Effect of Amlodipine and Trimetazidine on Gentamicin-Induced Nephrotoxicity in Rats

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Abstract: Background: Gentamicin is one of the aminoglycoside antibiotics that is used for treatment of gram-negative bacterial infections. Its clinical use is limited by its nephrotoxicity. Trimetazidine is a cytoprotector agent whose site of action, mechanism and chronological order of effect are not yet well known. Amlodipine is one of calcium channel blockers that plays an important role in treatment of hypertension. Objective: To study the effect of each of amlodipine and trimetazidine alone and in combination on gentamicin induced nephrotoxicity in rats. Methods: 50 albino rats were divided into 5 equal groups: Control untreated group, Gentamicin-induced nephrotoxicity group, Amlodipine + Gentamicin treated group, Trimetazidine + Gentamicin treated group, Amlodipine + Trimetazidine + Gentamicin treated group. Blood urea, serum creatinine, creatinine clearance, urinary gamma glutamyl transpeptidase, urinary N-acetyl beta-D-glucosaminidase, urinary protein, renal tissue malondialdehyde, tissue superoxide dismutase, tissue nitric oxide, tissue nitric oxide synthase, tissue reduced glutathione and mitochondrial complex I activity were determined. Kidneys were excised for histopathological examination. Results: Administration of trimetazidine or amlodipine alone or in combination induced significant increase in creatinine clearance, tissue superoxide dismutase, tissue reduced glutathione and mitochondrial complex I activity with significant decrease in blood urea, serum creatinine, urinary gamma glutamyl transpeptidase, urinary N-acetyl beta-D-glucosaminidase, urinary protein, tissue nitric oxide, tissue nitric oxide synthase and tissue malondialdehyde with improvement of the histopathological picture compared to gentamicin treated group. Conclusion: Gentamicin generated renal tubular toxicity by inducing the formation of reactive oxygen species, nitric oxide, producing renal tubular inflammation and necrosis and inducing mitochondrial dysfunction. It also demonstrated the renoprotective effect of each of amlodipine and trimetazidine alone and its additive antioxidant and cytoprotector effect in combination against gentamicin-induced nephrotoxicity.
[Mohamed Nabih Abdel-Rahman, and Mahmoud Kandeel. **Effect of Amlodipine and Trimetazidine on Gentamicin-Induced Nephrotoxicity In Rats.** J Am Sci 2012;8(6):328-335]. (ISSN: 1545-1003). http://www.americanscience.org. 40

Key words: amlodipine; trimetazidine; gentamicin; nephrotoxicity; rats.

1. Introduction

Kidneys are vulnerable to damage by toxins, infection, immune reactions and ischemia. Acute renal failure is a frequent complication of critical illness especially in the inpatient setting[(1)]. The prognosis of acute renal failure is complicated by secondary injuries induced by free radicals formed during ischaemia/reperfusion injury of the kidney [(2)].

Gentamicin (GM), being one of the aminoglycosides, is a very important agent for the treatment of gram-negative bacterial infections. However, its clinical use is limited by its nephrotoxicity [(3)]. Indeed, in addition to tubular toxicity, a part of gentamicin-induced renal damage is based on its glomerular effects, especially those altering the function of mesangial cells [(4)]. It had been demonstrated that the nephrotoxic effect of GM can be mediated by reactive oxygen species (ROS). Some studies suggested that hydroxyl and superoxide anions are mediators of ischemic tissue injury and of the pathophysiology of kidney disease [(3, 5)]. The value of GM in clinical practice would be greatly increased if some means could be found to protect the kidney from undesirable side effects. Thus, a therapeutic approach to protect or reverse renal GM damage would have very important clinical consequences [(6)].

Trimetazidine (TMZ) is one of the lines of treatment of coronary heart disease attenuating metabolic disturbances in ischemic cardiomyocytes. Its exact site of action, mechanism and chronological order of effect are not yet well known [(5)]. The mechanisms described include hemodynamic changes, reduction in the toxicity of oxygen-derived free radicals, decrease in the inflammatory reaction, optimization of the energetic metabolism and decrease in the utilization of fatty acids in favor of carbohydrates [(7)]. Moreover, TMZ stimulates ATP synthesis, normalizes ion balance, attenuates intracellular acidosis, inhibits platelet aggregation and suppresses accumulation and activation of neutrophils in the ischemic zone. TMZ decreases the content of lipid peroxidation and reactive oxygen species formation under conditions of ischemia-reperfusion.

Moreover, TMZ increases the resistance of the cells and tissues to prooxidants [8].

Dihydropyridine calcium antagonists, such as nifedipine, nitrendipine and amlodipine are cardiovascular agents used for treatment of hypertension, dependent on their calcium channel blocking activity. They have also been demonstrated to possess antioxidant activity and to reduce intracellular production of ROS, independent of calcium channel modulation [9]. It had been reported that treatment of the endothelium with dihydropyridine calcium antagonists resulted in increase in the release of nitrogen monoxide that is not due to a modulation of L-type calcium channels. This may cause vasodilatation and might contribute to the antithrombotic, antiproliferative, and antiatherosclerotic effects of dihydropyridine calcium antagonists [10]. Therefore, study of the effect of trimetazidine and amlodipine on nephrotoxicity caused by gentamicin is of particular interest. The aim of this work is to study the effect of each of amlodipine and TMZ alone and in combination on gentamicin induced nephrotoxicity in rats.

2. Materials and Methods:

Drugs used:

- Gentamicin sulphate 80 mg ampoule (Memphis, Schering-Plough) injected subcutaneously in a dose of 50 mg/kg b.wt./day [5].
- Trimetazidine dihydrochloride 20 mg tablets (Servier, Vastarel), dissolved in distilled water and given in a dose of 20 mg/kg/day orally [5].
- Amlodipine besylate 5 mg tablets (Pfizer, Norvasc), dissolved in distilled water and given in a dose of 5 mg/kg/day orally [11].

Animals and procedures:

Fifty albino Sprague-Dawley rats each weighing 100-120 grams (mean 112 ± 6.98 grams) were used throughout this study. All the experiments were conducted according to the National Research Council's guidelines. Animal handling was followed according to Helsenki declaration of animal ethics. The animals were kept under similar housing conditions and were subdivided into five groups of ten rats each as follows:

Group 1: rats were fed standard diet and served as normal control group.

Group 2: rats were subcutaneously injected with gentamicin for 7 days.

Group 3: rats were given trimetazidine by oral gavage for 7 days before administration of gentamicin and for 7 days concomitant with gentamicin.

Group 4: rats were given amlodipine by oral gavage for 7 days before administration of gentamicin and for 7 days concomitant with gentamicin.

Group 5: rats were given amlodipine and trimetazidine by oral gavage for 7 days before administration of gentamicin and for 7 days concomitant with gentamicin.

Rats were housed singly in individual metabolic cages during the period of the study. Twenty four hours urine collection from each isolated rat was done after the last injection of gentamicin. The collected 24 hours urine from each rat was analysed for protein content according to the sulfosalicylic acid colorimetric method [12], N-acetyl beta-D-glucosaminidase (NAG) activity according to the method of Price [13], γ-glutamyl transpeptidase (GGT) according to the method of Szasz [14] and creatinine concentration according to the method of Henry [15].

In the following day, blood samples were taken by retro-orbital method to estimate blood urea according to the method of Patton & Crouch [16] and serum creatinine according to the method of Henry [15]. According to the 24 hours urine volume, urinary creatinine and serum creatinine concentration, creatinine clearance was calculated by applying the following formula:

$$\text{Creatinine clearance (ml/min/day)} = \frac{\text{mg creatinine/dl urine X ml urine 24 hours}}{\text{mg creatinine /dl serum X 1440}} \quad [15]$$

Rats were sacrificed by decerebration and both kidneys were removed and sectioned for histopathological analysis [17]. The remaining kidney tissue was homogenized for determination of malondialdehyde (MDA) according to the method based on the reaction with thiobarbituric acid [18], superoxide dismutase (SOD) activity according to the method of Marklund & Marklund [19], reduced glutathione (GSH) according to the method of Beutler et al. [20], total protein content according to the method of Lowry et al. [21] and the concentration of nitric oxide (NO) and nitric oxide synthase (NOS) activity according to a colorimetric method based on Griess reaction [22].

Preparation of rat kidney mitochondria:

Part of the kidney of each rat was collected in the following medium (10 mM Tris–HCl, 1 mM EGTA, 0.32 M sucrose) obtained by dissolving 10.94 g sucrose, 1.21 g Tris–HCl, and 0.38 g EGTA in 100 ml distilled water and adjusted to pH 7.8. Homogenization was done in 9 volumes of this cold medium with three or four strokes using Teflon pestle homogenizer. Then the homogenate was centrifuged at 700 × g for 10 min at 4 °C, the supernatant was centrifuged for 20 min at 1000 × g to obtain mitochondria pellets that were washed once with the previous collecting buffer to remove microsomal and cellular contamination. Finally the mitochondria were resuspended in 9 volumes of the collecting buffer, pH 7.8 [23]. Mitochondrial protein was determined using Lowry method [21]. Then, mitochondrial complex I activity (NADH: coenzyme Q oxido-reductase

enzyme activity) was measured according to the method of Birch-Machin et al. [24], by following the decrease in the absorbance due the oxidation of NADH at 340 nm with the use of extinction coefficient = 6.81 l/mmol/cm.

Statistical analysis:

Data were presented as mean ± SEM, data were analyzed by one way analysis of normality of variance (ANOVA) using student t-test, differences between the means of different groups were considered significant at a level of $p< 0.05$.

3. Results:

I- Biochemical findings:

Gentamicin induced significant increase in blood urea, serum creatinine, urinary GGT, urinary NAG, urinary protein, tissue NO, tissue NOS and tissue MDA with significant decrease in creatinine clearance, tissue SOD, tissue GSH and mitochondrial complex I activity compared to the normal control group (Tables 1 & 2). Administration of either trimetazidine or amlodipine alone induced significant increase in creatinine clearance, tissue SOD, tissue GSH and mitochondrial complex I activity with significant decrease in blood urea, serum creatinine, urinary GGT, urinary NAG, urinary protein, tissue NO, tissue NOS and tissue MDA compared to gentamicin treated group (Tables 1 & 2). Concomitant administration of trimetazidine and amlodipine induced significant increase in creatinine clearance, tissue SOD, tissue GSH and mitochondrial complex I activity with significant decrease in blood urea, serum creatinine, urinary GGT, urinary NAG, urinary protein, tissue NO, tissue NOS and tissue MDA compared to gentamicin treated group which was more marked than each of trimetazidine and amlodipine alone (Tables 1 & 2).

II- Histopathological findings:

The kidneys of the control rats had normal morphology with normal appearance of the glomeruli, tubules and interstitium (Figure 1). Pathological evidence of kidney damage was observed in all rats treated with gentamicin (Figure 2) as indicated by diffuse tubular degeneration, focal necrosis, diffuse cellular infiltration and haemorrhage. This picture was significantly improved in rats given trimetazidine alone or amlodipine alone or both as evidenced by decrease in the number of infiltrated cells, haemorrhage, tubular degeneration and necrosis (Figures 3, 4, 5).

Table (1): Effect of administration of amlodipine, trimetazidine (TMZ) and their combination on blood urea, serum creatinine, creatinine clearance, urinary protein, urinary NAG and urinary GGT activity in gentamicin GM treated rats.

Parameters \ Group	Normal Control	GM	Amlodipine+GM	TMZ + GM	Amlodipine + TMZ + GM
Blood urea (mg/dl)	20.2 ± 0.67	77.12± 2.83*	52.41 ± 2.77 #	47.64 ±2.23#	37.4 ± 2.15#
Serum creatinine (mg/dl)	0.25 ± 0.03	1.23 ± 0.04*	0.69 ± 0.03#	0.57 ± 0.03#	0.47 ± 0.02#
Creatinine clearance (ml/min/day)	1.24 ± 0.02	0.44 ± 0.03*	0.68 ± 0.04#	0.73 ± 0.05#	0.87 ± 0.05#
Urinary GGT (U/L)	837.45 ± 22.7	2430 ±37.1*	1425.5± 34.12#	1354.41± 25.4#	1185.3± 19.24#
Urinary NAG (U/L))	0.23 ± 0.07	1.26 ± 0.12*	0.76 ± 0.09#	0.65±0.09#	0.49±0.11#
Urinary protein (mg/dl	0.56±0.02	1.69±0.07*	1.19±0.06#	0.98±0.03#	0.86±0.04#

Table (2): Effect of administration of amlodipine, trimetazidine (TMZ) and their combination on different oxidative stress biomarkers (Renal tissue SOD, GSH, MDA, NO and NOS) and mitochondrial complex I activity in gentamicin (GM) treated rats.

Parameters \ Group	Normal Control	GM	Amlodipine + GM	TMZ + GM	Amlodipine + TMZ + GM
Renal SOD (U/g protein)	742.3 ± 82.5	287.15 ± 42.4*	486.2 ± 38.21#	544.11±65.08#	595.51±58.15#
Renal GSH (mol/g)	4.3±0.04	2.44±0.07*	3.54±0.06#	3.41±0.09#	3.82±0.08#
Renal MDA (nmol/mg protein)	0.35±0.05	1.51±0.02*	0.63±0.03#	0.72±0.07#	0.58±0.03#
Renal NO (µmol/g protein)	1.34 ± 0.12	3.76 ± 0.08*	2.21 ± 0.09#	1.97 ± 0.13#	1.75 ± 0.06#
Renal NOS (nmol/min/g protein)	0.68 ± 0.05	1.87 ± 0.04*	1.22 ± 0.06#	1.15 ± 0.02#	0.95 ± 0.04#
Mitochondrial complex I activity nmol NADH/min/mg protein	94.7±2.13	36.92 ±1.45*	62.78±2.4#	53.62 ±3.2#	71.54 ±1.87#

Values are represented as mean ± SEM. Number of rats in each group = 10.

* Significant compared to the control group ($P < 0.05$). # Significant compared to gentamicin group ($P < 0.05$).

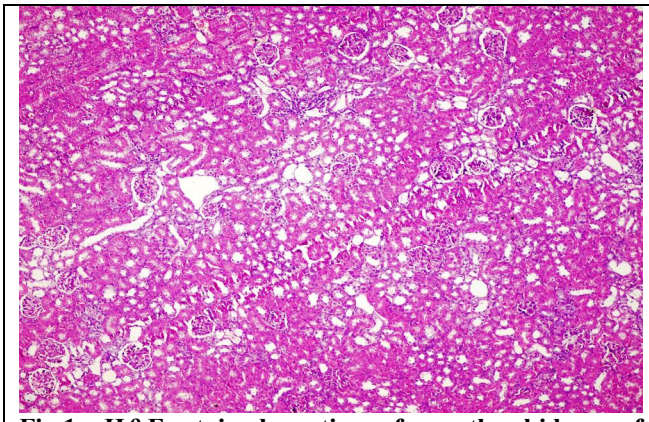

Fig.1: H&E stained sections from the kidney of normal control group with normal appearance of the glomeruli, tubules and interstitium

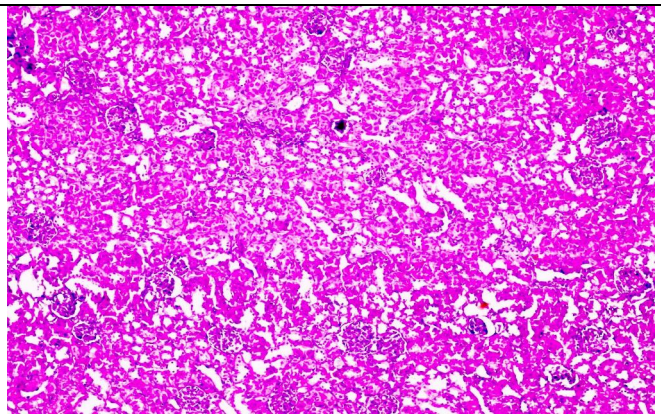

Fig.3: H&E stained sections from the kidney of Amlodipine + GM treated group with apparently normal glomeruli, focal tubular degeneration and minimal tubular necrosis

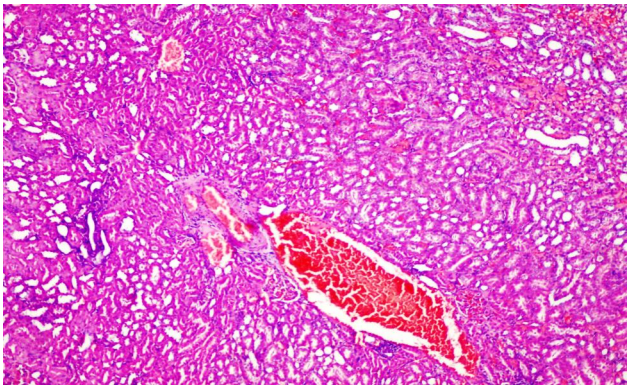

Fig. 2: H&E stained sections from the kidney of GM treated group with diffuse tubular degeneration, necrosis and haemorrhage

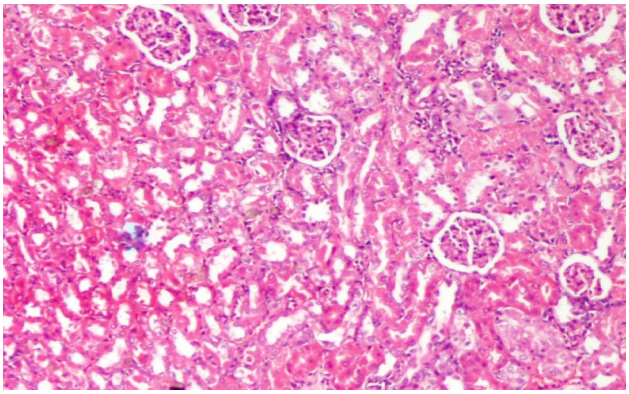

Fig.4: H&E stained sections from the kidney of TMZ + GM treated group with apparently normal glomerul, mild dilatation and necrosis of the tubules

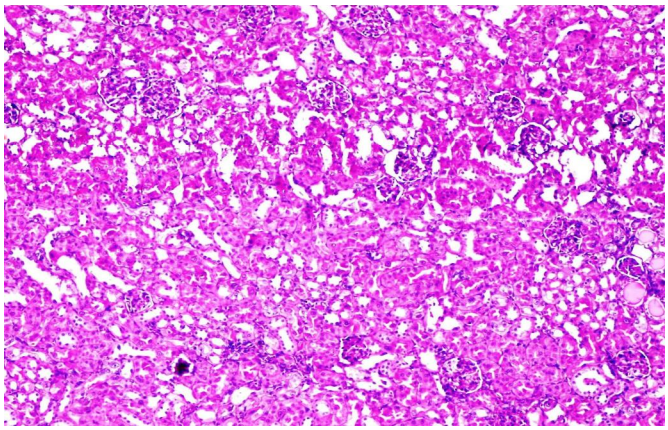

Fig.5: H&E stained sections from the kidney of Amlodipine + TMZ + GM treated group with apparently normal glomeruli and mild dilatation of the tubules.

4. Discussion:

Gentamicin (GM) is one of the aminoglycoside antibiotics that is used for treatment of gram-negative bacterial infections. However, its clinical use is limited by its nephrotoxicity [(3)]. GM is a strongly cationic drug that binds to the negatively charged acidic phosphoinositide components of the brush border membrane of the proximal tubule, and they act on the megalin receptors located at the base of the brush border villi. The receptor–drug complex is taken up by lysosomes, where lysosomal phospholipidosis occurs that disrupts the renal intracellular processes [(25)]. GM-induced nephrotoxicity is associated with the

occurrences of cellular desquamation, glomerular atrophy, tubular necrosis, tubular fibrosis, epithelial oedema of proximal tubules, glomerular hypertrophy, perivascular edema and inflammation and glomerular congestion [26]. Previous studies suggested that reactive oxygen species play an important role in the tubular effects of GM [27]. It had been reported that gentamicin increased lipid hydroperoxides and suppressed superoxide dismutase, catalase and glutathione peroxidase activities [4]. Moreover, reactive oxygen species scavengers had been proven to be beneficial at reducing the development of GM nephrotoxicity [28]. GM increased macrophage infiltration and elevated TGF-β levels that led to the progression of tubulointerstitial nephritis [29]. Moreover, it had been revealed that GM-nephrotoxicity is associated with renal over-expression of p38-mitogen activated protein kinase (p38MAPK) and nuclear factor kappa B (NFkB) pathways [26].

NO plays an important role in cellular signalling, cellular energetics, host defence mechanisms, inflammation, systemic and intrarenal haemodynamics and renal tubular function and is involved in the pathophysiology of nephrotoxicity. The role of NO is thought as controversial, although many studies reported that it increased renal injury through its reactions with a superoxide radical and generation of a cytotoxic peroxynitrite [30]. Rats treated with GM exhibited decrease in renal function and also had increased NO in plasma. This probably occurred to counterbalance the effects of vasoconstrictor agents such as angiotensin and/or prostaglandins, or it was a reaction against the vasoconstrictor action of GM, or the generation of free radicals (such as peroxynitrite) [31].

NO is produced by NOS, of which there are three isoforms (endothelial eNOS, neuronal nNOS and inducible iNOS). The last is known to aggravate renal failure, and its selective inhibition would be expected to ameliorate signs of GM nephrotoxicity. Intravenous administration of the selective iNOS inhibitor, N-imino-ethyl lysine (L-NIL), together with GM significantly ameliorated biochemical and renal histological indices caused by GM. On the other hand, N-omega-l-arginine methyl ester (l-NAME), a non-selective NOS inhibitor, aggravated GM nephrotoxicity [30]. Also, administration of L-arginine, an immediate precursor of NO, showed beneficial effects against gentamicin-nephrotoxocity in rats [32].

Complex I (NADH dehydrogenase) is an enzyme located in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q. It is the entry enzyme of oxidative phosphorylation in the mitochondria [33]. Morales et al. [3] demonstrated that GM induced marked mitochondrial dysfunction that largely precede cell necrosis and acute renal failure in the form of marked decrease of mitochondrial complex I activity and leakage of cytochrome c into the cytosol.

The present study showed that GM administration induced severe damage to renal tissues, most likely caused by reactive oxygen species generation evidenced by decreased activities of the antioxidant enzymes, mitochondrial complex I activity and creatinine clearance while increased blood urea, serum creatinine, urinary GGT, urinary NAG, urinary protein, tissue NO, tissue NOS and tissue MDA with marked histopathological changes of renal tubules. These results were consistent with the results reported by Bledsoe et al. [29] and Li et al. [11].

GM-induced decrease in glomerular filtration rate occurs as a result of contraction of mesangial cells due to the high cytosolic Ca^{2+} levels. GM markedly raised intracellular Ca^{2+} levels by activating both calcium influx from external source and calcium release from internal stores, and this was suggested to be responsible for renal mesangial cellular contraction and proliferation [31]. Therefore, calcium channel blockers (CCBs) were thought to have ameliorative effects on GM nephrotoxicity. CCBs are widely used in treatment of a variety of cardiovascular diseases. Among CCBs, amlodipine plays an important role in treatment of hypertension [34]. However, research of their non-antihypertensive effects had attracted more attention, especially their effect on GM-induced nephrotoxicity [35]. In the present study, we investigated the effect of amlodipine on GM-induced nephrotoxicity. Results showed that amlodipine significantly alleviated GM-induced increase in urinary protein, urinary GGT, urinary NAG, serum creatinine, blood urea, tissue NO, tissue NOS, tissue MDA and renal tubular histopathological changes while increased activities of SOD, GSH and mitochondrial complex I in renal tissues and creatinine clearance. The above results indicated that the renoprotective effects of amlodipine were due to reduced GM-induced reactive oxygen species generation by increasing activities of antioxidant enzymes & reduction of the concentraton of NO and NOS activity in renal tissues.

These results were in agreement with Li et al. [11] who reported that nifedipine and amlodipine reversed GM-induced nephrotoxicity. Moreover, nitrendipine, a diisopyridine derived calcium channel blocker, showed marked protection against GM nephrotoxicity in rats[36]. Although the antioxidant mechanisms of amlodipine had not been fully elucidated, there was evidence suggesting that it belongs to the chain breaking group of antioxidants. Lacidipine, lercanidipine, and amlodipine are dihydropyridine calcium antagonists which had already been shown to possess antioxidant activity in in vitro studies. It is likely that the dihydropyridine ring can donate

electrons to the propagating radicals to reduce it to a non-reactive form [37]. It had been suggested that the intrinsic structural characteristics of dihydropyridine calcium antagonists are important in determining their antioxidant properties [10]. Amlodipine has an alkaline amino 3-oxygen group on the dihydropyridine ring which presents itself in ionization state under physiological conditions. This special group can form electrostatic interaction between amlodipine and membrane phospholipids. High-lipophilia and steady chemical structure of amlodipine can inhibit membrane lipid-peroxidative damage. Furthermore, it had been reported that this effect of amlodipine does not depend on modulation of calcium channels. Previous reports showed that amlodipine can prevent renal dysfunction and affect NOS expression as well as reduced the free radical production resulting in improvement of renal function [11].

On the contrary, Li et al. [11] observed that nitrendipine had little effect, or even aggravated GM nephrotoxicity. Additionally, GM nephrotoxicity in rats was not modified or even exacerbated by verapamil [38]. These results suggested that CCBs may have different mode of action in modulating GM nephrotoxicity, and further studies are needed to explore their defensive action against GM-induced nephrotoxicity.

Trimetazidine (TMZ) is a cytoprotector agent whose mechanism of action is not yet well known. It was suggested that TMZ induces hemodynamic changes, decreases toxicity of oxygen-derived free radicals, decreases inflammatory reactions and decreases utilization of fatty acids in favor of carbohydrates [7]. The protective effect of TMZ on ROS-induced renal failure during ischemia/reperfusion in rats had been attributed to its antioxidant and oxygen free radical scavenger activity [39].

In the present study, we investigated the effect of TMZ on GM-induced nephrotoxicity. Results showed that TMZ significantly decreased GM-induced increase in urinary protein, urinary GGT, urinary NAG, serum creatinine, blood urea, tissue NO, tissue NOS and tissue MDA and alleviated renal tubular histopathological changes while increased activities of SOD, GSH and mitochondrial complex I in kidney tissues and creatinine clearance. With these results, we concluded that previous treatment with TMZ exerted a protective effect on GM-induced nephrotoxicity. These results were in accordance with Hauet et al. [40] and De la Cruz Rodriguez et al. [5] who explained the cytoprotective effect of TMZ by that it may act at the level of the brush border, preventing reabsorption and accumulation of GM in the membranes of the renal tubular cells [5].

TMZ had been described as a cellular anti-ischemic agent. It was demonstrated that TMZ prevented the harmful effects of ischemia-reperfusion at both the cellular and mitochondrial levels and exerted potent antioxidant activity on various tissues [41]. TMZ inhibited the excessive release of oxygen-free radicals, increased glucose metabolism, limited intracellular acidosis, protected ATP stores, reduced membrane lipid peroxidation and inhibited neutrophil infiltration after ischemia-reperfusion [40]. Other studies suggested that TMZ may also be beneficial in attenuation of glycerol, cyclosporine, tacrolimus and ferric nitrillotriacetic-induced renal damage. Addition of TMZ to organ preservation solutions may improve renal function after transplantation [41].

In the present study, the combined administration of amlodipine and TMZ induced augmented renal protective effect which is more marked than each of these drugs alone, as evidenced by significant decrease in urinary protein, urinary GGT, urinary NAG, serum creatinine, blood urea, tissue NO, tissue NOS, tissue MDA and alleviated renal tubular histopathological changes with significant increase in the activities of SOD, GSH and mitochondrial complex I in kidney tissues and creatinine clearance. This can be explained by the combined antioxidant effect of amlodipine and TMZ with their abilities to prevent depletion and regenerate endogenous antioxidants and protect against the harmful effects of reactive oxygen species, NO and NOS.

In conclusion, the present study indicated that GM generated renal tubular toxicity by inducing the formation of reactive oxygen species, NO, producing renal tubular inflammation and necrosis and inducing mitochondrial dysfunction. It also demonstrated the renoprotective effect of each of amlodipine and TMZ alone and its additive antioxidant and cytoprotector effect in combination against GM-induced nephrotoxicity.

Acknowledgment:

Many thanks to Prof. Dr. Karima El-Desoky, Department of Pathology, Faculty of Medicine, Tanta University, for her valuable help and revising the pathological study.

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