and convoluted tubules stained for PARP. In the medulla, the collecting tubules and Henle's loop (HL) were positive to PARP stain. (X20)

dependent effect of tenofovir supports an involvement of TFV in KTD. Horberg et al (2010) performed a retrospective cohort analysis in Kaiser Permanente for years 2002 to 2005 comparing renal function among antiretroviral naïve patients initiating a tenofovir-containing regimen (964 patients) or tenofovir-sparing

regimens (683 patients). They evaluated glomerular filtration rate (GFR), serum creatinine, and the development of renal proximal tubular dysfunction in these patients. Tenofovir-exposed patients had greater development of proximal tubular dysfunction over time, reduced GFR and had greater risk of medication disco-

ntinuation, especially as renal function worsened. Accordingly, in the present study, long term high dose TDF administration resulted in damage to the tubules and glomerulus. However, we observed no alterations in plasma creatinine levels and urea levels, the indicators of glomerular function.

Although several recent studies have revealed the nephrotoxicity of tenofovir, the mechanism of nephrotoxicity of TDF is not clear. Studies have suggested that mitochondrial damage may play an important role in TDF induced renal damage (Saumoy et al., 2004; Vidal et al., 2006; Birkus et al., 2002; Kohler et al., 2009; Lebrecht et al., 2009) accordingly.

In the present study electron microscopic examination of the kidneys of TDF treated rats revealed damage to the mitochondria of the proximal tubules. The mitochondria were swollen, cristae were disrupted, and amorphous deposits were observed in the matrix. Our findings are similar to those reported earlier. Mitochondrial swelling is considered to be a characteristic feature of deteriorated function of this organelle. Mitochondria are the energy source of the cell, with two membranes, one of which limits the organelle and the other of which is inside the organelle and is thrown into folds that project inward in a tubular nature called cristae. The components in the electron transport chain, which play the central role in ATP synthesis, are found in the cristae. Thus damage to the cristae can result in the disruption of electron transport chain and hence decrease ATP production by the mitochondria. Although long term TDF administration has been shown to cause mitochondrial damage, the precise mechanism by TDF induced mitochondrial damage results in renal damage is not known.

It is well known that damaged mitochondria are the main sources of reactive oxygen species. To overcome ROS induced damage to the lipids and proteins, cells are equipped with antioxidant defense systems that minimize the susceptibility to ROS. These defense mechanisms include antioxidants such as reduced luthathione and protein thiol, and antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S transferase. In the present study we observed increase in protein carbonyl content, a sensitive indicator of oxidative damage to proteins, decrease in the level of the major intracellular antioxidant glutathione and decrease in the activities of glutathione related enzymes namely glutathione peroxidase, glutathione reductase and glutathione S transferase. Besides, the activity of SOD, an important antioxidant enzyme was decreased in the kidneys of TDF treated rats as compared with control.

Protein carbonyl content (Pco) is reported to be a sensitive and early marker of oxidative stress to tissues as compared with lipid peroxidation (Levine et al., 1990). The present study shows for the first time an increase in Pco content in the kidneys following treatment with TDF. It is well documented that protein

oxidation marks the protein for degradation (Rivette et al., 1985). Proteins (enzymes) regulate various metabolic pathways and damage to proteins may result in the alteration of normal metabolic pathway resulting in cell death and tissue damage.

In general, besides their direct damaging effects on the tissues, reactive oxygen species trigger the accumulation of leukocytes in the tissues involved, and thus aggravates tissue injury indirectly through activated neutrophils. The activated neutrophils secrete myeloperoxidase (MPO) and other proteases (Zimmerman et al., 1992). In turn, MPO plays important role in oxidant production by neutrophils. In the present study a marked elevation (9 fold) in MPO activity was observed after TDF treatment of rats, indicating that neutrophil accumulation contributes to TDF induced small renal damage. The generation of oxidants by neutrophils is critical to host defenses against microbial pathogens (Güngör et al., 2007; Klebanoff et al., 1978). Oxidant production begins with a cytoplasmic membrane associated NADPH oxidase, which reduces molecular oxygen to superoxide. Dismutation of superoxide then yields hydrogen peroxide (H_2O_2), but both superoxide and H_2O_2 are relatively nontoxic to bacteria. However, activated neutrophils also secrete the heme protein MPO (Karras et al., 2003). MPO plays a fundamental role in oxidant production by neutrophils and has been used as an effective quantitative index of inflammation due to correlation between MPO activities and histological analysis of neutrophil infiltration (Sekizuka et al., 1988). A unique activity of MPO is its ability to use chloride as a co-substrate with hydrogen peroxide to generate chlorinating oxidants such as hypochlorous acid, a potent antimicrobial agent. However, evidence has emerged that MPO-derived oxidants contribute to tissue damage and the initiation and propagation of acute and chronic vascular inflammatory disease. The MPO-hydrogen peroxide-chloride system leads to a variety of chlorinated protein and lipid adducts that in turn may cause dysfunction of cells in different compartments of the kidney. Hypochlorous acid reacts readily with amino acids, proteins, carbohydrates, lipids, nucleobases and antioxidants (Pattison et al., 2006). Proteins are the major molecular target for hypochlorous acid. Of primary importance among the hypochlorous acid-mediated protein modifications are tyrosine chlorination, formation of chloramines and carbonyls (Winterbourn et al., 2000), and in some cases cross-linking, (Pattison et al., 2001). Protein carbonyl is a biomarker of oxidative hypochlorous acid attacks on proteins, and in the inflamed lung, for instance, a high correlation between protein carbonyl concentration and MPO activity was observed, Buss et al (2000) Thus, carbonyl groups represent an irreversible protein modification, often leading to the inactivation of the proteins, (Den et al., 2002; Haaften et al., 2001; Dalle-Donne et al., 2006). It is noteworthy to mention that in the present study, a significant increase in MPO activity (9 fold) and protein

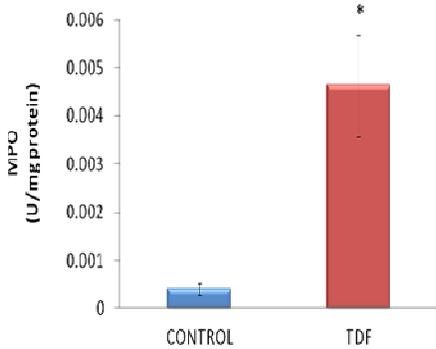


Figure 4. MPO activity in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. * $p < 0.05$ compared with controls.

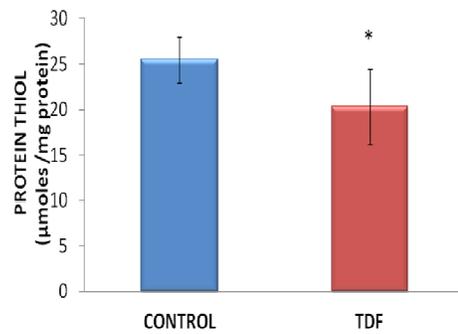


Figure 7. Protein thiol levels in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. * $p < 0.05$ compared with controls.

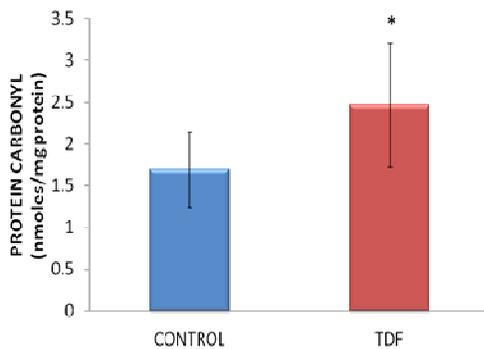


Figure 5. Protein carbonyl content in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group, * $p < 0.05$ compared with controls.

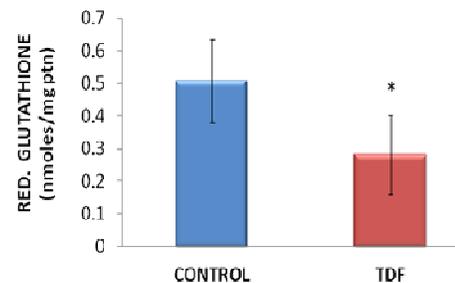


Figure 8. Reduced glutathione levels in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. * $p < 0.05$ compared with controls.

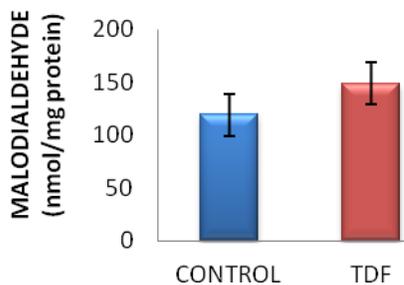


Figure 6. Malodialdehyde levels in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group.

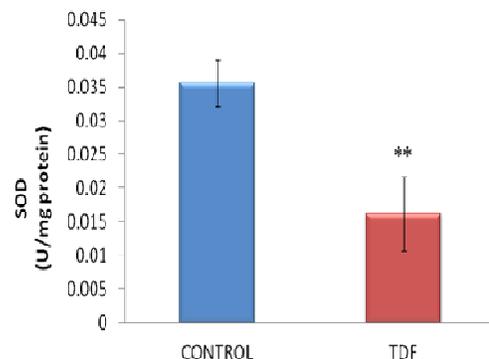


Figure 9. Superoxide dismutase activity in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. ** $p < 0.005$ compared with controls.

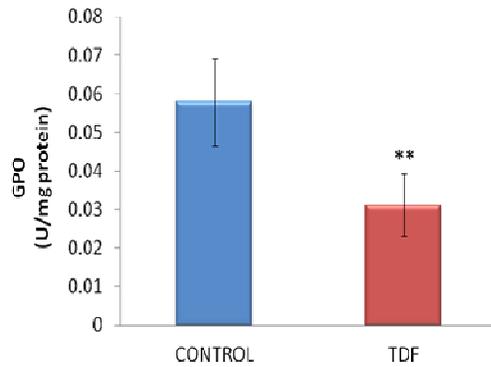


Figure 10. Glutathione Peroxidase activity in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. ** $p < 0.005$ compared with controls.

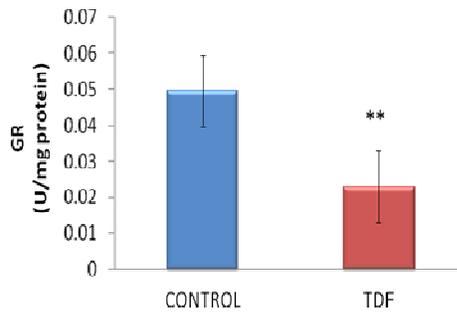


Figure 11. Glutathione Reductase activity in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. ** $p < 0.005$ compared with controls.

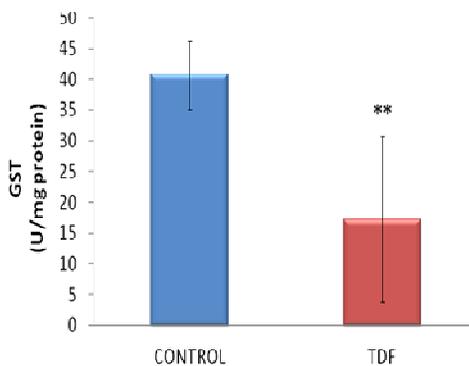


Figure 12. Glutathione S-transferase activity in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. ** $p < 0.005$ compared with controls.

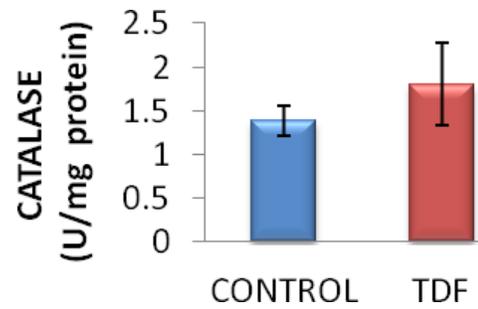


Figure 13. Catalase activity in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group.

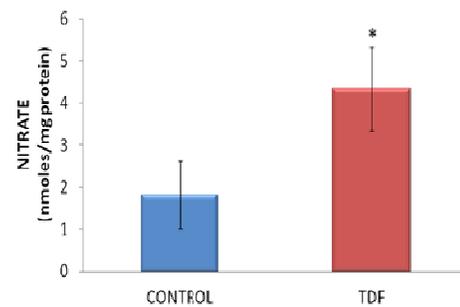


Figure 14. Nitrate levels in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group. * $p < 0.05$ compared with controls.

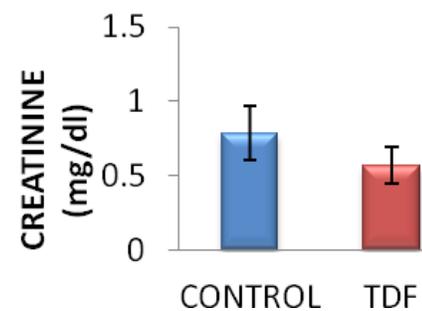


Figure 15. Creatinine levels in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group.

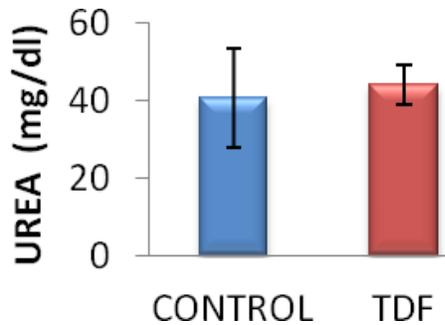


Figure 16. Urea levels in the kidney of control and experimental rats treated with TDF for 35 days. Data represent means \pm SD, 5–7 animals in each group.

carbonyl content (2 fold) was observed in the kidneys of TDF-treated rats. This finding suggests that activated neutrophils contribute to increased ROS generation and oxidative stress observed in TDF treated rat kidneys.

In the present study, significant decrease in the levels of reduced glutathione in the kidneys was observed following treatment with TDF. It is well established that depletion of reduced glutathione in tissues promotes oxidative stress and tissue injury (Sies et al., 1999). The decrease in the activities of the free radical detoxifying enzymes, GPO and GSTase in the kidneys of TDF treated rats observed in the present study may be due to lack of availability of sufficient amounts of reduced glutathione as a coenzyme for these enzymes.

The activity of glutathione reductase, the enzyme crucial for the regeneration of reduced glutathione from oxidized glutathione was significantly less in the kidneys of TDF treated as compared with that of control. The reduced activity of this enzyme may account for the decreased availability of reduced glutathione for scavenging reactive oxygen species, thereby rendering the cells to increased oxidative stress and tissue injury. Thus significant decrease in reduced glutathione levels promoted by TDF, leads to a reduction of effectiveness of the antioxidant enzyme defense system, thereby sensitizing the cells to reactive oxygen species, (Babiak et al., 1998).

With respect to the activities of other antioxidant enzymes, superoxide dismutase and catalase, a significant decrease in the activity was observed with respect to SOD only. SOD provides the first line of defense against superoxide generated in mitochondria. SOD competes with nitric oxide for reaction with superoxide and prevents generation of peroxynitrite, a potent oxidant that can modify proteins to form 3-nitrotyrosine (Johnson et al., 2005). Thus, sufficient amounts of catalytically competent SOD are required to prevent tissue damage. Inactivation of SOD could lead to self-amplification of oxidative stress in the tissues progressively enhancing peroxynitrite production and secondary damage.

In the present study, long term administration of TDF to rats resulted in tubular damage and glomerular damage. This was accompanied by increased oxidative stress and depletion of reduced glutathione and reduced activities of glutathione dependent and other antioxidant enzymes in the kidney. Besides, there was marked increase in the activity of myeloperoxidase, a marker of neutrophil infiltration. Based on these observations it is concluded that oxidative stress, glutathione depletion, decrease in the activities of (Krambovitis et al., 2005) antioxidant enzymes and neutrophil infiltration contribute to TDF induced renal damage in rats. TDF induced oxidative stress in the kidneys may be due to the overproduction of ROS as well as the depletion of cellular antioxidant system. (Krambovitis et al., 2005). The sources of ROS may be damaged mitochondria and activated neutrophils. At present we are investigating whether pretreatment with melatonin prevents TDF induced oxidative stress and renal damage.

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