

Research Article

Simvastatin Attenuates Contrast-Induced Nephropathy through Modulation of Oxidative Stress, Proinflammatory Myeloperoxidase, and Nitric Oxide

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Contrast media- (CM-) induced nephropathy is a serious complication of radiodiagnostic procedures. Available data suggests that the development of prophylaxis strategies is limited by poor understanding of pathophysiology of CM-induced nephropathy. Present study was designed to determine the role of oxidative stress, myeloperoxidase, and nitric oxide in the pathogenesis of iohexol model of nephropathy and its modification with simvastatin (SSTN). Adult Sprague Dawley rats were divided into seven groups. After 24 h of water deprivation, all the rats except in control and SSTN-only groups were injected (10 ml/kg) with 25% glycerol. After 30 min, SSTN (15, 30, and 60 mg/kg) was administered orally, daily for 4 days. Twenty-four hours after the glycerol injection, iohexol was infused (8 ml/kg) through femoral vein over a period of 2 min. All the animals were sacrificed on day 5 and blood and kidneys were collected for biochemical and histological studies. The results showed that SSTN dose dependently attenuated CM-induced rise of creatinine, urea, and structural abnormalities suggesting its nephroprotective effect. A significant increase in oxidative stress (increased lipid hydroperoxides and reduced glutathione levels) and myeloperoxidase (MPO) and decreased nitric oxide in CM group were reversed by SSTN. These findings support the use of SSTN to combat CM-induced nephrotoxicity.

1. Introduction

Contrast media (CM) are frequently used for various diagnostic procedures including X-rays, computed tomography and magnetic resonance imaging. CM-induced nephropathy is a leading cause of acute kidney injury and is associated with significant mortality and morbidity [1, 2]. The mechanism of CM-induced renal impairment at cellular level is not fully understood; recent reports suggested both direct and indirect effects on renal tubules including biochemical and hemodynamic disturbance due to CM-induced prerenal dehydration, hypotension, and medullary ischemia [3, 4]. Jost et al. [5] also reported that contrast media cause impairment of renal perfusion leading to hypoxic conditions

resulting in acute tubular necrosis. It has been reported that patients with diabetes and preexisting renal insufficiency may be at a higher risk of CM-induced nephrotoxicity compared to patients with normal renal function [6].

Several experimental and clinical studies suggested beneficial effects of statins against CM-induced nephrotoxicity [7, 8]; however, this claim has been refuted by some of the recent clinical trials [9, 10]. Meta-analysis of available data about the renoprotective effect of statins against CM-induced nephropathy is still conflicting and inconclusive. It has been suggested that the development of highly effective prophylaxis strategies is limited by poor understanding of the pathophysiology of CM-induced renal toxicity. In this paper, attempt has been made to study the role of oxidative

stress, proinflammatory enzyme myeloperoxidase (MPO), and vasoregulatory nitric oxide in the pathogenesis of CM-induced nephropathy and its modification with simvastatin (SSTN). Iohexol (CM) plus glycerol was administered in rats as a model for CM-induced nephrotoxicity according to the method described by Duan et al. [11]. Pretreatment with glycerol was used to simulate preexisting renal insufficiency in clinical setting; this protocol provides for an optimal nephropathy on exposure of rats to contrast media.

2. Material and Methods

2.1. Radio Contrast Medium and Drugs. Contrast medium, iohexol, was obtained from Amersham Health, Cork, Ireland. Glycerol was purchased from Sigma Chemical Co., St. Louis, USA. Simvastatin was purchased from Calbiochem, CA, USA.

2.2. Animals and Treatment Groups. Adult Sprague Dawley male rats (250–300 g) raised in our animal breeding facility were used in this study. The experimental protocol of this study was approved by Hospital Research and Ethical Committee. The animals were randomly divided into 7 groups (G) of 7 animals each; G1 (Control), G2 (Glycerol only), G3 (Glycerol + CM), G4 (Glycerol + CM + SSTN 15 mg/kg), G5 (Glycerol + CM + SSTN 30 mg/kg), G6 (Glycerol + CM + SSTN 60 mg/kg), and G7 (SSTN 60 mg/kg).

2.3. Study Protocol. The protocol suggested by Duan et al. [11] was followed in this study. After 24 h water deprivation, all rats except the control group (G1) and SSTN only group (G7) were injected intramuscularly with 10 mL/kg body weight of 25% glycerol; drinking water and food were then resumed ad libitum. After 30 minutes of glycerol injection, SSTN was administered orally to the animals in groups G4, G5, G6, and G7; the different doses of SSTN were prepared in a volume of 10 mL/kg and administered by gavage using an intubation needle. SSTN treatment was continued daily for 4 days. Twenty-four hours after the glycerol injection, CM were infused through femoral vein in the dosage of 8 mL/kg of body weight over a period of 2 min to the animals in groups G3, G4, G5, and G6. All the animals were weighed and sacrificed on day 5. Animals were killed under deep ethyl ether anaesthesia. Blood was collected through heart puncture and serum separated for renal function test. The kidneys were excised and weighed immediately; one kidney was stored at -70°C for biochemical analysis and the other kidney was preserved in 10% formalin for histopathological studies as shown in Table 1.

2.4. Renal Function Test. Blood urea nitrogen (BUN), serum creatinine (SCr), and serum calcium levels were measured spectrophotometrically (APEL PD-303S Japan) using the commercial kits from United Diagnostics Industry, Riyadh, Saudi Arabia. These values are expressed as mg/dL (Figure 1).

2.5. Determination of Lipid Peroxidation. The level of lipid peroxidation in the kidney tissue was assessed as thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. [12]. The kidney tissue (0.5 g) was homogenized in 1.15% cold KCl using an Ultraturrax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 3000 g for 5 min and an aliquot of supernatant was mixed with 2 mL of reaction mixture (15% trichloroacetic acid and 0.375% thiobarbituric acid in 0.25 N HCl) and heated for 5 minutes in a boiling water bath. The tubes were cooled at room temperature and centrifuged at 1000 g for 10 minutes. The absorbance of supernatant was read at 535 nm on a UV visible spectrophotometer (UV-160A, Shimadzu, Japan), against a blank that contained all reagents except homogenate. Tissue lipid peroxide levels were calculated as nanomoles of MDA using tetramethoxypropane as standard.

2.6. Determination of Glutathione. The reduced glutathione (GSH) in the kidney tissue was measured enzymatically according to the method of Mangino et al. [13]. The kidney tissue was homogenized with 1 mL of 0.1 M perchloric acid plus 0.005% EDTA in a ratio of 1:20 (wt/vol). The homogenate was centrifuged at 4000 rpm for 10 min and the supernatant was used for GSH assay. The reaction mixture consisted of the following freshly prepared solutions: solution 1, 0.3 mM, NADPH (Sigma) and solution 2, 6 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (Sigma), and solution 3, 50 U/mL glutathione reductase (Sigma). All three solutions were prepared with a stock buffer consisting of 125 mM NaH_2PO_4 and 6.3 mM EDTA at pH 7.5. For GSH assay, 800 μL of solution 1, 100 μL of solution 2, and 10 μL of solution-3 were mixed in a quartz cuvette and placed in a UV-VIS spectrophotometer at 30°C . The enzymatic reaction was started by the addition of 100 μL of the sample (the supernatant) and the absorbance was monitored for 3 min at 412 nm on a UV-visible Spectrophotometer (UV-160A, Shimadzu, Japan). The slope of the change in absorbance was used to quantitate GSH by comparing the slope of the samples with a standard curve prepared with pure GSH (Sigma).

2.7. Determination of Myeloperoxidase. The activity of inflammatory marker myeloperoxidase (MPO) in the tissue was measured according to the method of Barone et al. [14]. Preweighed tissue was homogenized (1:20 wt/vol) in 5 mM potassium phosphate buffer (pH 6.0) using an Ultraturrax homogenizer. The homogenate was centrifuged at 17000 g for 15 min at 4°C . The supernatant was discarded, and the pellet was extracted with 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM potassium phosphate buffer (pH 6.0). Three freeze-thaw cycles were performed, with sonication (10 s, 25°C) between cycles. After the last sonication, the samples were incubated at 4°C for 2 minutes and centrifuged at 12500 g (15 min, 4°C). MPO in the supernatant was assayed by mixing 0.1 mL of supernatant with 2.9 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride

TABLE 1: Drug treatment protocol.

Groups	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Control (G-1)	WD	Saline	Saline	—	—	Sacrificed
Glycerol (G-2)	WD	Gly +	Saline	—	—	Sacrificed
CM + Gly (G-3)	WD	Gly	CM	—	—	Sacrificed
CM + Gly + SSTN 15 (G-4)	WD	Gly + SSTN	CM + SSTN	SSTN	SSTN	Sacrificed
CM + Gly + SSTN 30 (G-5)	WD	Gly + SSTN	CM + SSTN	SSTN	SSTN	Sacrificed
CM + Gly + SSTN 60 (G-6)	WD	Gly + SSTN	CM + SSTN	SSTN	SSTN	Sacrificed
SSTN 60 (G-7)	WD	SSTN	SSTN	SSTN	SSTN	Sacrificed

Abbreviations: WD (Water deprived), Gly (Glycerol), CM (Contrast Media), and SSTN (Simvastatin).

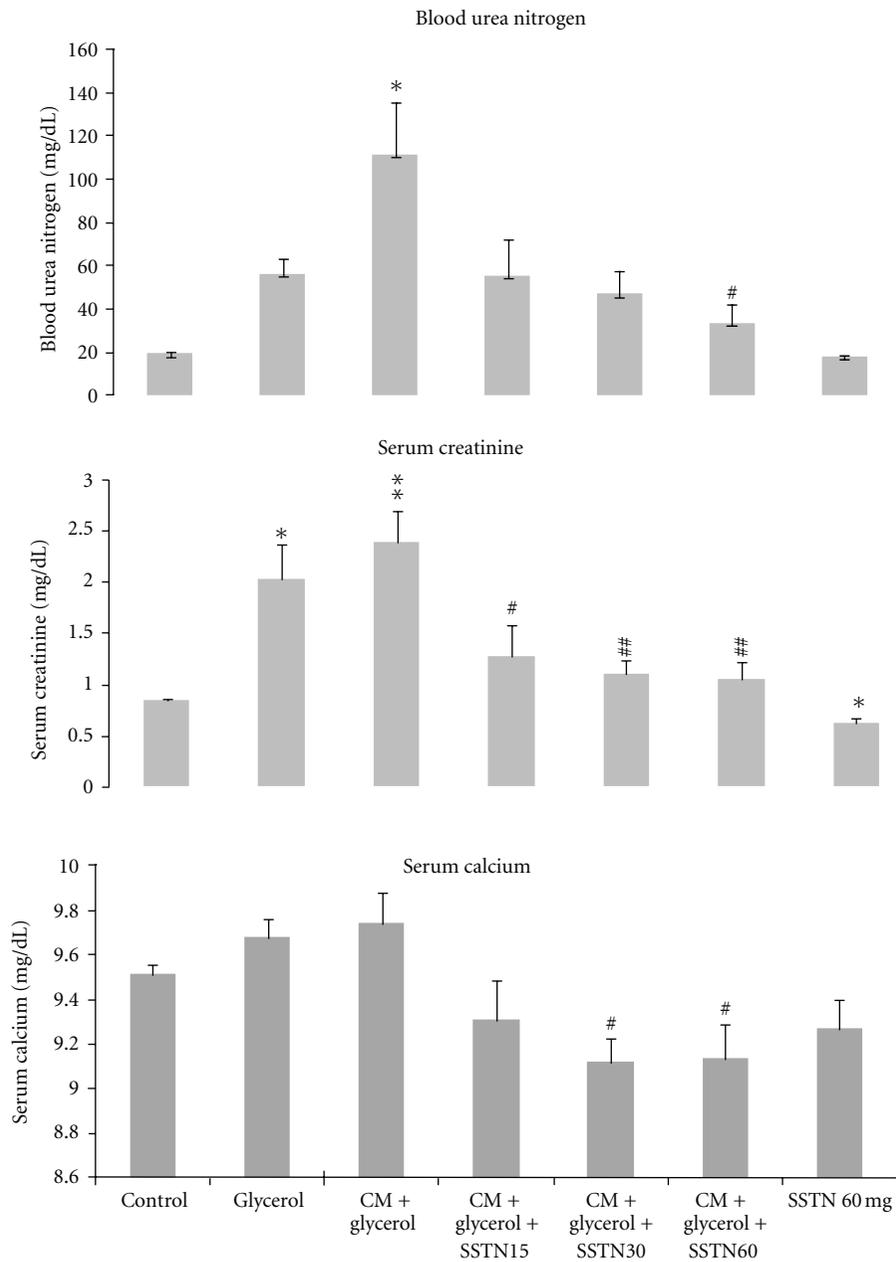


FIGURE 1: Effect of Simvastatin treatment on blood urea nitrogen, serum creatinine, and calcium in a rat model of contrast media-induced nephropathy. Values are \pm SEM. * $P < 0.05$, ** $P < 0.01$ as compared with control group, and # $P < 0.05$, ## $P < 0.01$ versus glycerol + CM using Tukey's test. CM: contrast media; SSTN: simvastatin, 15, 30, and 60 mg/kg.

(Sigma) and 0.0005% hydrogen peroxide (ICN Pharmaceuticals, Irvine, CA). The change in absorbance at 460 nm was measured for 3 min by using a UV-visible spectrophotometer (UV-160A, Shimadzu, Japan).

2.8. Determination of Nitric Oxide. The levels of nitric oxide (NO) in kidney tissues were determined indirectly using the Griess reaction for simultaneous evaluation of nitrite and nitrate concentrations as described by Moshage et al. [15]. Kidney tissue was homogenized in 0.1 M sodium phosphate buffer pH 7.4 in 1 : 10 (w/v) and centrifuged at 8000 g for 10 minutes. Deproteinization of the sample was carried out by the mixing of 40 μ L of 30 g/100 mL zinc sulphate, in 800 μ L of sample using vortex and the solution was allowed to stand for 10 min. The tubes were centrifuged at 4000 g for 10 min. For nitrite assay, 500 μ L of the clear deproteinized supernatant were transferred into the tubes containing 400 μ L distilled water, 500 μ L glycine buffer, and 1 mL of Griess reagent (1 g/L sulfanilamide, 25 g/L phosphoric acid, and 0.1 g/L N-1-naphthylethylenediamine). After 15–20 min the absorbance was measured at 545 nm against a blank containing all the reagents except the homogenate sample. For nitrate estimation the same steps were followed but before addition of Griess reagent 2.5–3 g cadmium granules were added in every tube and the samples were kept for 2 hours at room temperature and the absorbance was read at 545 nm on a UV-visible Spectrophotometer (UV-160A, Shimadzu, Japan). Calculation of nitric oxide was based on nitrite and nitrate values.

2.9. Kidney Weight-to-Body Weight Ratio. At the time of sacrifice the body weights and the kidney weights of rats were recorded. The kidney weight body weight ratio was calculated by simple arithmetic calculation of the kidney weight divided by body weight and then converted to percent.

2.10. Histopathological Examination. After recording the kidney weight and morphological examination, the kidney was fixed immediately in 10% formalin, embedded in paraffin, sectioned at 3 μ m thickness and the sections were stained with hematoxylin and eosin. The extents of tubular injury, dilatation, vacuolation, and necrosis were evaluated semiquantitatively with a slight modification of the method of Raij et al. [16]. Briefly, the extent of tissue damage was graded from 0–4 according to the severity of tubular necrosis, tubular vacuolation, and tubular dilatation. The scoring system was as follows: 0 = no change in the tubules, 1 \leq 25% of tubular injury (mild), 2 = 25% to 50% of tubular involvement (moderate), 3 = 50% to 75% of tubules showing characteristic change (severe), and 4 \geq 75% of tubular damage (very severe). Fifty fields were counted from each slide. All the assessments were done in a blinded fashion.

2.11. Statistics. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS (Version 15) statistical package. *P* values <0.05 were considered as statistically significant.

3. Results

Treatment of rats with SSTN alone did not produce any significant change in serum BUN, SCr, and calcium levels (Figure 1). Administration of Glycerol alone increased the serum BUN levels which were significantly potentiated by CM (ANOVA $F = 3.820$, $P < 0.01$). Treatment with SSTN dose dependently attenuated CM-induced BUN levels (Figure 1). Serum creatinine was significantly increased by glycerol as well as glycerol + CM (ANOVA $F = 7.259$, $P < 0.001$). Treatment with SSTN significantly and dose dependently reduced the SCr levels induced by glycerol + CM and also in SSTN-treated control animals (Figure 1). Treatment of rats with glycerol alone and glycerol plus CM produced a nonsignificant increase in serum calcium. Both the medium (30 mg/kg) and high doses (60 mg/kg) of SSTN were equipotent in reducing serum calcium levels in glycerol + CM treated rats (ANOVA $F = 3.702$, $P < 0.01$) (Figure 1).

Administration of Glycerol with or without CM significantly increased TBARS levels in kidneys of rats which were significantly attenuated by medium and high doses of SSTN (ANOVA $F = 15.239$, $P < 0.001$) (Figure 2). The level of kidney GSH significantly decreased in rats exposed to glycerol or glycerol plus CM. Treatment with medium and high doses of statin significantly and dose dependently reversed the depletion of GSH caused by glycerol and CM (ANOVA $F = 16.223$, $P < 0.001$). Although glycerol alone produced a nonsignificant increase of the activity of MPO in kidneys, combined treatment with glycerol plus CM significantly increased MPO activity (Figure 2) which was significantly reduced by high dose of SSTN (ANOVA $F = 6.380$, $P < 0.001$). A significant reduction in kidney NO level was observed in rats exposed to glycerol alone or in combination with CM. Treatment with medium and high doses of SSTN significantly reversed the effects of Glycerol and CM on kidney NO levels (ANOVA $F = 6.008$, $P < 0.001$).

Administration of glycerol alone or in combination with CM significantly increased kidney weight to body weight ratio (ANOVA $F = 10.228$, $P < 0.001$). Concomitant treatment with SSTN reduced glycerol and CM-induced increase in kidney/body weight ratio; however, the reduction was not significant (Table 2).

3.1. Histological Findings. The light microscopic findings in the kidneys of different treatment groups are given in Table 2. The groups injected with only glycerol produced tubular dilatation and mild tubular vacuolation and necrosis, whereas treatment with glycerol and CM produced significant tubular dilatation, tubular vacuolation, and tubular necrosis. Treatment of rats with SSTN dose dependently reversed CM-induced histological changes (Table 2, Figure 3).

4. Discussions

The findings of this study clearly showed a significant increase in the biomarkers of renal injury including BUN and SCr in the rats exposed to CM plus glycerol (Figure 1). The increase in SCr and BUN following exposure of rats

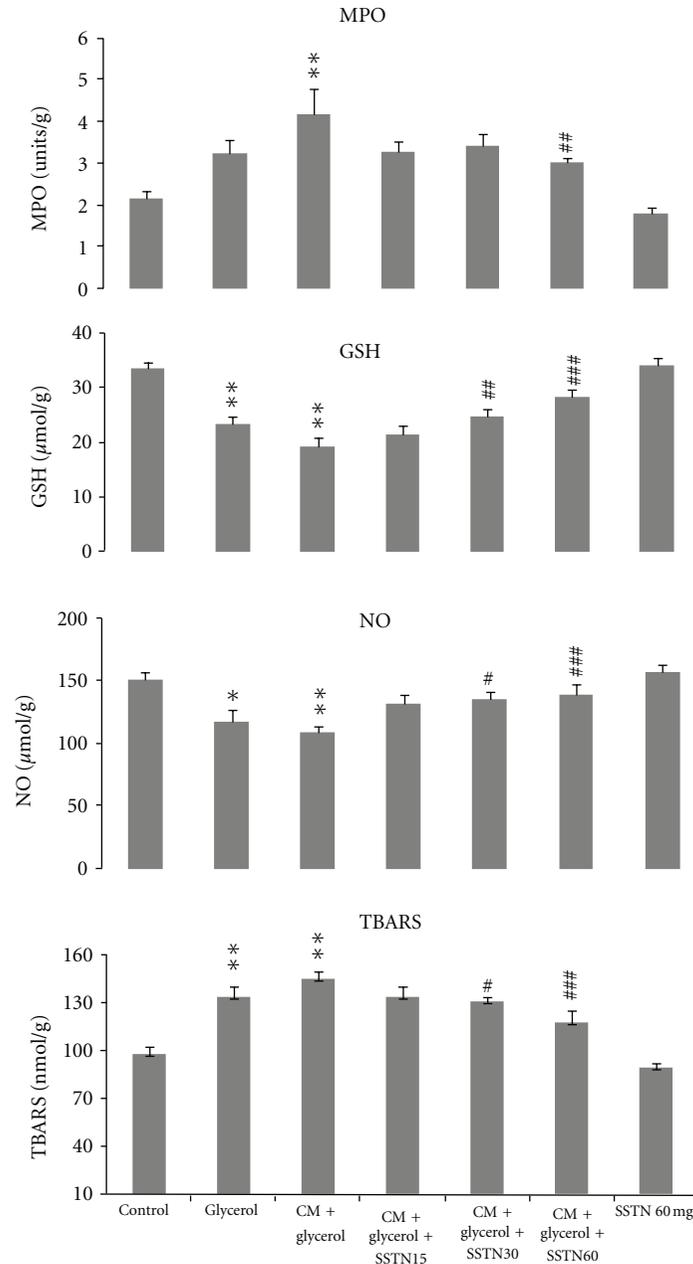


FIGURE 2: Effect of simvastatin on TBARS, NO, GSH, and MPO in a rat model of contrast media-induced nephropathy. Values are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ as compared to control group and # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ versus glycerol + CM group using Tukey's test. CM: contrast media; SSTN: simvastatin 10, 30, and 60 mg/kg.

to CM and glycerol has been reported earlier [17, 18]. Our histopathological studies also confirm significant tubular vacuolar transformation, interstitial edema, and tubular degeneration in this nephropathy model. Histologic alterations including proximal tubular vacuolar transformation, interstitial edema, and tubular degeneration following contrast administration have also been reported earlier [19]. Development of edema and enlargement of kidney by CM which was evident by significant increase in kidney weight-to-body weight ratio also confirm significant renal toxicity (Figure 3). Pretreatment with SSTN significantly and dose

dependently reversed the effects of CM on serum markers of renal injury (Figure 1). Treatment of animals with SSTN has been shown to exert a favorable effect on renal blood flow and glomerular filtration rate by earlier investigators [20], that is evident from a decrease in serum creatinine and blood urea nitrogen levels even in control rats treated with SSTN (Figure 1). There was a nonsignificant increase in the level of serum calcium following exposure to contrast media (Figure 1). On the other hand, treatment with SSTN prevented contrast induced rise in serum calcium. Calcium plays an important role in maintaining cell viability and

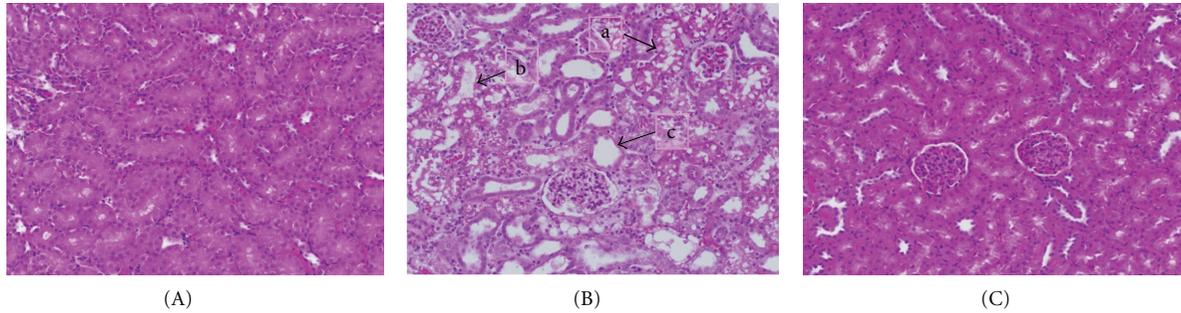


FIGURE 3: Light microscopy of renal tissue of rats stained with hematoxylin and eosin ($\times 200$). (A) Normal histology of kidney tissue. (B) Marked tubular necrosis (a), dilatation (b), and vacuolation (c) in CM model of nephropathy. (C) SSTN-induced reversal of CM-induced nephropathy showing minimal tubular abnormalities.

TABLE 2: Effect of simvastatin on tubular injury scores in a rat model of CM nephropathy.

Groups	Tubular dilatation	Tubular vacuolation	Tubular necrosis	Kidney wt/Body wt ratio (%)
Control	0	0	0	0.33 ± 0.01
Glycerol	$2.7 \pm 0.28^{***}$	$0.6 \pm 0.20^*$	$0.70 \pm 0.18^{**}$	$0.58 \pm 0.04^{***}$
CM + glycerol	$3.4 \pm 0.32^{***}$	$2.9 \pm 0.29^{***}$	$3.0 \pm 0.26^{***}$	$0.61 \pm 0.04^{***}$
CM + glycerol + SSTN 15	$2.3 \pm 0.28^\#$	$1.0 \pm 0.01^{###}$	$0.40 \pm 0.20^\#$	$0.50 \pm 0.03^\#$
CM + glycerol + SSTN 30	$1.9 \pm 0.22^{###}$	$0.9 \pm 0.01^{###}$	$0 \pm 0^{###}$	$0.48 \pm 0.03^\#$
CM + glycerol + SSTN 60	$1.2 \pm 0.2^{###}$	$0.4 \pm 0.24^{###}$	0	$0.47 \pm 0.03^\#$
SSTN 60	0	0	0	0.31 ± 0.00

Values are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control group; $^\#P < 0.05$, $^\#^\#P < 0.01$, and $^\#^\#^\#P < 0.001$ versus glycerol + CM group using Tukey's test.

High scores indicate severe injury.

integrity and alteration of calcium homeostasis may lead to cellular injury through activation of phospholipases, impairment of ion permeability, and their subsequent effect on cytoskeletal structure and function. Calcium channel blocker amlodipine has been shown to attenuate CM-induced nephropathy [18].

The pathogenesis of CM-induced nephropathy is poorly understood; however, numerous pathways have been suggested to participate in the injury process. While some studies support a role for direct action of CM on tubular toxicity, other investigators suggested that the final pathway for contrast-induced renal injury might be due to medullary hypoxia conditioned by medullary ischemia (due to volume depletion and prostaglandin inhibition) affecting metabolically active and hyperfiltering nephrons. CM infusion induces renal vasoconstriction [3], a process that is thought to arise from an imbalance between endothelium-derived vasoconstrictive and vasodilatory factors [21, 22]. Depending on the severity of CM induced vasoconstriction, ischemic tubular injury may result in cell detachment, apoptosis, or necrosis [23, 24]. The ischemic insult is often accompanied by reperfusion injury involving excessive generation of potentially toxic-free radicals and inflammatory mediators [24].

We observed a significant increase in TBARS and decrease in GSH in kidneys of rats exposed to CM, indicating a state of oxidative stress (Figure 2). Treatment of rats with SSTN reversed CM-induced oxidative stress. A significant

increase in lipid peroxides has been observed in kidneys of rats treated with CM [11]. Earlier studies also suggested that oxidative stress plays an important role in CM-induced nephrotoxicity [25–27]. The antioxidant, N-acetylcysteine (NAC), has been used clinically for prevention of iohalamate meglumine contrast-induced acute renal failure [28–32]. The protective effect of SSTN against CM can be attributed to its antioxidant property.

There was a significant increase in the proinflammatory enzyme MPO levels in the kidneys of CM-treated rats; this rise in MPO levels was attenuated by SSTN (Figure 2). Increased MPO concentration in the kidney is an indicator of neutrophil infiltration and it is directly proportional to the tissue inflammation caused by CM. MPO activation has been shown to cause glomerular morphologic changes, endothelial and mesangial cell injury, and activation of platelets as well as glomerulonephritis [33]. Higher activity of MPO has been reported in primed macrophages under inflammatory conditions in both patients with chronic kidney disease and experimental studies [34]. The protective effect of SSTN against CM-induced increase in MPO levels can be attributed to its anti-inflammatory property [35]. It has been reported that SSTN may prevent loss of kidney function to a greater extent in individuals with evidence of increased inflammation than in those who show no inflammation [36].

Biochemical analysis of kidney showed a significant decrease in NO levels in our contrast media model of

renal toxicity (Figure 2). A significant decrease in kidney NO following exposure of rats to iodixanol has also been reported by Sendeski et al. [37]. Decreased eNOS expression and increased plasma endothelin-1 have been implicated in nonionic CM-induced endothelial dysfunction and kidney injury [17]. Aggravation of CM-induced nephrotoxicity by hypercholesterolemia has been associated with the reduced production of NO [38]. Administration of L-arginine (NO donor) significantly attenuated hypercholesterolemia-induced aggravation of CM nephrotoxicity in rats [39]. Statins exert a positive influence on NO resulting in improved microcirculation, reduction in platelet aggregation, and enhancing cell proliferation [40–43]. Preservation of renal function by simvastatin in cyclosporine-induced ischemic/reperfusion injury has also been attributed to its vasodilator properties mediated by NO [44]. In conclusion SSTN offers significant nephroprotection against CM-induced nephropathies by inhibiting oxidative stress and inflammation and by improving renal microcirculation. Further clinical trials by using higher doses of statins alone or in combination with other nephroprotective agents should be tested as prophylactic agents to combat contrast-induced nephropathy.

5. Conclusion

In conclusion, the results of this study suggest a significant role of oxidative stress, proinflammatory myeloperoxidase and vasoregulatory nitric oxide in the pathogenesis of contrast-induced nephropathy. Treatment with high dose of SSTN prevents contrast-induced biochemical and structural changes in kidney. Therefore, further studies are warranted to test high doses of SSTN alone or in combination with other antioxidants and/or anti-inflammatory drugs to prevent CM-induced nephropathy.

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