

## Effect of silymarin on renal levels of free calcium and trace elements in gentamicin-induced nephrotoxicity in rats

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### Summary:

**Background:** Aminoglycosides antibiotics are nephrotoxic, with most of the damage confined to the proximal convoluted tubules. But the mechanism for cellular toxicity is not clear; whether inhibition of mitochondrial respiration and calcium transport or lipid peroxidation were claimed to be the causes of irreversible cell damage.

Silymarin as a natural remedy for diseases of the liver necessitates the evaluation of the efficiency of this compound and its possible mode of action on the ameliorating the injury induced by xenobiotics or drugs on tissues other than the liver.

**Objectives:** The aim of this study was designed to investigate the possible effect of silymarin on amelioration of renal injury induced by gentamicin and the consequences of nephrotoxicity by measuring changes in zinc, copper and calcium homeostasis, which may play a role in gentamicin-induced nephrotoxicity.

**Materials and methods:** Kidney tissue homogenate from normal controls, gentamicin-treated, and silymarin 250 mg/kg pre-treated before the induction of renal toxicity with gentamicin in *Rattus norvegicus* rats were obtained, and processed for the estimation of free calcium levels, trace elements, (zinc and copper) levels using atomic absorption spectrophotometry. (AAS).

**Results:** Analysis of data revealed significant decrease in the levels of free calcium, copper and in copper over zinc ratio, while there is an increase in the level of zinc in kidney tissue homogenate in the animals pre-treated with silymarin when compared to gentamicin-treated animals.

**Conclusion:** These findings suggest that, silymarin is effective in ameliorating the consequences of renal cell injury evoked by gentamicin as evidenced by decreasing the levels of free calcium and copper with the reduction in copper/zinc ratio, and elevation in the level of zinc in kidney tissue homogenate.

**Key words:** Silymarin, gentamicin, calcium, trace elements, nephrotoxicity

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### Introduction:

Aminoglycosides have long been one of the commonest causes of drug-induced nephrotoxicity<sup>(1)</sup>. Higher doses, (40 mg/kg or more for gentamicin) are necessary in animals to induce extended cortical necrosis and overt renal dysfunction. At this stage, a large number of structural, metabolic and functional alterations are observed in tubular cells.<sup>(2)</sup> The mechanism of this necrosis remains unsettled, and can not be traced to a single, well-determined causes. One of the hypotheses is the impairment of calcium homeostatic mechanism<sup>(3)</sup>. The second hypotheses is the involvement of highly reactive radical species which are supposed to play a role in the nephrotoxic effect of gentamicin, and are considered as a possible intermediates that may contribute to cellular damage<sup>(4, 5)</sup>. Oxidants can interact with both cellular metabolites and

structural elements and modify their properties by changing calcium ( $\text{Ca}^{+2}$ ) code and modify essential life processes<sup>(6)</sup>. Oxygen free radicals are normally neutralized by very efficient systems in the body including anti-oxidant enzymes like superoxide dismutase (SOD)<sup>(7)</sup>, essential elements like zinc and copper, co-factors in some metabolic pathways including many cellular enzymes<sup>(8, 9)</sup>.

Silymarin, a mixture of flavonolignans derived from milk thistle *Silybum marianum*, comprised mainly of silybinin A and B, isosilybinin, silychristine, silydianin, and taxifoline having anti-oxidant and free radical scavenging activities.<sup>(10, 11, 12)</sup>

This study was undertaken to assess of silymarin on gentamicin-induced nephrotoxicity by measuring changes in zinc (zn), copper (cu) and calcium ( $\text{ca}^{+2}$ ) homeostasis in kidney tissue homogenate.

### Methods:

Fourteen rats, *Rattus norvegicus* of either sex, weighing 180-220g allocated into 3 groups, housed in the animal house-college of pharmacy under standard laboratory conditions and had free access to water and fed standard chow *ad libitum*. Animal

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groups are treated as follows:

Group I- Four rats treated orally with corn oil (vehicle) and injected intraperitoneally. (I.P.) with normal saline, served as control.

Group II- Four rats treated twice daily with 250mg/kg silymarin in corn oil, given orally 7 days before induction of renal toxicity by subcutaneous (S.c) injection of 40mg/kg gentamicin twice daily for 5 days<sup>(13)</sup>.

Group III- Six rats treated with corn oil orally for 7 days and then renal toxicity was induced by S.c injection of 40mg/kg gentamicin twice daily for 5 days.

All animals were sacrificed on day 6 after gentamicin administration by cervical dislocation. Their renal tissues were obtained and utilized for preparation of tissue homogenate. by standard procedure, and utilized for the estimation of levels of free calcium, trace elements (zn and cu) using atomic absorption spectrophotometry, (AAS), in which each sample was diluted with de-ionized water to bring the metal concentration within the working range of AAS by direct aspiration of diluted sample using different concentrations of  $ca^{+2}$ , zn and cu for the curve preparation.<sup>(14)</sup>

Statistical analysis of data performed utilizing students' t-test. 95% confidence of data was considered for significance.

### Results:

Subcutaneous injection of twice daily dose of gentamicin (40mg/kg for 5 days) to rats showed significant higher levels ( $P < 0.05$ ) of free  $ca^{+2}$  in kidney tissue homogenate than controls as shown in table 1. The levels being 1.2 fold higher than that of controls.

Pre-treatment with oral dose of silymarin 250mg/kg to animals produces 1.16 fold decrease in free  $ca^{+2}$  levels in kidney tissue homogenate when compared to gentamicin-treated group Table 1

Gentamicin-treated rats had significant higher and lower levels of cu and elemental zn, respectively in kidney tissue homogenate when compared to controls as shown in table 2. Moreover, there were a significant increase in cu/zn ratio in kidney tissue homogenate in animals treated with gentamicin compared to control group. ( $P < 0.05$ ) Table 3

Pre-treatment of rats with 250mg/kg silymarin orally, resulted in significant lower and higher levels of cu and elemental zn, respectively when compared to gentamicin-treated animals ( $P < 0.05$ ) as shown in table 2.

In addition, there were a significant decrease in cu/zn ratio in kidney tissue homogenate in animals pre-treated with silymarin 250mg/kg orally when compared to gentamicin-treated group. ( $P < 0.05$ ) Table 3.

### Discussion:

Aminoglycosides cause structural, functional and metabolic alteration within the renal proximal tubules.<sup>(2)</sup> Impairment of calcium homeostasis may be implicated in gentamicin-induced nephrotoxicity and cellular damage<sup>(3)</sup>.

In the present study, rats treated with toxic dose of gentamicin (40mg/kg for 5 days), showed significant increase in the levels of free  $ca^{+2}$  in kidney tissue homogenate when compared to control group. (Table 1).

Previous reports proposed that, extra cellular  $ca^{+2}$ -sensing receptor (caR) distribution, are abundance in rats' proximal tubules is consistent with the site of aminoglycoside endocytosis and nephrotoxicity which is mostly confined to the  $S_1$  and  $S_2$  segments<sup>(11, 14)</sup>.

Martinez-Salgado, C et al in 2000<sup>(15)</sup> proposed that, gentamicin directly raise intracellular  $ca^{+2}$  activating both calcium influx from external media and calcium release from internal stores in cultured mesangial cells, that is responsible of cellular activation and proliferation. Moreover, Ward, DT et al in 2005<sup>(16)</sup> used a proximal tubular-derived cell line model, (ok cells), which are responded to neomycin, tobramycin and gentamicin with an increase in cytosolic [ $ca^{+2}$ ] and activated the extracellular signal regulated kinase, ERK<sub>1</sub> and ERK<sub>2</sub> and thus could play a role in the direct effect of aminoglycosides-induced nephrotoxicity.

Aminoglycosides may indirectly impair calcium homeostasis, through the involvement of reactive oxygen species<sup>(17)</sup>, where oxidative stress causes calcium influx into the cytoplasm from extra cellular environment and from endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) through the cell membrane and ER/SR channels, respectively<sup>(18)</sup> resulting in increase [ $ca^{+2}$ ] in cytoplasm which in turn causes  $ca^{+2}$  influx into mitochondria and nuclei<sup>(19)</sup> leading to disruption of normal metabolism resulting in cell death.

In this study, pre-treatment of rats with oral silymarin 250mg/kg was effective in preventing the rise in free calcium levels induced by gentamicin in kidney tissue homogenate. (Table 1).

**Table 1: Effect of 250mg/kg silymarin on free calcium levels in the kidney homogenate of control and gentamicin-treated rats. Data are given as mean $\pm$ SD.**

Animal group	Free $ca^{+2}$ levels in kidney homogenate $\mu g/g$ tissue
Control N=4	3.5 $\pm$ 0.19 <sup>(a)</sup>
Gentamicin-treated N=6	4.3 $\pm$ 0.65 <sup>(b)</sup>
Gentamicin + silymarin 250mg/kg N=4	3.7 $\pm$ 0.12 <sup>(c)</sup>

Values with non-identical superscripts (a,b,c) within each parameter are significantly different. ( $P < 0.05$ )  
N= no. of animals.



A study performed by Farghali, H. et al in 2000<sup>(20)</sup> using perfused rat hepatocytes, shown that the hepatoprotective effect of silymarin against tert-butyl hydroperoxide (TBH) and D-galactosamine (D-Gal) intoxication was attributed to inhibition of lipid peroxidation and that the modulation of hepatocyte intracellular  $Ca^{+2}$  plays a pivotal role in a protective effect. In view of this biochemical mechanism, silymarin was expected to exhibit similar effect on other cell types, like the cells of renal tissue.

The present work showed that, rats received gentamicin (40mg/kg for 5 days) produced significant increase and decrease in the levels of Cu and Zn, respectively and a significant increase in Cu/Zn ratio in kidney tissue homogenate (Table 2). Additionally, rats pre-treated with silymarin orally, one week before S.c injection of toxic dose of gentamicin, resulted in significant decrease and increase in the levels of renal Cu and Zn, respectively and a decrease in Cu/Zn ratio compared to gentamicin-treated rats (Table 3)

**Table 2: : Effect of 250mg/kg silymarin on the levels of trace elements (copper and zinc) in the kidney homogenate of control and gentamicin-treated rats. Data are given as mean±SD.**

Animal group	Elemental zinc level in kidney homogenate $\mu\text{g/g}$ tissue	Copper levels in kidney homogenate $\mu\text{g/g}$ tissue
Control N=4	2 ± 0.78	2.3 ± 0.62 ( a )
Gentamicin-treated N=6	1.63 ± 0.81	2.7 ± 0.46 ( b )
GN + silymarin 250mg/kg N=4	1.22 ± 0.4	1.3 ± 0.12 ©

Values with non-identical superscripts (a,b,c) within each parameter are significantly different. ( $P < 0.05$ )  
N= no. of animals.

**Table 3 Effects of 250mg/kg silymarin on the copper/zinc ratio in the kidney homogenate of control and gentamicin-treated rats. Data are given as mean±SD.**

Animal group	Cu/Zn ratio in kidney homogenate $\mu\text{g/g}$
Control N=4	1.518 ± 0.37 ( a )
Gentamicin-treated N=6	1.829 ± 0.58 ( b )
GN + silymarin 250mg/kg N=4	1.068 ± 0.6 ©

Values with non-identical superscripts (a,b,c) within each parameter are significantly different. ( $P < 0.05$ )  
N= no. of animals.

Trace elements, Cu and Zn are important in the function of many cellular enzymes and are critical components of anti-oxidant enzyme SOD<sup>(7)</sup> Where copper can adapt distinct redox states oxidized Cu II or reduced Cu I allowing the metal to play a pivotal role in cell physiology as a catalytic co-factor in the redox chemistry of enzymes, mitochondrial respiration, iron absorption and free radical

scavenging.<sup>(9, 21)</sup> If present in excess, free copper ions can cause damage to cellular components with the production of  $O\cdot H^{\cdot}$ <sup>(22, 23)</sup>.

Considering Zn ions, it possesses two additional anti-oxidant mechanisms, it may induce synthesis of metallothionein, sulfhydryl-rich protein chelator that protect against free radicals<sup>(24)</sup> and may replace redox active molecule such as iron and copper, at critical sites in cell membrane and proteins.<sup>(25)</sup>

Highly reactive radical species are supposed to be one of the proposed mechanism of gentamicin-induced nephrotoxicity and are considered as a possible intermediates that may contribute to cellular damage<sup>(4,5,26)</sup> where the second proposed mechanism is the significant change found among trace elements levels in renal tissue.

The results of this study are consistent with previous report, in which a significant change found among trace elements deposit in renal homogenate, might be the mechanism for kidney toxicity of gentamicin similar to that induced by the nephrotoxic agent realgar in renal tissue of rats<sup>(27)</sup>.

Pre-treatment of rats with silymarin, attenuates the changes in trace elements in kidney tissue homogenate and decrease Cu/Zn ratio. The effect produced by silymarin, may be due to its powerful anti-oxidant effect and may act as potent chelating agents. Previous report showed that silybinin, one of the active constituents of silymarin, acts as a potent iron chelating agent<sup>(28)</sup>, though to block the formation of  $O_2$  free radicals through iron chelation; and this may provide a speculation for the expanding the chelating property of silymarin other than iron like copper, which is considered to be an essential catalyst for the production of  $O\cdot H^{\cdot}$  similar to iron and thus preventing gentamicin nephrotoxicity.

This study showed that, silymarin increases the kidney levels of Zn, which is an essential element of the anti-oxidant enzyme SOD<sup>(7)</sup>; in addition it induces the synthesis of metallothionein, a ubiquitous, cysteine-rich metal binding protein, which play a role in detoxifying of heavy metals and reactive oxygen species<sup>(29, 30)</sup>. Thus silymarin may indirectly ameliorating gentamicin nephrotoxicity via the induction of renal tubular metallothionein synthesis.

In conclusion, changes of free calcium level and trace elements (Cu and Zn) in rat kidney homogenate, might explain the possible mechanisms for renal toxicity and the protective effect of silymarin in ameliorating nephrotoxicity induced by gentamicin.

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