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Carvedilol and trimetazidine attenuates ferric nitrilotriacetate-induced oxidative renal injury in rats

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Abstract

Intraperitoneal (i.p.) injection of ferric nitrilotriacetate (Fe-NTA) induces acute proximal tubular necrosis as a consequence of lipid peroxidation and oxidative tissue damage, which eventually leads to high incidence of renal adenocarcinoma in rodents. This study was designed to investigate the effect of carvedilol, an antihypertensive and trimetazidine, an antiischemic, both the drugs with additional antioxidative potentials, on Fe-NTA induced nephrotoxicity in rats. One hour after a single i.p. injection of Fe-NTA (8 mg iron per kg), a marked deterioration of renal architecture and renal function as evidenced by a sharp increase in blood urea nitrogen (BUN) and serum creatinine was observed. Fe-NTA induced a significant renal oxidative stress demonstrated by elevated thiobarbituric acid reacting substances (TBARS) and reduction in activities of renal catalase, superoxide dismutase (SOD) and glutathione reductase (GR). Pretreatment of animals with carvedilol (2 mg/kg, i.p.) as well as with trimetazidine (3 mg/kg, i.p.), 30 min before Fe-NTA administration markedly attenuated renal dysfunction, reduced elevated TBARS, restored the depleted renal antioxidant enzymes and normalised the renal morphological alterations. These results clearly demonstrate the role of oxidative stress and its relation to renal dysfunction, and suggest a protective effect of carvedilol and trimetazidine on Fe-NTA-induced nephrotoxicity in rats.

Keywords: Ferric-nitrilotriacetate (Fe-NTA); Oxidative stress; Carvedilol; Trimetazidine

1. Introduction

Ferric-nitrilotriacetate (Fe-NTA), an iron chelate of nitrilotriacetic acid, causes iron mediated free radical injury and cancer in the rodent kidney (Okada, 1996). A high incidence of renal cell carcinoma is prevalent in mice and rats following repeated intraperitoneal (i.p.) injections of Fe-NTA (Ebina et al., 1986; Li et al., 1987). Redox-active iron, responsible for generating oxygen free radicals (OFR) has been detected in the serum of rats treated with Fe-NTA (Liu et al., 1993; Zhang et al., 1995).

Various studies have shown that Fe-NTA causes high levels of lipid peroxidation products [thio-

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barbituric acid-reactive substances, malondialdehyde (MDA), hydroxynonenal (HNE) and HNE-modified proteins] and oxidative DNA damage such as 8-hydroxydeoxyguanosine (8-OH-dG) in rat kidney (Preece et al., 1985; Umemura et al., 1990a,b Toyokuni et al., 1994).

Carvedilol, a novel vasodilating β-blocker, a potent competitive antagonist of β_1, β_2-adrenoceptors and α_1-adrenoceptors is used in the treatment of hypertension, angina and congestive heart failure. Carvedilol and certain of its metabolites (SB 211475 and SB 209995) are potent antioxidants and this activity has been attributed to the carbazole moiety of the drug (Feuerstein et al., 1992; Yue et al., 1992a,b,c, 1993; Feuerstein and Yue, 1994; Maggi et al., 1996; Tadolini and Franconi, 1998). This antioxidative function would serve as an additional benefit given that carvedilol is used in the treatment of patients with reno-vasuclar hypertension. However, the mechanism of its antioxidative action is still unclear. It scavenges the free radicals as well as forms a complex with iron ion (Oettl et al., 2001). The protective effects of this drug have been demonstrated in a variety of in vitro and in vivo systems (Noguchi et al., 2000; Cargnoni et al., 2000; Yue et al., 1992a).

Trimetazidine is an anti-ischemic drug that restores the ability of the ischemic cells to produce energy and reduces the generation of oxygen derived free radicals (Inci et al., 2001). Various experimental studies have shown that trimetazidine has preserved the intracellular concentrations of ATP and inhibited the extracellular leakage of potassium during cellular ischemia. Additionally, it prevents excessive release of free radicals, which are particularly toxic to phospholipids membranes and are responsible for both the fall in the intracellular ATP concentration and the extracellular leakage of potassium (Inci et al., 2001).

In order to substantiate the in vivo antioxidant and renoprotective activity of carvedilol and trimetazidine, the present study was designed to investigate their effect on Fe-NTA-induced oxidative damage in the rat kidney.

2. Materials and methods

2.1. Animals

Male Wistar rats (150–200 g) bred in the central animal house of Punjab University (Chandigarh, India) were used. The animals were housed under standard conditions of light and dark cycle with free access to food (Hindustan Lever Products, Kolkata, India) and water. The experimental protocols were approved by the institutional animal ethics committee of Punjab University, Chandigarh.

2.2. Drugs

Trimetazidine and Carvedilol were kind gifts from Themis Labs and Zydus-Medica (Mumbai, Ahmedabad, India), respectively. Disodium Nitrilotriacetate (Na_2NTA) was purchased from Sigma (St. Louis, MO, USA) and Ferric nitrate ($Fe(NO_3)_3$) was purchased from E. Merck (India) Ltd. (Mumbai, India).

2.3. Preparation of ferric nitrilotriacetate (Fe-NTA) solution

The Fe-NTA solution was prepared by the method of Awai et al. (1979). Briefly, for the preparation of Fe-NTA, ferric nitrate (0.16 mmoles/kg body weight) solution was mixed with a 4-fold molar excess of disodium salt of NTA (0.64 mmoles/kg body weight) and the pH was adjusted to 7.4 with a sodium bicarbonate solution. The solution was prepared immediately before its use.

2.4. Study design

Four groups were employed in the present study, each consisting of five to seven animals. Group I served as control group and received saline only. Group II animals received Fe-NTA (8 mg iron per kg body weight i.p.). Group III animals received carvedilol (2 mg/kg i.p.) 30 min prior to the administration of Fe-NTA and Group IV animals were given trimetazidine (3 mg/kg, i.p.). One hour after the Fe-NTA administration,

all animals were sacrificed with a high dose of anaesthesia and the blood was collected in heparinised centrifuge tubes through the abdominal aorta. The blood samples were centrifuged and plasma was collected.

2.5. Assessment of renal function

Plasma samples were assayed for blood urea nitrogen (BUN) and serum creatinine by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

2.6. Post mitochondrial supernatant preparation (PMS)

The animals were sacrificed 1 h after Fe-NTA treatment. Their kidneys were quickly removed, perfused immediately with ice cold saline (0.9% sodium chloride) and homogenised in chilled potassium chloride (1.17%) using a Potter Elvehjem homogeniser. The homogenate was centrifuged at $800 \times g$ for 5 min at 4 °C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at $10\,500 \times g$ for 20 min at 4 °C to get the post mitochondrial supernatant which was used to assay reduced glutathione (GSH), glutathione reductase (GR), catalase, and superoxide dismutase (SOD) activity.

2.7. Estimation of lipid peroxidation

The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) (Ohkawa et al., 1979). In brief the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10%(w/v) of PMS. The mixture was brought up to 4.0 ml with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of *n*-butanol and pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm. TBARS were quantified using an extinction coefficient of 1.56×10^5 per M cm and expressed as nmol of TBARS per mg protein. Tissue protein was estimated using Biuret method (Varley, 1988) of protein assay and the renal MDA content expressed as nanomoles of MDA per milligram of protein.

2.8. Estimation of reduced glutathione

GSH in the kidney was assayed by the method of Jollow et al. (1974). Briefly 1.0 ml of PMS (10%) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and than subjected to centrifugation at $1200 \times g$ for 15 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot and 2.7 ml phosphate buffer (0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm on a spectrophotometer.

2.9. Glutathione reductase activity

GR was assayed by the method of Carlberg and Mannerviek (1975) as modified by Mohandas et al. (1984). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidised glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml PMS (10%) in a total volume of 2.0 ml. The enzyme activity was quantitated at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as units/l.

2.10. Catalase activity

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml PMS (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of k/min.

2.11. SOD activity

SOD activity was assayed by the method of Kono (1978). The assay system consisted of EDTA

0.1 mM, sodium carbonate 50 mM and 96 mM of nitro blue tetrazolium (NBT). In the cuvette 2 ml of above mixture, 0.05 ml hydroxylamine and 0.05 ml of PMS were taken and the auto-oxidation of hydroxylamine was observed by measuring the absorbance at 560 nm.

2.12. Renal histology

The right kidney was isolated immediately after sacrificing the animal and washed with ice-cold saline. It was then fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. 5 m thick sections were cut, deparaffinised, hydrated and stained with hematoxylin and eosin. The renal sections were examined in blind fashion for tubular brush border loss, interstitial oedema, tubular dilatation, and necrosis of epithelium and hyaline casts in all treatments. A minimum of 10 fields for each kidney slide were examined and assigned for severity of changes using scores on a scale of none (−), mild (+), moderate (++) and severe (+++) damage.

2.13. Statistical analysis

Values are expressed as means ± S.E.M. One way analysis of variance (ANOVA) followed by Dunnett's test was applied to calculate the statistical significance between various groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of carvedilol and trimetazidine on Fe-NTA-induced renal dysfunction

The Fe-NTA treatment significantly increased the levels of serum creatinine and BUN. Carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) produced a significant decrease in BUN and plasma creatinine (Fig. 1a, b).

3.2. Effect of carvedilol and trimetazidine on Fe-NTA- induced lipid peroxidation

TBARS levels were increased significantly by Fe-NTA treatment as compared with the control group. Treatment with carvedilol (2 mg/kg, i.p.) as well as trimetazidine (3 mg/kg, i.p.) produced a significant reduction in TBARS in Fe-NTA treated rats (Fig. 2).

3.3. Effect of carvedilol and trimetazidine on Fe-NTA-induced changes in the antioxidant pool

Treatment with Fe-NTA significantly decreased the enzymatic activity of GSH, GR, catalase (CAT), and SOD. This reduction was significantly improved by treatments with carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) (Figs. 3–6).

3.4. Effect of carvedilol and trimetazidine on Fe-NTA- induced changes on renal morphology

The histopathological changes were graded and summarised in Table 1. The control group did not show any morphological changes. By contrast, the kidneys of rats treated with Fe-NTA showed marked histological changes in the cortex and outer medulla. The renal sections showed tubular brush border loss, interstitial oedema, tubular dilatation, necrosis of epithelium and hyaline casts. Treatment with carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) preserved the normal morphology of the kidney (Fig. 7), and shows normal glomeruli, no cast formation and slight oedema of the tubular cells.

4. Discussion

Intraperitoneally injected Fe-NTA is absorbed into the portal vein through mesothelium and passes into circulation via the liver (Umemura et al., 1990b). The low molecular weight Fe-NTA is easily filtered through the glomeruli into the lumen of the renal proximal tubules where Fe^{3+}-NTA is reduced to Fe^{2+}-NTA by the glutathione degradation products cysteine or cysteinglycine (Tsao and Curthoys, 1980; Guder and Ross, 1984;

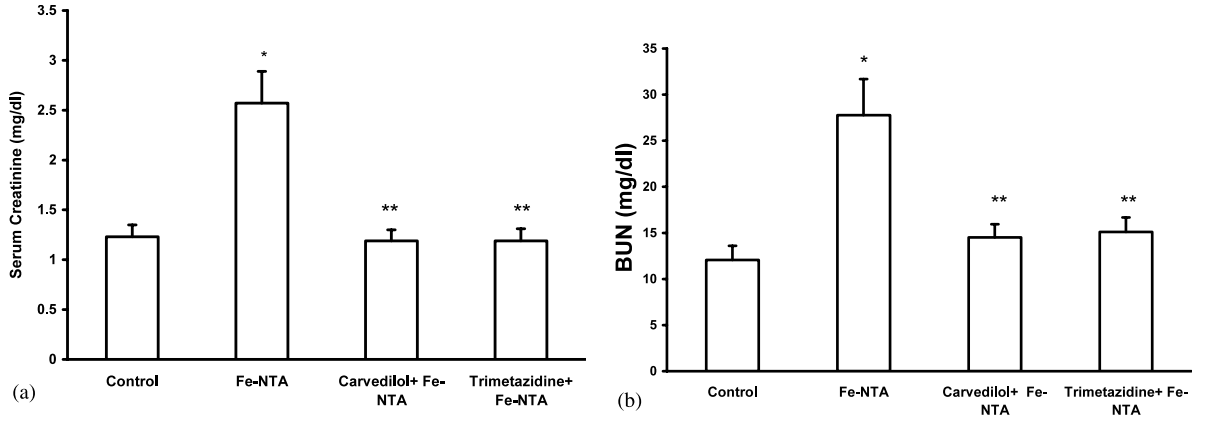

Fig. 1. Effect of carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) on plasma creatinine (a) and BUN (b). The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ as compared with the control group; $^{**}P < 0.05$ as compared with the Fe-NTA treated group (one-way ANOVA followed by Dunnett's test).

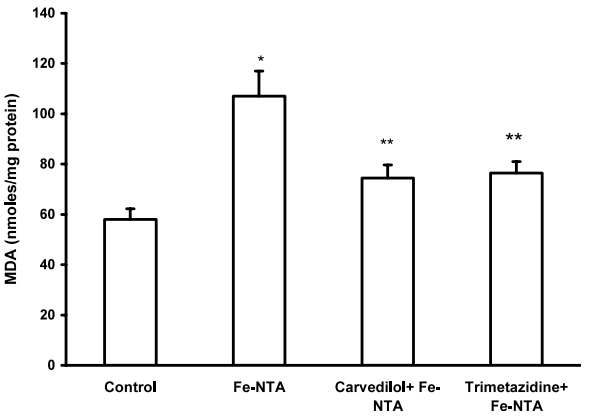

Fig. 2. Effect of carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) on Fe-NTA induced lipid peroxidation (MDA). The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ as compared with the control group; $^{**}P < 0.05$ as compared with the Fe-NTA treated group (one-way ANOVA followed by Dunnett's test).

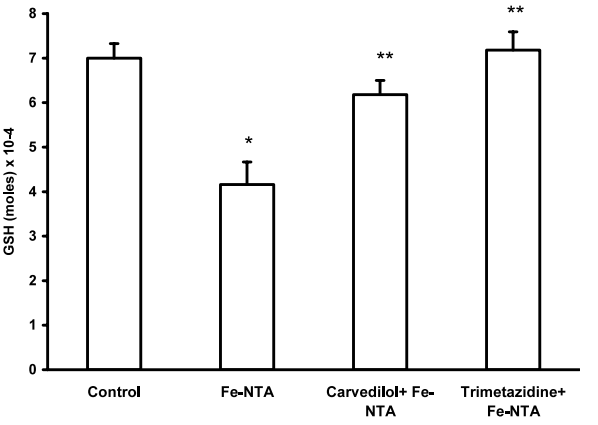

Fig. 3. Effect of carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) on GSH, in Fe-NTA treated rats. The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ as compared with the control group. $^{**}P < 0.05$ as compared with the Fe-NTA group (one-way ANOVA followed by Dunnett's test).

Hamazaki et al., 1989). In the brush border surface of the renal proximal convoluted tubules, a gamma glutamyl-traspeptidase hydrolyses glutathione to cysteinylglycine which is rapidly degraded to cysteine and glycine by dipeptidase (Hahn et al., 1978). Cysteine and cysteinylglycine are the proposed thio reductants that reduce Fe^{3+}-NTA to Fe^{2+}-NTA. The auto-oxidation of Fe^{2+}-NTA generates superoxide radicals (O_2^{-}) which subsequently potentiate the iron catalysed Haber-Weiss reaction to produce hydroxyl radical ($OH^{\bullet}$), leading to induction of lipid peroxidation and oxidative DNA damage (Aruoma et al., 1989; Umemura et al., 1990b).

The acute nephrotoxicity of Fe-NTA is produced by a single large dose and it varies from 5 to 15 mg iron per kg body weight. However, the sub acute nephrotoxicity is produced by repeated dosing of Fe-NTA and it is either 1 or 2 mg iron per kg body weight and is repeated daily for 10 or 12 days (Eguchi et al., 1999; Qi et al., 1999; Shimoi

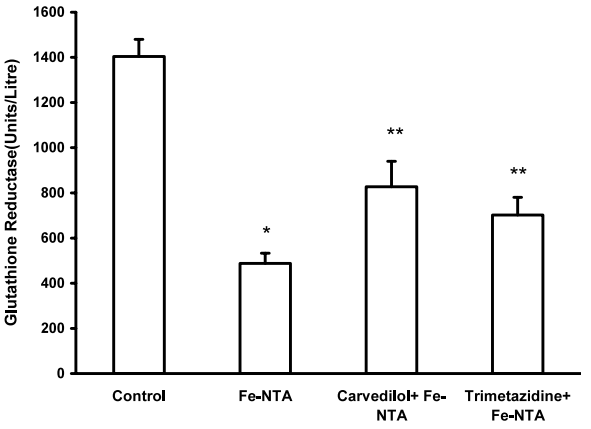

Fig. 4. Effect of carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) on catalase in Fe-NTA treated rats.

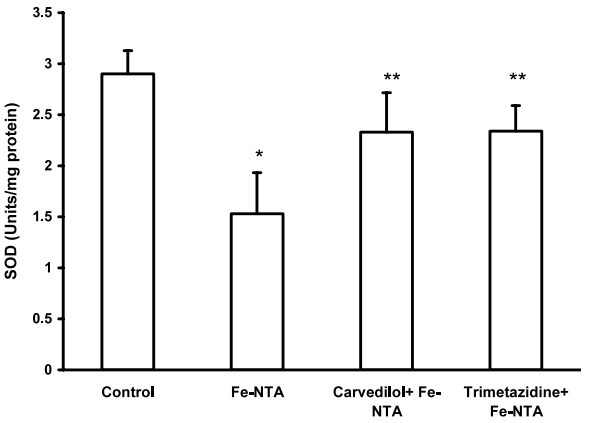

Fig. 5. Effect of carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) on GR in Fe-NTA treated rats.

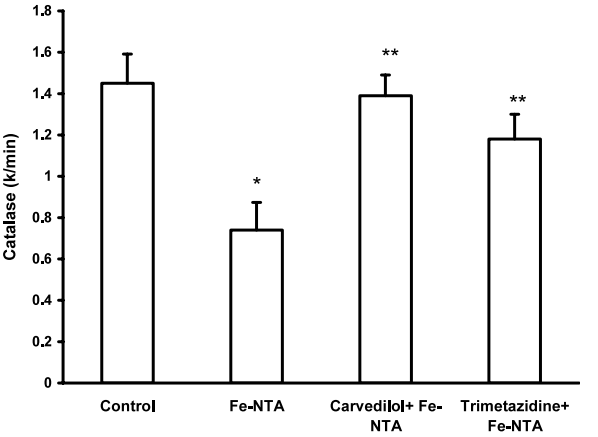

Fig. 6. Effect of carvedilol (2 mg/kg, i.p.) and trimetazidine (3 mg/kg, i.p.) on SOD in Fe-NTA treated rats.

et al., 1997). In the current study, single injection of Fe-NTA (8 mg iron per kg body weight) increased the renal TBARS levels and the antioxidant enzyme pool was depleted as is evident from the declined levels of GSH, catalase, GR, SOD enzymes. Fe-NTA-induced oxidative stress was associated with impaired renal function leading to a marked increase in serum creatinine and BUN. Moreover, the kidney of Fe-NTA treated rats showed characteristic morphological changes such as tubular brush border loss, interstitial oedema, tubular dilatation, necrosis of epithelium and hyaline casts. Oxidative stress can promote the formation of a variety of vasoactive mediators that can affect renal function directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient, and thus reduce the glomerular filtration rate (Baud and Ardaillou, 1993; Bomzon et al., 1997).

Interestingly, both the drugs, carvedilol as well as trimetazidine, as pretreatments produced a significant attenuation of lipid peroxidation (as is evident from MDA levels), and prevented the severe depletion of renal antioxidant enzyme pool (as evidenced by levels of GSH, GR, Catalase and SOD) in Fe-NTA treated rats. Moreover, the renal functional and morphological damage was significantly improved. The protective effect of carvedilol may be related to any of the following actions: (1) Carvedilol forms a complex with Fe^{3+} ions in a 1:1 ratio (Tadolini and Franconi, 1998). (2) Moreover, carvedilol has a remarkable inhibitory action on Fe^{2+}-induced lipid peroxidation (Yue et al., 1992a). It has been suggested that carvedilol may interact with the membrane lipids and interrupt the free radical chain reactions (Mak and Weglicki, 1988). (3) Carvedilol is also reported to scavenge superoxide anion and $OH^{\bullet}$ radicals (Yue et al., 1992b,c). Fe-NTA induced nephrotoxicity is believed to be partly due to iron-stimulated production of free radicals including $OH^{\bullet}$ which in turn leads to initiation of lipid peroxidation and to the formation of the DNA oxidative products, 8-OH-dG (Preece et al., 1985; Umemura et al., 1990a). A combination of these

Table 1
Effect of carvedilol (2 mg/kg) and trimetazidine (3 mg/kg) treatment on morphological changes as assessed by histopathological examination of kidney in Fe-NTA treated rats

Group	Tubular brush border loss	Interstitial oedema	Tubular dilatation	Necrosis of epithelium	Hyaline casts
Control	−	−	−	−	−
Fe-NTA	++	+++	+++	+++	+++
Carvedilol	+/−	−	−	−	−
Trimetazidine	+/−	+/−	−	−	−

−, None; +, Mild; ++, Moderate; +++, Severe.

actions might have contributed to carvedilol's ability to reduce Fe-NTA induced oxidative damage in vivo in the rat kidney.

Trimetazidine, (1-[2,3,4-trimethoxybenzyl]-piperazine) is a piperazine compound and has been demonstrated to protect against ischemia-reperfusion injury. Various studies have shown that trimetazidine has preserved the intracellular concentration of ATP and inhibited the extracellular leakage of potassium during cellular ischemia. It reduces the intracellular accumulation of sodium and calcium and inhibits platelet adhesion-aggre-

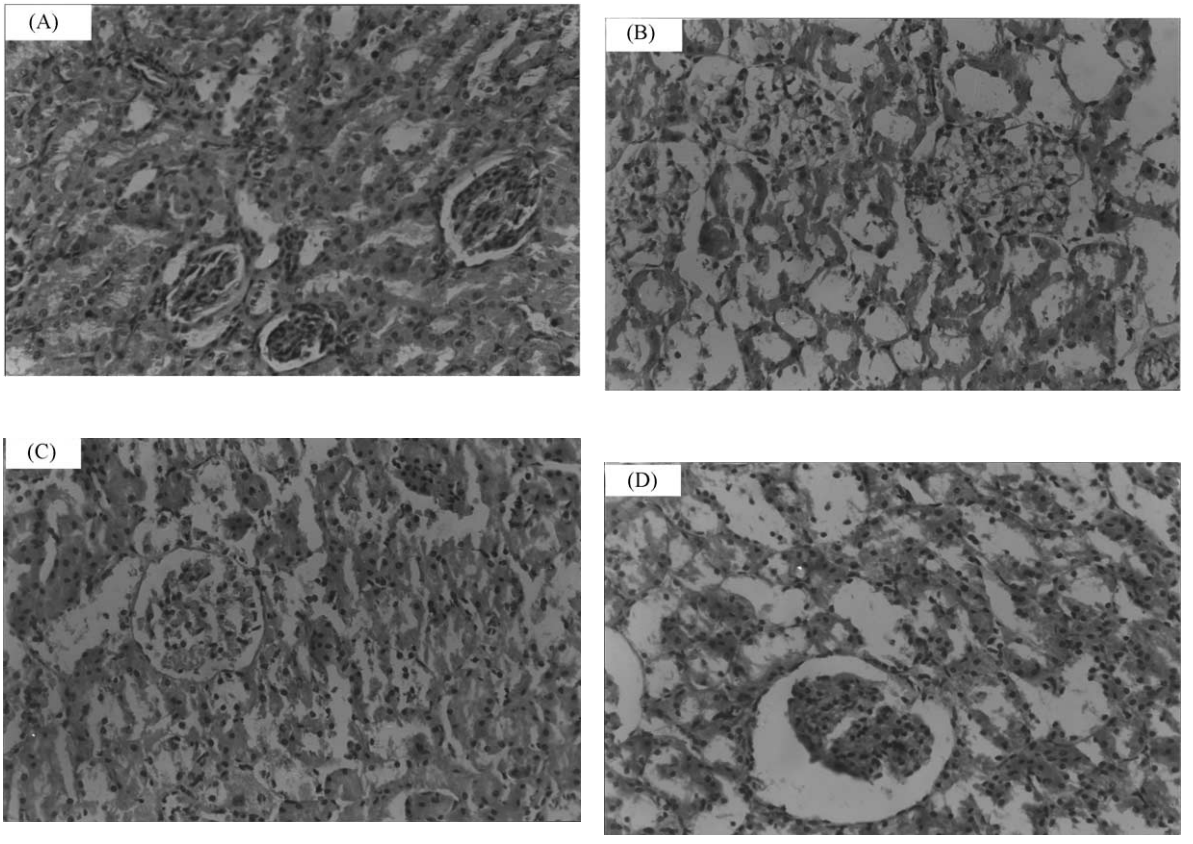

Fig. 7. Hematoxylin and Eosin stained sections of rat kidneys: (A) Normal kidney section. (B) Kidney section of Fe-NTA treated rat showing tubular brush border loss, interstitial oedema, tubular dilatation, necrosis of epithelium and hyaline casts. (C) Kidney section of carvedilol (2 mg/kg, i.p.)+Fe-NTA treated rat showing normal morphology. (D) Kidney section of trimetazidine (3 mg/kg, i.p.)+ Fe-NTA treated rat showing a near normal morphology.

gation, neutrophil infiltration, and the generation or activity of oxygen derived free radicals (Harpey et al., 1989; Williams et al., 1993; El Banani et al., 2000). The OFR cause the loss of intracellular potassium, which has been attributed to an increase of the passive potassium permeability of the cell membrane following peroxidation of membrane lipids (Halliwell and Gutteridge, 1985; Labrid, 1986). Moreover, trimetazidine counteracts the vasoconstriction mediated by the vasoactive mediators released by OFR. However, the exact role of trimetazidine needs to be explored.

With the data in hand we could postulate that it is not only the iron that is a causal agent in the Fe-NTA induced nephrotoxicity, the cellular homeostasis and maintenance of ionic gradients is also as much involved. The findings imply that the toxicity of Fe-NTA relates to its ability to generate free radicals and carvedilol as well as trimetazidine's ability to protect against this agent probably is due to the free radical scavenging and antioxidant activities of these drugs. Considering the apparent virtual absence of either acute or chronic toxicity of carvedilol and trimetazidine, their clinical application against oxidative damage due to a variety of biological toxins (such as Fe-NTA) should be considered.

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