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A reliable and reproducible rodent model of Tenofovir disoproxil fumarate (TDF) (anti-HIV drug) nephrotoxicity that resembles human TDF tubulopathy.

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Abstract

Tenofovir disoproxil fumarate (TDF) is recommended as a first-line therapy in HIV treatment. However, TDF is nephrotoxic especially with long-term use. Early detection of nephrotoxicity and its prevention are key to avoid irreversible renal damage. This requires knowledge of the mechanism of TDF nephrotoxicity. A reliable animal model is therefore necessary to study the mechanism of TDF nephrotoxicity. To standardize a rodent model of TDF nephrotoxicity resembling humans, adult male Wistar rats were used for the studies. Initially the optimal dose and the duration of treatment required to produce nephrotoxicity in rats was established and it was found that a dose of 600 mg/kg body weight for 5 weeks p.o. was required. The histological changes (in the kidney) and biochemical changes (in the serum and urine) were recorded after treatment at different doses of TDF. At this dose the TDF treated rat kidneys showed structural and functional alterations in the proximal tubule resembling that of HIV patients on TDF therapy. The proximal convoluted tubules were distorted and their lining epithelium was absent. Biochemically, the rats exhibited Fanconi syndrome characterized by bicarbonate wasting, phosphaturia, kaluria, low serum bicarbonate and phosphate. TDF dose of 600 mg/kg body wt. which is 12 x the human dose and the treatment period of 5 weeks induces proximal tubular damage and dysfunction that is very similar to that seen in TDF treated humans. Thus, the rat model appears to be a suitable model for the study of TDF nephrotoxicity.

Keywords: Tenofovir disoproxil fumarate (TDF), Nephrotoxicity, Rat, Proximal tubular dysfunction.

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Introduction

Tenofovir disoproxil fumarate (TDF) is an oral prodrug of tenofovir, which exhibits activity against HIV-1 reverse transcriptase [1, 2]. It is at present the only nucleotide analogue reverse-transcriptase inhibitor (NRTI) approved by the US Food and Drug administration (FDA) for the treatment of AIDS [3]. It is primarily excreted through the kidney via glomerular filtration and active tubular secretion. The 300-mg/day oral TDF regimen [3] is preferred to other anti-retrovirals such as adenofir and cidofir because of its convenience, efficacy, safety, and tolerability [4-6]. However recent studies show that TDF has serious side effects, especially with long-term use.

Nephrotoxicity due to tenofovir treatment of HIV patients has been reported over the past few years [7-11]. Among the principal side effects associated with TDF use were hypophosphatemia, [11-13], renal failure, [14,15] and tubular toxicity [16]. The main site of toxicity appears to be the proximal tubule, and in more severe cases, patients can develop Fanconi syndrome (which is characterized by tubular proteinuria, aminoaciduria, phosphaturia, glycosuria, and bicarbonate wasting (leading to metabolic acidosis) or acute kidney injury [17]. Several case reports, observational studies, animal models and cell culture data support that tenofovir is nephrotoxic for proximal tubular cells [18-21]. It has been suggested that TDF can cause direct proximal tubular damage, which may lead to renal failure [22].

It is well established that TDF targets the mitochondria of the proximal tubular epithelium. Morphological evidence of mitochondrial toxicity has been reported in human biopsies of tenofovir treated HIV patients [13,18]. In the TDF treated HIV patients who underwent kidney biopsy, the main abnormality on light microscopy was acute proximal tubule damage, and eosinophilic intracytoplasmic inclusions formed of giant mitochondria [13]. Electron microscopy showed mitochondria of marked variations in size and shape and disruption of cristae, mitochondrial swelling, and accumulation of crystals in the mitochondrial matrix [18]. However, how TDF causes

mitochondrial damage is not clear. In order to investigate TDF nephrotoxicity, a reliable animal model is necessary. Although a few models have been proposed earlier, to date there is no standard animal model that resembles that of humans histologically and biochemically. In the present study we describe a reliable and reproducible rat model of TDF nephrotoxicity which resembles that of humans both histologically and biochemically. This model may be useful to study the nephrotoxicity of TDF as well as to carry out intervention studies.

Methods

Animals and animal treatment

The rat is proven to be a useful model for the study of nephrotoxicity of a number of agents including gentamicin, an antibiotic, cisplatin, a chemotherapeutic drug, and acetaminophen, an antipyretic drug. Therefore, we chose to standardise a rat model of TDF nephrotoxicity.

Adult male Wistar rats (200-250 g) were used for the studies. They were housed in standard rat cages (421 × 290 × 190 mm). All animals were exposed to 12 hour light-dark cycles and allowed access ad libitum to water and rat chow. The experiments done were approved by the institutional animal ethics committee and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

Study 1

The daily dose of TDF in humans is 300 mg/60 kg [3]. Therefore to remain clinically relevant, we administered TDF daily by gavage (morning) to adult male Wistar rats at doses that resembled human therapy on an mg/kg/day basis i.e., 5 mg/kg; -250 g rat=1.25 mg/day) for 5 weeks. Based on a number of NRTI treatment protocols used by other workers, maximum treatment duration of 5 weeks was used in this study as the duration of treatment for 5 weeks is suggested to model chronic human treatment [23]. The rats were sacrificed 24 hrs. After the final dose of TDF, the kidneys processed for histological studies.

Glomeruli and tubules appeared to be intact in the TDF-treated rats. EM studies showed normal renal proximal tubular epithelial cells with characteristic, oval mitochondria having densely packed cristae (Figure not shown).

Study 2

We next tried the dose that was used by Lebrecht et al. [21]. Three adult male Wistar rats were treated by oral gavage once a day for 8 weeks with 100 mg/kg body wt. of TDF. Based on area under the curve exposure, the TDF dose used in these rats is about twice the clinical dose used in patients [3]. We could not find any renal abnormalities when examined by light microscopy and electron microscopy. Therefore we tried a higher dose that was used by Biesecker et al. [24].

Study 3

Biesecker et al., [24] have tried a higher dose of TDF -300 mg/kg body weight/day (which is 6 × the human dose) over a period of 4 weeks. Treatment related clinical observations reported by them were slight increase in blood urea nitrogen (BUN) with normal creatinine, decrease in urinary phosphorus and increase calcium. Minimal renal proximal tubular epithelial karyomegaly was observed but they could not find any mitochondrial abnormalities. When we tried 300 mg/kg body weight/day for 4 weeks on 3 adult male Wistar rats we also could not find any proximal tubular abnormalities (figure not shown). Therefore, we next checked whether a longer duration of treatment i.e., 300 mg/kg body wt. per day for 8 weeks produces renal tubular toxicity. No mitochondrial injury was observed by electron microscopy in the rats at this dose and duration of treatment also (Figure 1).

Study 4

Tenofovir has been shown to cause bone toxicity in animal models, when given at 6–12 times higher dose than recommended for humans [25]. Therefore, we tried 600 mg/kg body weight/day (corresponds to 12 × human dose). Based on a number of NRTI treatment protocols used by other workers, maximum treatment duration of 5 weeks was used in these studies as the duration of treatment for 5 weeks is suggested to model chronic human treatment [29].

We administered 600 mg TDF/kg body weight/day in two divided dose of by gavage for 5 weeks to 3 adult male Wistar rats. TDF treatment for 5 weeks showed

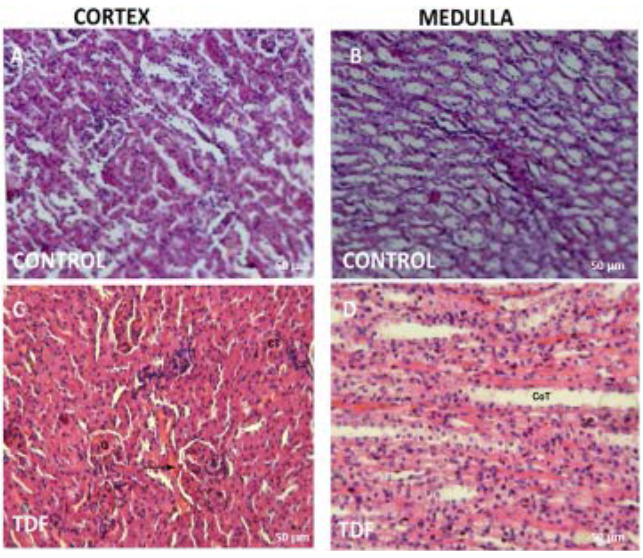

Figure 1: *Representative light micrographs of rat kidney. (A) Renal cortex of a control rat showing normal architecture [H & E, 200X]; (B) Renal medulla of a control rat showing normal architecture [H & E, 200X]; (C) Renal cortex of a 300 mg/kg/d TDF treated rat showing almost normal structure, (H & E, 200X); (D) Renal medulla of a 300 mg/kg/d TDF treated rat showing normal architecture (H & E, 200X).*

severe morphologic abnormalities in proximal tubule mitochondria, such as variations in size and shape (giant mitochondria), disruption of cristae, mitochondrial swelling, and presence of amorphous deposits in the mitochondrial matrix, the findings that were in close comparison with human kidney biopsies from TDF treated HIV patients.

Standardisation of rat model of TDF tubulopathy

The rats were assigned randomly into 2 groups and were treated as follow.

Group I (control): The rats in this group (n=3) received sterile water.

Group II: The rats (n=6) in this group received 600 mg/kg body weight TDF divided into two doses, one in the morning and one in the evening by gavage for 5 weeks.

Control animals were treated with sterile water on the same schedule as TDF treatment and were killed at the same time point for TDF treated and control rats.

Mortality check and body weights

Animals were checked daily for morbidity or death. Body weights were measured daily just before gavage dosing.

Collection of blood, urine and kidneys

Twenty-four hours before sacrifice, the rats were placed individually in metabolic cages, and urine was collected for biochemical analysis. On the 36th day, after overnight fast, blood samples were collected from the rats under halothane anesthesia, by cardiac puncture into tubes and allowed to clot at room temperature. Thereafter, serum was separated by centrifugation at 1200 g for 15 min at 4°C for clinical chemistry. Then the animals were sacrificed by over dose of halothane anesthesia. The abdomen was opened by midline incision and kidney was dissected out carefully and cleaned off the extraneous tissue and weighed. Half of left kidney was cut in cross-section and fixed in 10% buffered formalin for light microscopy, and the remaining half was fixed in 3% glutaraldehyde for electron microscopy.

Morphological examination of the kidney

After fixation of kidney tissues in 10% buffered formalin for 24 h at room temperature, the slices were embedded in paraffin and then sectioned. Four micrometer-thick paraffin sections were stained with hematoxylin and eosin for light microscope examination using conventional protocol [26]. A minimum of 8 fields for each kidney section were examined and assigned for severity of changes by an observer blinded to the treatments of the animals. Since the tenofovir-induced morphological abnormalities in rat kidney are mainly localized in the proximal tubules, and the other structures of the kidney do not exhibit major histological alterations, only the renal cortex was examined in detail.

Examination of the ultrastructural changes in the kidney tissues by electron microscope (EM)

Electron microscopy was done based on the methods employed routinely in the Lewis lab [27]. The kidney tissues were fixed in 3% glutaraldehyde and washed in buffer, post fixed by 1% osmium tetraoxide 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. Sections (0.5 μ) were cut with glass knives and stained with Toluidine Blue for orientation. Ultrathin (900 Å) sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined by EM, evaluated and photographed. Each EM photomicrograph was reviewed independently by two investigators. Parameters included presence of structurally abnormal mitochondria, numbers of mitochondrial profiles per field, mitochondrial swelling, abnormal cristae density, cristae disruption, and accumulation of intra-mitochondrial crystals [28].

Serum clinical chemistry

Serum was separated out and used for the estimation of phosphate, potassium, bicarbonate, glucose, urea and creatinine by standard spectrophotometric methods.

Urinalysis

Characteristic features associated with mitochondrial dysfunction in proximal tubular cells include phosphaturia, bicarbonate wasting, tubular proteinuria, glycosuria, and aminoaciduria - acquired Fanconi Syndrome [22]. Urine samples were centrifuged to remove suspended material, and the supernatants were used for the estimation of bicarbonate, phosphate, and potassium by standard spectrophotometric methods. Glucose and protein were semi quantified by dipstick. Low molecular weight proteins in urine were detected by SDS PAGE.

Detection of low molecular weight proteins in urine by SDS PAGE

Urine proteins were measured by Lowry's method and fractionated by SDS-PAGE using 8% resolving gel and 5% stacking gel [29]. Each sample containing 100 μg of urinary protein was mixed with protein dissociation buffer in the ratio of 1:1 and kept in a boiling water bath for 5 mins. Samples were briefly centrifuged; they were then loaded onto wells. Running gel buffer (pH 8.6) was added to electrophoresis tank. The apparatus was connected to the power pack and was run at 70V till the sample reached the separating gel. The voltage applied was increased to 90V at this point. Electrophoresis was stopped when the marker dye reached near the end of the gel. After electrophoretic separation, the gel was stained with Coomassie blue solution (0.01% Coomassie brilliant blue R 250, 50% (v/v) methanol and10% (v/v) glacial acetic acid) for 3 h at room temperature and subsequently destained in the destaining solution (50% (v/v) methanol and 10% (v/v) acetic acid) for 2 h. The gel image was captured and analysed by a gel documentation system (Alpha Innotech).

Assessment of mitochondrial function using respiratory control ratio (RCR)

The respiratory control ratio is the single most useful general measure of function in isolated mitochondria. High RCR indicates good function, and low RCR usually indicates dysfunction. Therefor the RCR ratio was carried out on mitochondria isolated from the kidney.

Isolation of kidney mitochondria

The kidney tissues were homogenized (5%) using the homogenizing buffer consisting of 220 mM Mannitol/70 mM sucrose/5 mM Tris/1 mM EGTA; pH 7.4. The homogenates were centrifuged at 4000 × g for 10 min, and the nuclear pellet was discarded. Crude mitochondrial fractions were obtained by centrifuging at 12,000×g for 20 min, and the pellet was washed thrice with wash buffer containing 220 mM mannitol/70 mM sucrose/20 mM HEPES; pH 7.4 [30]. The final pellet was suspended in the same buffer. The purity of the mitochondria was established by enrichment of marker enzyme, succinate dehydrogenase. The activity of succinate dehydrogenase was assayed using INT as an electron acceptor, which forms formazan crystals on reduction [31]. The isolated mitochondria were used for assessing RCR.

Measurement of oxygen uptake (RCR)

Oxygen uptake was determined polarographically using a Clark-type electrode in 2 ml of respiratory buffer (225 mM sucrose, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 20 mM KCl, 10 mM Tris, and 5 mM HEPES pH 7.4), containing 5 mM succinate as a respiratory substrate [32]. About 2 mg/ml of mitochondrial protein was introduced into the oxygen electrode compartment (Rank oxygen electrode). The electrode output was connected to an appropriate recorder, and the recorder was set such that 100% full range corresponded to the total oxygen content of the mixture. Oxygen uptake was stimulated with 0.3 mM ADP, and the rate of states 3 and 4 respirations was measured. Oxygen uptake was calculated from the decrease in the percentage saturation of the mixture. The ratio of state 3/state 4 respiratory rates was calculated for RCR.

Statistical Analysis

The results are expressed as mean ± SD. Significant statistical differences between the two groups were evaluated using Student's t test. P value ≤ 0.05 was taken as statistically significant.

Results

Effect of TDF administration on morbidity or death of rats

All the rats (control and TDF treated) survived the treatment period of 5 weeks. The rats did not show any signs of morbidity.

Chronic TDF treatment decreases the body wt. and kidney weight of rats

There was significant difference in body weight and kidney weights between control and TDF treated rats at the time of sacrifice (Figure 2). The kidney/body weight ratio was significantly lower in the TDF treated rats as compared with control.

Chronic TDF treatment results in proximal tubular atrophy and degeneration

Since the tenofovir-induced morphological abnormalities in are mainly localized in the proximal tubules , and the other structures of the kidney do not exhibit major histological alterations, only the renal cortex was examined in detail. The kidneys of control rats showed normal morphology. Sections from control group showed normal histological structure of the glomeruli and renal tubules in the cortex (Figure 3A) and normal tubules in the medulla (Figure 3B). TDF induced renal damage involved mainly the cortex and to a lesser extent the medulla. The proximal convoluted tubules were distorted and their lining epithelium was destroyed. Interstitial edema was present. However, there was no evidence of necrosis (Figure 3C). The glomeruli were reduced in number. Sections from

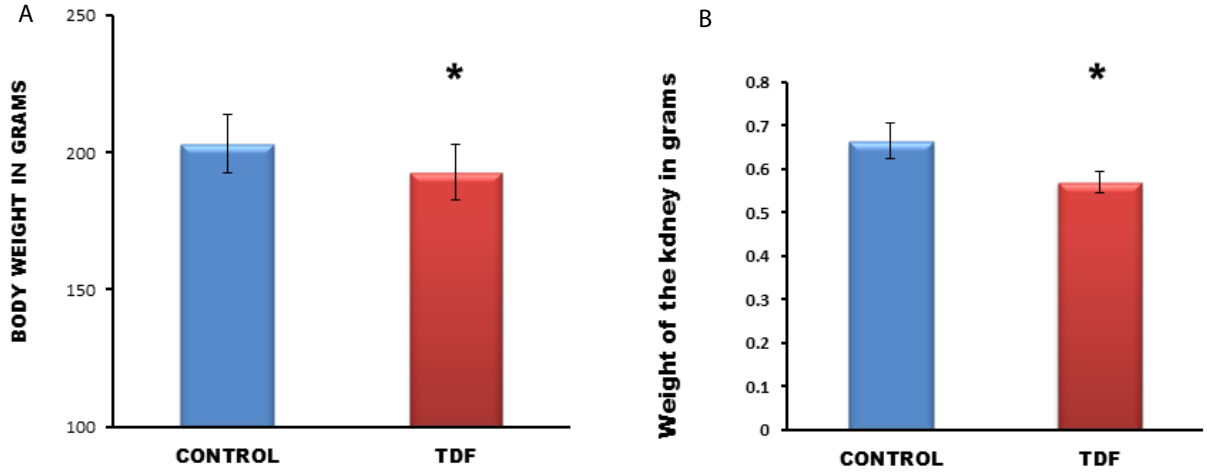

Figure 2: *Body weight and kidney weight of control rats and TDF treated rats. Significant reduction in body weights and kidney weights between control rats and TDF treated rats. Values represent mean ± S.D., n=6. *P<0.05 vs. control.*

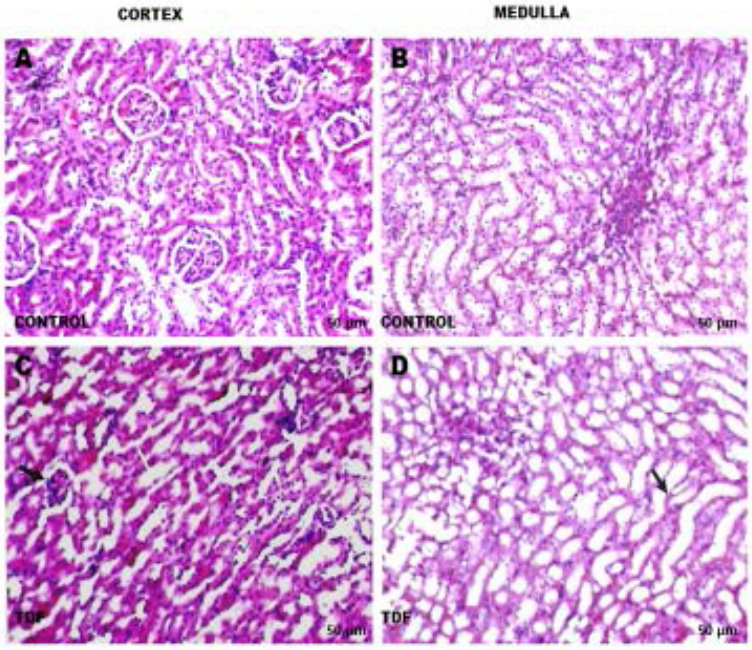

Figure 3: *Representative light micrographs of rat kidney. (A) Renal cortex of a control rat-shows normal architecture [H& E, 200X]; (B) Renal medulla of a control rat shows normal architecture [H & E, 200X]; (C) renal cortex of a TDF treated rat. The proximal convoluted tubules were distorted and their lining epithelium was destroyed (white arrow, H & E, 200X). Some glomeruli were shrunken (black arrow); (D) Renal medulla of a TDF treated rat–There was mild destruction of the lining epithelium of the loops of Henle and the convoluted tubules (black arrow) H & E, 200X.*

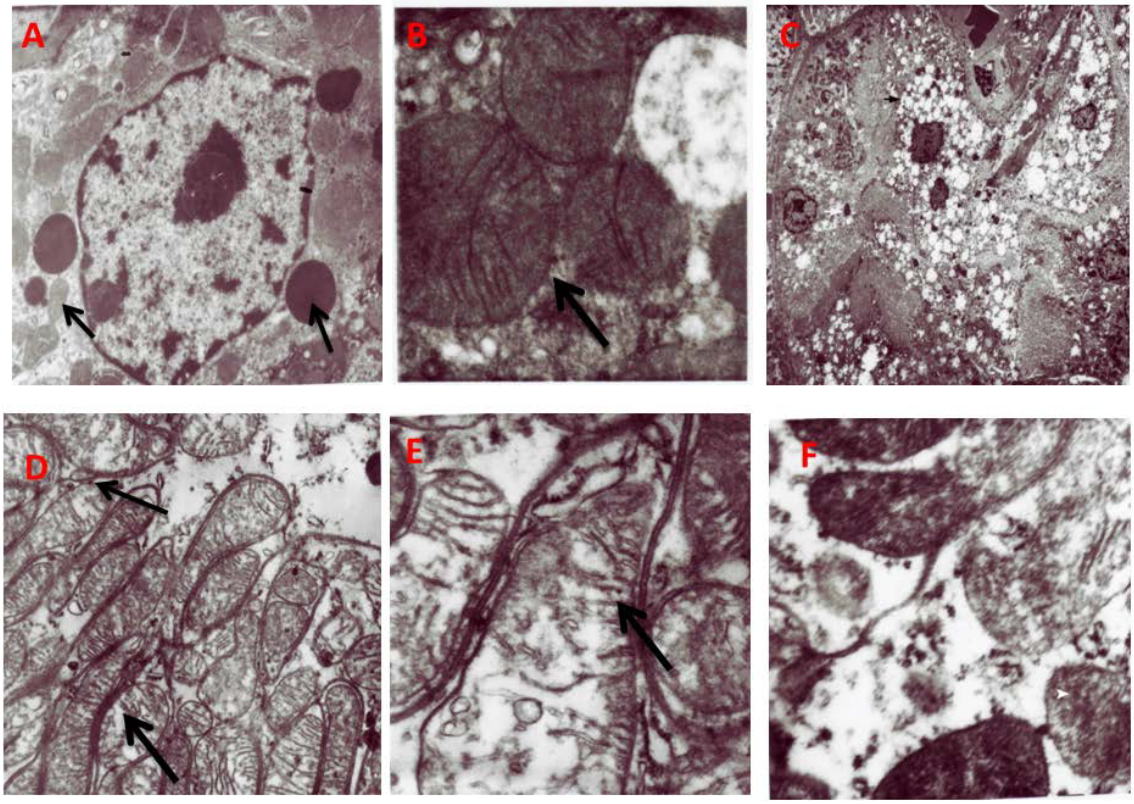

Figure 4: *Representative electron micrographs of tubular mitochondria. A) Normal Kidney tubules (original magnification × 22000); (B) Normal mitochondrial structure (black arrow) in the renal tubules of control rats (original magnification × 22000); (C) Vacuoles seen in the cytoplasm of the kidney tubule (black arrow) Less number of lysosomes (white arrow); (D) Swollen mitochondria; (M) black arrow(original magnification × 22000) E; Disruption of mitochondrial cristae (black arrow) in the renal tubules of TDF treated rats; (F) Amorphous deposits in the mitochondrial matrix (white arrow) × 22,000.*

TDF treated rat kidney medulla showed mild distortion of renal tubular epithelium. There was a diffuse epithelial cell shrinkage observed, suggestive of apoptotic changes (Figure 3D).

TDF treatment causes severe damage to the proximal tubular mitochondria

Vehicle-treated rats showed normal tubular structure with numerous mitochondria and lysosomes (black arrows) (Figure 4A). Oval mitochondria with densely packed cristae were observed (Figure 4B). Proximal tubular epithelia of TDF-treated rats showed moderate to severe damage to the mitochondria. The cytoplasm showed increased number of vacuoles and reduced number of lysosomes. Nucleus appeared shrunken (Figure 4C). The mitochondria showed marked variations in size and shape. Mitochondrial toxicity included swollen (giant) mitochondria (Figure 4D), disrupted cristae (Figure 4E), and accumulation of amorphous deposits in the mitochondrial matrix (Figure 4F). An increase in the number of mitochondria with irregular shape and fragmented cristae was observed in the cytoplasm of basal part of tubule cell. In some epithelial cells, the mitochondria were reduced in number. These findings suggest that TDF targets mainly the proximal tubular mitochondria and to a lesser extent the lysosomes and nucleus.

TDF treatment causes mitochondrial dysfunction

RCR was reduced by 44% in the kidneys of TDF-treated rats, suggesting mitochondrial dysfunction (Figure 5). Decreased RCR indicates uncoupling of oxidative phosphorylation and suggests extensive mitochondrial damage.

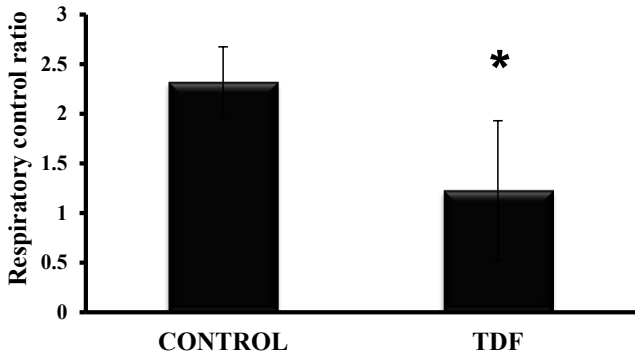

Figure 5: *RCR in the mitochondria isolated from TDF-treated kidneys and control rat kidneys. Values represent mean ± S.D., n=6. *P<0.05 vs. control.*

Table 1: *Serum and urine levels of potassium, phosphate, bicarbonate, and glucose in TDF treated rats and control rats.*

Parameter	Control (n=6)	Test (n=6)
Phosphate (mmol/L)	5.88 ± 0.78	4.10 ± 0.36*
Potassium (mmol/L)	6.31 ± 0.47	5.58 ± 0.51*
Glucose (mg %)	94.33 ± 12.6	90.8 ± 15.4
Urine		
Bicarbonate (mmol/L)	0.25 ± 0.058	2.56 ± 1.21**
Potassium (mmol/L)	2.68 ± 1.3	10.38 ± 2.9**
Phosphate (mmol/L)	0.27 ± 0.1	4.68 ± 1.52*

Values are represented as mean ± S.D., n=number of rats. *P<0.05, **P<0.01 vs. control

TDF treatment causes proximal tubular dysfunction - acquired Fanconi syndrome

Chronic TDF treatment has been shown to induce proximal renal tubular dysfunction that resembles Fanconi syndrome (Table 1), which is characterized by increased urinary losses of bicarbonate, phosphate, amino acids, glucose and other nutrients due to decreased reabsorption at the proximal renal tubuli. Proximal tubular function was impaired in TDF-treated rats, as evidenced by increased urinary excretion of phosphate, potassium and bicarbonate and a considerable reduction in serum phosphate, bicarbonate, and potassium.

TDF causes tubular proteinuria

We found no proteinuria or glycosuria using dipstick. SDS electrophoresis is a useful technique as it identifies low molecular proteins i.e., tubular proteins with mol. wt. less than 55,000 using a molecular weight marker protein. In SDS-PAGE proteins are separated based on their molecular weight. Individual proteins can then be identified within these patterns.

Urine from normal rats when subjected to electrophoresis yielded an identifiable protein band that corresponded to approximately Mr.60, 000, suggestive of albumin (Figure 6). In addition, faint bands were also observed in some controls corresponding to molecular weight less than 55 KDa. α1 microglobulin is also detectable in normal urine. Thus, normal rats appear to excrete detectable amount of albumin (by electrophoresis), and negligible amount of low molecular weight tubular proteins in urine.

Tubular proteinuria is characterized by the dominant excretion of low-molecular-weight proteins such as alpha 1-microglobulin or retinol-binding protein (RBP), which correlate better with the extent of tubulo-interstitial damage than does the determination of total 24-h protein levels. The urine protein pattern in the TDF treated rats revealed at least two bands of molecular weight lesser than 55 KDa suggesting tubular dysfunction.

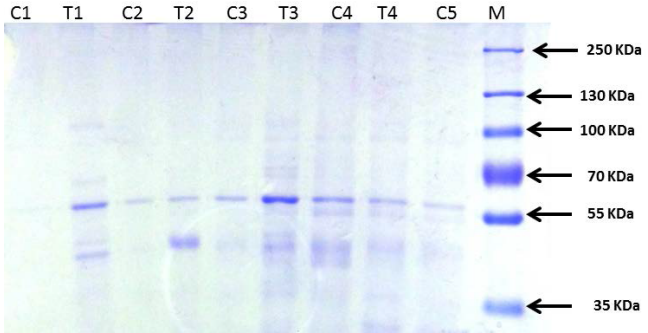

Figure 6: *Urine protein separation by SDS-PAGE electrophoresis showing low molecular weight proteins (<55 KDa) in the TDF treated rats.*

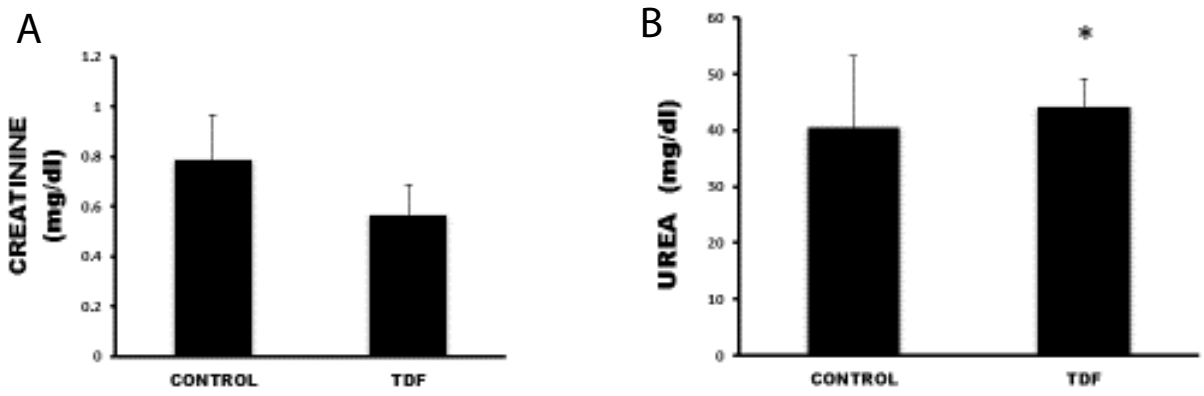

Figure 7: *Serum creatinine levels and urea levels in control rats and TDF treated rats. Values represent mean ± S.D., n=6. *P<0.05 vs. control.*

TDF causes no significant change in serum creatinine levels but increases urea levels mildly

Serum urea levels were increased slightly in the TDF treated rats as compared with control (Figure 7). TDF treatment had no significant effect on the serum creatinine values. It has been demonstrated that renal tubular dysfunction can occur in patients with or without a decrease in glomerular filtration rate [33] as decline of renal function is evident only after persistent tubular injury.

Discussion

Long term exposure to TDF is associated with an increased risk over time of kidney tubular abnormalities in the absence of significant impaired glomerular function (serum creatinine, GFR etc.]. The main site of toxicity seems to be the proximal tubule, and in more severe cases, patients can develop Fanconi syndrome (which is characterized by tubular proteinuria, aminoaciduria, phosphaturia, glycosuria, and bicarbonate wasting [17] or acute kidney injury. In order to investigate TDF nephrotoxicity a reliable animal model is necessary. Although a few models have been proposed earlier, to date there is no standard animal model that is available for the study of mechanism of TDF induced nephrotoxicity. The rat is proven to be a useful model for the study of nephrotoxicity of a number of agents, including gentamicin, an antibiotic, cisplatin, a chemotherapeutic drug, and acetaminophen, an antipyretic drug. Therefore, we chose to standardise a rat model of TDF nephrotoxicity.

We tried different doses of TDF and different time period of treatment. We tried 300 mg/60 kg body weight/day (human dose) [3] for 5 weeks, 100 mg/kg body weight/day for 8 weeks [21], 300 mg/kg body weight/day for 8 weeks [24], and finally 600 mg/kg body weight/day for 5 weeks. We could find proximal tubular damage and dysfunction only in those rats that were treated with 600 mg/kg body wt. TDF/day. Histologically we were able to observe proximal tubular damage, and mitochondrial abnormalities as seen in human biopsies. Proximal tubular function was also impaired in TDF-treated rats, as evidenced by increased urinary excretion of phosphate, potassium and bicarbonate and a considerable reduction in serum phosphate, bicarbonate, and potassium. However, there was no significant change in serum creatinine values between TDF treated rats and control rats, suggesting that glomerular functions were unaffected upon TDF treatment, just as seen in humans on TDF therapy [33].

In the present study we have used high dose of TDF (12 × human dose) to produce proximal tubular damage and dysfunction in rats. The requirement of very high dose of TDF to produce a rat model of TDF nephropathy may be attributed to 1. Usage of normal rats instead of HIV infected ones as HIV itself is known to affect the renal functions in humans; 2. The administration of TDF only and no other drug unlike humans who may be on other antiretroviral drugs or any other drug that may affect renal function or clearance; 3. Induction of renal tubular toxicity within a short period of time. This may justify the requirement of high dose of TDF in order to produce TDF tubulopathy in rats.

Conclusion

This rat model is a good model for the study of TDF nephrotoxicity as it resembles that of TDF nephrotoxicity in humans both structurally and biochemically. The model will be useful not only for the study of nephrotoxicity of TDF but also for carrying out intervention studies. This model will also permit to study drug interactions by overcoming the limitations of cell culture and the difficulties of obtaining human kidney samples. A limitation of this model in our opinion is the usage of 12 × dose of TDF used in humans in order to produce proximal tubular nephropathy.

Acknowledgments

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