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Silibinin: A Bioactive Flavanone in Milk Thistle Ameliorate Gentamicin Induced Nephrotoxicity in Rats

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ABSTRACT

Background and Objectives: The nephrotoxicity due to gentamicin is well established in man and experimental animals. Silibinin a bioactive flavanone in milk thistle possess anti-inflammatory and antioxidant activity. Therefore, the present study investigated the renoprotective effect of silibinin against gentamicin induced nephrotoxicity in rats. **Method:** Thirty rats were randomly divided into five equal groups (n = 6). Group I served as a control and treated orally with vehicle and Group II as a gentamicin control and administered vehicle two days before and then treated with gentamicin intraperitonially (100 mg/kg/day) for eight days. Group III-V were received silibinin orally at three dose levels (20, 40 and 80 mg/kg/day) two days before and eight days concomitantly with gentamicin intraperitonially (100 mg/kg/day). The silibinin was suspended in CMC (1% w/v). At the end of the treatment urine and blood was collected to assess the kidney functions as well as renal tissue processed for antioxidant and histopathological study. **Results:** Eight days of gentamicin treatment significantly increased levels of BUN, serum creatinine and decreased urinary creatinine and creatinine clearance. Further it increased MDA levels and decreased SOD and CAT activity as well as GSH levels. Silibinin treatment (40 and 80 mg kg^{-1}) reversed the gentamicin induced alterations dose dependently. Further necrosis and degenerative changes in glomeruli and tubules observed in gentamicin treated rats were significantly restored with silibinin treatment at a dose of 40 and 80 mg kg^{-1}. **Conclusion:** Silibinin dose dependently protected the kidney functions and normalize biochemical parameters and histopathological changes.

Key words: Silibinin, gentamicin, creatinine, antioxidant, milk thistle

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INTRODUCTION

Gentamicin is a powerful antibiotic intravenously administered to the patients in hospitals, nursing homes and home healthcare settings to combat severe infections. Acute tubular necrosis is a common complication of gentamicin therapy, occurring in 10 to 20% of patients (Moore *et al*., 1984). The nephrotoxicity of gentamicin is well established in man and experimental animals. Aminoglycosides are freely filtered across the glomerulus and then partially taken up and concentrated in proximal tubular cells (Laurent *et al*., 1990). Renal tubular cell injury produced by gentamicin evolves sub-acutely over several days and clinical manifestation of gentamicin toxicity is manifested by an increase in creatinine, urea and electrolyte alterations (Werner *et al*., 1995; Mingeot-Leclercq and Tulkens, 1999; Rougier *et al*., 2003). The generation of free radical species and alteration of mitochondrial function in the renal proximal convoluted tubules are associated with gentamicin therapy. In addition, induction of acute tubular necrosis, glomerular damage and renal inflammation are the major events implicated in gentamicin nephrotoxicity (Yanagida *et al*., 2004). Several reports indicate antioxidants significantly protect the rats against this toxicity (Morales *et al*., 2002).

Flavonoids are phenolic compounds widely distributed in fruits, vegetables; plant extracts as well as plant derived beverages. These have generated interest because of their broad pharmacological effects. Many of these effects are related to their antioxidant properties which may be due to their ability to scavenge free radicals. Silibinin, a flavanone is the major and most active component, constitutes about 60-70% in silymarin (Saller *et al*., 2001). Various preclinical reports suggest the myriad pharmacological activities of silibinin. It has been reported for antioxidant and hepatoprotective in nonalcoholic steatohepatitis (Haddad *et al*., 2011). Silibinin markedly improves endothelial dysfunction in

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db/db mice by reducing circulating and vascular ADMA levels (Li *et al*., 2011). Recently, Marrazzo *et al*. (2011) reported its neuroprotective effect due to DNA protection and antioxidant activity in diabetic mice (Marrazzo *et al*., 2011). In addition, several recent studies have shown the potential cancer preventive and therapeutic efficacy of silibinin in different animal models and cell culture systems (Raina *et al*., 2007; Singh *et al*., 2008).

Therefore, the present investigation was carried out to study the possible protective effect and to elucidate the mechanism of action of silibinin on gentamicin induced renal dysfunction, in order to gain new insights into the prophylaxis of gentamicin nephrotoxicity which is a common problem in its use.

MATERIALS AND METHODS

Drugs and chemicals: Gentamicin was purchased from local market of Pune (Genticyn, Piramal Healthcare, India), silibinin, malondialdehyde (MDA), tetrabutyl ammonium and superoxide dismutase (Sigma-Aldrich, St. Louis), Catalase (Hi Media Laboratories Pvt. Ltd., Mumbai) and all other reagents and chemical were of analytical grade and purchased from local suppliers of Pune.

Animals: Sprague Dawley (SD) rats (150-200 g) were procured from National Institute of Biosciences, Pune. Rats were placed separately in polypropylene cages with paddy husk as bedding. The animals were maintained under standard laboratory conditions at temperature 23±2°C with relative humidity 55±10% in a 12 h light and 12 h dark cycle throughout the experiment. Animals had free access to water and standard laboratory feed *ad libitum* (Nutrivet Lab, India). All the experimental procedures and protocols used in this study were reviewed and approved (IAEC/2011-12/33) by the Institutional Animal Ethics Committee (IAEC). Ethical guidelines were strictly followed during all the experimental procedures.

Experimental design: Thirty rats were randomly divided into five equal groups (n = 6). Group I served as a control and treated orally with vehicle (CMC, 1% w/v in water) and Group II as a gentamicin control and administered vehicle two days before and then treated intraperitonially with gentamicin (100 mg/kg/day) for eight days. Group III-V received silibinin orally at three different dose levels (20, 40 and 80 mg/kg/day) two days before and eight days concomitantly with gentamicin intraperitonially (100 mg/kg/day). The silibinin was suspended in CMC (1% w/v) (Parlakpinar *et al*., 2006; Harlalka *et al*., 2007).

One day before sacrifice each rat was individually placed in metabolic cage for 24 h urine collection. Urine was centrifuged at 1000 rpm for 10 min to remove cells and debris. Blood was collected from retro orbital plexus under light anesthesia and the serum was separated by centrifugation at 3000 rpm for 15 min. At the end of the experiment rats were killed by cervical dislocation under ether anesthesia. The abdominal cavity was immediately opened and both kidneys were removed and processed for antioxidant activity as well as histological examinations.

Body and kidney weight change: The body weight of all animals after the experiment was taken and their difference was expressed as body weight change. After sacrificing the animal one of the kidneys was rinsed in chilled saline, decapsulated blotted on filter paper and quickly weighed. For standardization, total kidney weight was normalized as kidney/body-weight ratio:

$$\text{Relative kidney weight (\%)} = \frac{\text{Absolute kidney weight}}{\text{Body weight at sacrifice}} \times 100$$

Biochemical estimations in serum and urine: Blood Urea Nitrogen (BUN) (Fawcett and Scott, 1960), serum and urinary levels of creatinine (Bartels *et al*., 1972) were estimated as per the instruction of commercial diagnostic kits. Whereas creatinine clearance was calculated as per the following equation:

$$\text{Ccr (mL/min/kg)} = (\text{urinary Cr (mg dL}^{-1}) \times \text{urinary volume (mL)/serum Cr (mg dL}^{-1})) (1000/\text{body weight (g)}) \times (1/1440\ \text{(min)})$$

Determination of oxidative stress biomarkers in renal tissues: Left kidneys was rinsed, decapsulated, blotted on filter paper and quickly weighed. Then, it was homogenized in chilled 50 mM phosphate buffer saline (pH 7.4) in volume of nine times of its weight to yield 10% (w/v) tissue homogenate. The homogenates were centrifuged at 10500 rpm for 15 min at 4°C. The homogenate was then used for determination of the levels of Malondialdehyde (MDA) (Ohkawa *et al*., 1979), reduced glutathione (GSH) (Beutler *et al*., 1963) and activities of SOD (Sun *et al*., 1988) and catalase (CAT) (Luck, 1971). Protein concentrations of homogenates were determined according to Lowry *et al*. (1951).

Histopathological studies: Right kidney of individual rat stored in 10% formalin solution were embedded with paraffin and stained with Haematoxylin-Eosin (HE). HE stained sample was observed under light microscope (100x).

Statistical analysis: All the data were expressed as the Mean±SEM (n = 6). Data were subjected to one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. Level of significance was set at $P<0.05$ and the analysis were made using computerized Graph Pad Prism version 5.0 (Graph pad software, USA).

RESULTS

Body and kidney weight change: Gentamicin treatment produced significant loss in body weight and increase in kidney weight compared to control ($p<0.01$). Treatment with silibinin (40 and 80 mg kg^{-1} body weight) resulted in significant increase in body weight ($p<0.05$ and $p<0.01$, respectively) and decrease in kidney weight ($p<0.01$ and $p<0.001$, respectively) compared to gentamicin control rats. However, silibinin at dose of 20 mg kg^{-1} body weight could not produce significant changes in body weight and kidney weight compared to gentamicin control rats (Table 1).

Biochemical estimations in serum and urine: As shown in Table 2, gentamicin produced significant elevation in blood urea nitrogen and serum creatinine levels as well as decrease urinary creatinine levels compared to control rats ($p<0001$). Treatment with silibinin at dose of 40 and 80 mg kg^{-1} significantly reduced elevated levels of BUN ($p<0.001$) and serum creatinine ($p<0.01$ and $p<0.001$, respectively) and increased levels of urinary creatinine ($p<0.01$ and $p<0.001$, respectively) compared to gentamicin control rats. Further, gentamicin induced decrease in creatinine clearance was significantly increased by silibinin treatment at a dose of 80 mg kg^{-1} ($p<0.001$) (Fig. 1).

Renal antioxidant biomarkers: Gentamicin control rats exhibited increased renal MDA levels ($p<0.001$). Treatment with silibinin (20, 40 and 80 mg kg^{-1}) attenuated MDA levels associated with gentamicin treatment ($p<0.01$, $p<0.01$ and $p<0.001$, respectively). Moreover, gentamicin treatment decreased the activity of renal SOD ($p<0.001$) and CAT ($p<0.01$) as well as levels of GSH ($p<0.001$) compared to control rats. Treatment with silibinin increased the activity of SOD ($p<0.05$ and $p<0.001$, respectively) and CAT ($p<0.05$ and $p<0.01$, respectively) as well as GSH levels ($p<0.01$ and $p<0.001$, respectively) compared to gentamicin control rats (Table 3).

Histopathological studies: Gentamicin treated animals showed more extensive and marked tubular necrosis, inflammation, blood vessel congestion and disintegrated nucleus. However, no abnormalities were observed in control rats. Treatment with silibinin (40 and 80 mg kg^{-1}) dose dependently attenuated these progressions. Accordingly, there were no marked microscopical differences among the control and silibinin treated group (80 mg/kg/day) (Fig. 2).

Table 1: Effect of silibinin on body weight, kidney weight change in gentamicin treated rats

Groups	Body wt. (g)	Kidney wt. (g)	Relative kidney wt. (%)
Control	217.67±2.36	0.89±0.01	0.42±0.09
Gentamicin control	200.83±3.59[a] (8.20%)	1.21±0.03[a] (35.96%)	0.61±0.02[a]
Silibinin (20)	203.35±2.83 (1.29%)	1.12±0.06 (7.43%)	0.58±0.03
Silibinin (40)	214.78±2.27[*] (8.57%)	0.93±0.04[#] (23.14%)	0.43±0.02[c]
Silibinin (80)	217.71±3.86[#] (9.42%)	0.91±0.02[b] (24.79%)	0.41±0.01[b]

[a]$p<0.01$ compared to control. [*]$p<0.05$, [#]$p<0.01$, [b]$p<0.001$ compared to gentamicin control. Numbers in ( ) indicates percentage decrease in body weight as compared to control, percentage increase in body weight as compared to gentamicin control, percentage increase in kidney weight as compared to control and percentage decrease in kidney weight as compared to gentamicin control

Table 2: Effect of silibinin on serum and urinary biochemical estimations in gentamicin treated rats

Groups	BUN (mg dL^{-1})	Sr. Creatinine (mg dL^{-1})	Ur. Creatinine (mg dL^{-1})
Control	24.50±1.10	1.12±0.08	82.68±2.26
Gentamicin control	47.24±2.18[b]	3.53±0.21[b]	33.77±2.44[b]
Silibinin (20)	38.27±1.92	3.13±0.19	42.98±5.62
Silibinin (40)	27.17±1.14[a]	2.34±0.28[#]	56.89±3.29[#]
Silibinin (80)	22.00±2.09[a]	1.07±0.15[a]	82.40±3.07[a]

[b]$p<0.001$ compared to control. [*]$p<0.05$, [#]$p<0.01$, [a]$p<0.001$ compared to gentamicin control

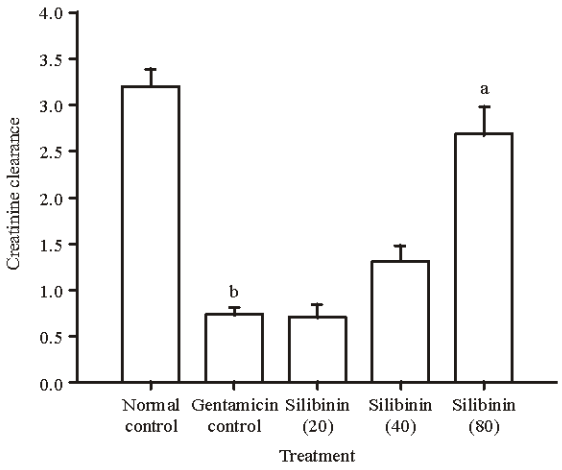

Fig. 1: Effect of silibinin on creatinine clearance in gentamicin treated rats. [b]$p<0.001$ compared to control; [a]$p<0.001$ compared to gentamicin control

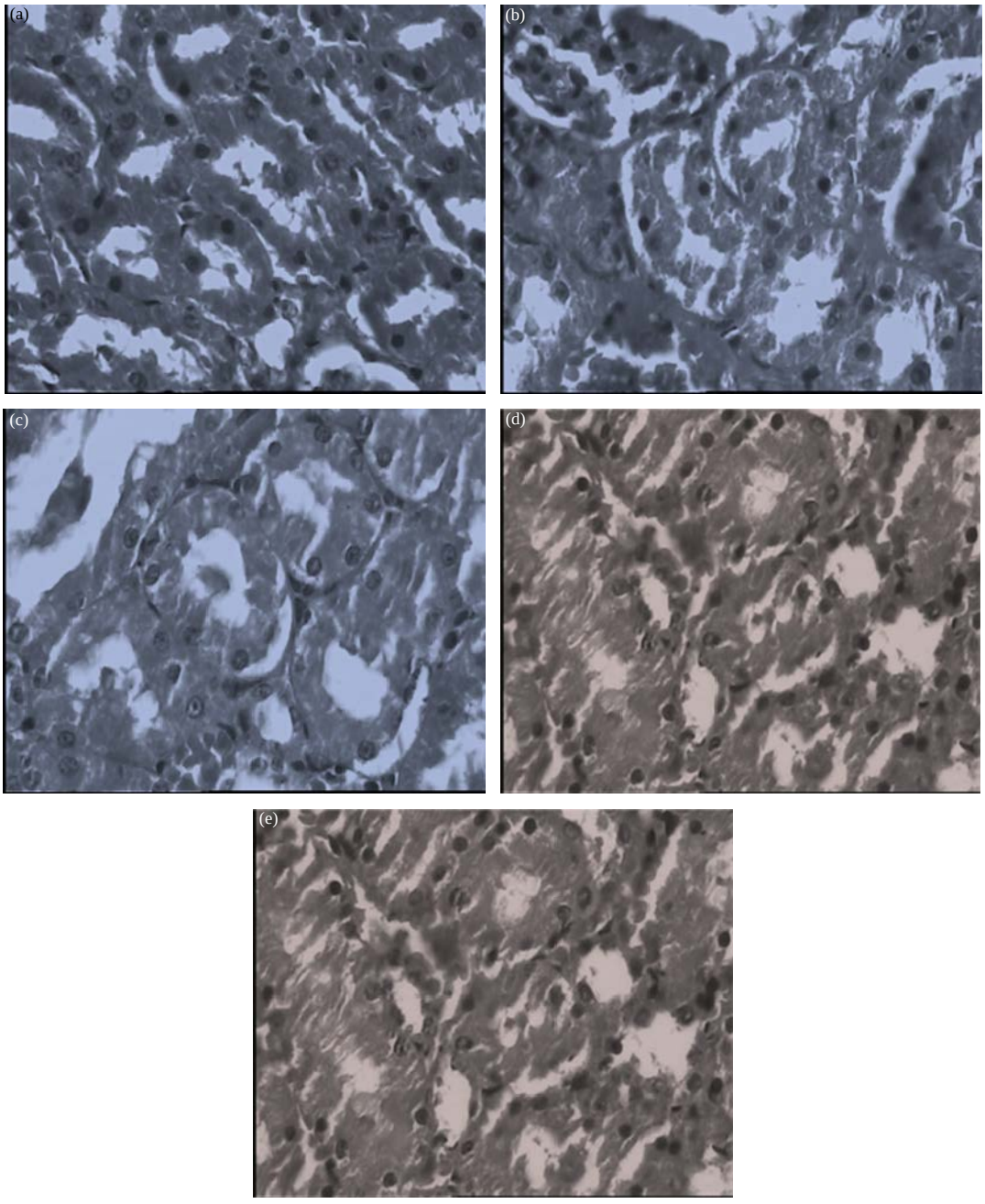

Fig. 2(a-e): Histological section of kidneys stained with Haematoxylin and Eosin (100x). (a) Control (NC) showing normal kidney cells, (b) Rats treated with gentamicin showing tubular necrosis, inflammation, disintegrated nucleus and blood vessel congestion, (c) Rats treated with gentamicin+silibinin (20 mg kg^{-1}) showing moderate to severe necrosis, (d) Rats treated with gentamicin+silibinin (40 mg kg^{-1}) showing mild necrosis and (e) Rats treated with gentamicin+silibinin (80 mg kg^{-1}) showing normal kidney cell

Table 3: Effect of silibinin on renal antioxidant biomarkers in gentamicin treated rats

Groups	MDA (nmol mg^{-1})	SOD (U mg^{-1})	GSH (nmol mg^{-1})	CAT (U mg^{-1})
Control	7.17±0.48	32.31±1.19	45.64±2.44	18.98±2.28
Gentamicin control	11.58±1.08[b]	23.72±1.47[b]	30.88±1.27[b]	13.03±1.27[a]
Silibinin (20)	7.58±1.12[#]	24.29±1.28	34.36±1.19	16.86±1.36
Silibinin (40)	7.95±0.46[#]	28.84±1.05[*]	42.70±1.37[#]	17.78±0.78[*]
Silibinin (80)	5.94±0.54[c]	31.54±1.54[c]	44.91±1.77[c]	18.60±0.65[#]

[a]p<0.01, [b]p<0.001 compared to control. *p<0.05, [#]p<0.01, [c]p<0.001 compared to gentamicin control

DISCUSSION

Therapeutic use of aminoglycosides, gentamicin for more than seven days being used in clinical practice to combat severe infections is the commonest cause of nephrotoxicity in 20-30% of patient (Pedraza-Chaverri *et al*., 2003). Clinically, renal failure with a slow rise in serum creatinine, urea nitrogen along with reduction in Glomerular Filtration Rate (GFR) is the characteristic manifestation of gentamicin induced nephrotoxicity. The generation of free radical species and alteration of mitochondrial function in the renal proximal convoluted tubules are associated with gentamicin therapy. Therefore, antioxidants associated with renoprotective activity have been extensively studied against gentamicin induced nephrotoxicity.

In the present investigation intraperitoneal administration of gentamicin (100 mg kg^{-1}) produced significant reduction in body weight which is in agreement of previous reports (Lakshmi and Sudhakar, 2010). In acute renal failure increased catabolism results in acidosis which is accompanied by anorexia responsible for decreased food intake and causes body weight loss (Ali *et al*., 1992). Further, renal tubular injury leads to subsequent loss of the tubular cells that take part in renal water reabsorption leading to dehydration and loss of body weight (Ali *et al*., 2005). The increase in the kidney weight of gentamicin treated rats probably resulted from the edema caused by drug induced acute tubular necrosis (Erdem *et al*., 2000). Treatment with silibinin at a dose of 40 and 80 mg kg^{-1} significantly restored the change in body weight and kidney weight associated with gentamicin.

Several reports suggest serum creatinine concentration is a more potent indicator than urea in the first phases of kidney disease (Gilbert *et al*., 1989). It was reported that gentamicin produced prominent kidney damage as evidenced by significantly higher levels of serum creatinine, blood urea nitrogen and decreased urine creatinine as well as marked reduction in creatinine clearance (Silan *et al*., 2007; Soliman *et al*., 2007). In the present study, we observed the significantly higher levels of serum creatinine, blood urea nitrogen and decreased urine creatinine as well as marked reduction in creatinine clearance following gentamicin treatment which is in agreement of the previous reports. On the other hand administration of silibinin at dose levels of 40 and 80 mg kg^{-1} two days prior and eight days concomitant with gentamicin provided marked improvement in renal functions. Silibinin dose dependently restored the elevated levels of serum creatinine, blood urea nitrogen and decreased urinary creatinine levels.

Gentamicin has been found to increase the generation of Reactive Oxygen Species (ROS) like superoxide anions, hydroxyl radicals and hydrogen peroxides and Reactive Nitrogen Species (RNS) in the renal cortex that eventually lead to renal damage and necrosis via several complex mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage (Nagai and Takano, 2004; Nagai, 2006). In the present study we observed that gentamicin induced oxidative stress as a result of marked elevation in MDA levels and decreased SOD and CAT activity as well as GSH levels. Free radical scavengers or agents interfere with the production of ROS have been used successfully to ameliorate gentamicin nephropathy. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase protect the cells against oxidative stress mediated cellular injury by converting the toxic radicals to non-toxic end products. Treatment with silibinin at dose levels of 40 and 80 mg kg^{-1} significantly restored the oxidative stress by decreased MDA formation and increased GSH concentration as well as SOD and CAT activity. Moreover, histopathological examination of gentamicin treated rats supported the biochemical results indicating the structural abnormalities revealed by the presence of tubular necrosis, inflammation, blood vessel congestion and disintegrated nucleus in the gentamicin treated rats. Treatment with silibinin (40 and 80 mg kg^{-1}) was found to reduce such changes induced by gentamicin.

Thus, treatment with silibinin at dose levels 40 and 80 mg kg^{-1} showed dose dependant renoprotective effect against gentamicin induced nephrotoxicity and the effect may be related to the antioxidant properties, since it has been found that reactive oxygen species may be involved in the impairment kidney function.

CONCLUSION

In conclusion, co-administration of silibinin (40 and 80 mg kg^{-1}) along with gentamicin protect both functional and histological changes through inhibiting free-radical formation and restoration of the antioxidant systems.

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