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THE PROTECTIVE EFFECTS OF ZINC IN EXPERIMENTAL GENTAMICIN INDUCED ACUTE RENAL FAILURE IN RATS

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This study investigates the effects of zinc in acute kidney injury induced by gentamicin (Ge). We used Wistar male rats distributed in 4 groups of 12 animals each, treated intraperitoneally as follows: Group I (Control) treated with distilled water; Group II (Ge) with experimental induced acute renal failure with Ge; Group III (Ge + Zn) administration of $ZnCl_2$ in animals with experimental induced renal failure with Ge, Group IV (Zn) treated with $ZnCl_2$ as positive control. We measured serum levels of urea, creatinine, total antioxidant status, superoxide dismutase, glutathione peroxidase and urinary proteins before the nephrotoxicity induction (baseline) and 3, 7 and 10 days after Ge administration. The renal histopathological analysis was also done. The results showed an increase of urea and creatinine values in Ge + Zn group after 7 days compared to baseline, but less accentuated than those in Ge group. Zn supplementation was associated with an increase of the total antioxidant status in Ge + Zn group compared to Ge group ($P < 0.01$). It was also revealed a significant reduction of proteinuria in Ge + Zn group compared to Ge group ($P < 0.001$). The histopathological investigation highlighted the tubular necrosis affecting more than 90% of proximal tubules in Ge group. In Ge + Zn group it was observed a milder degree of tubular necrosis (influencing less than 25% of proximal tubules), a moderate inflammation and the presence of tubular regeneration. In conclusion, Zn administration proved a to have a protective role in experimental gentamicin-induced acute renal failure.

Key words: *zinc, gentamicin, acute renal failure, oxidative stress, urea, creatinine, superoxide dismutase, glutathione peroxidase*

INTRODUCTION

The kidneys are key organs with important functions in regulating of the hydro-electrolyte equilibrium, in elimination of the waste products from the blood, and in maintaining of the organism's acid-base balance (1). In acute renal failure, the kidney filtration capacity is altered, which results in an accumulation of the excessive amounts of wastes and also in a marked deterioration of renal structure with cell necrosis and apoptosis. The development of chronic renal failure occurs as a result of severe metabolic changes in the body, and it is associated with kidney histopathological changes, characterized by hypertrophy of the basement membrane, glomerular endothelial cell proliferation, interstitial edema, intertubular capillary congestion (2, 3). This pathological state is usually accompanied by the decrease of plasmatic zinc values and the increase of urinary zinc elimination. The serum depletion of zinc induces the reduction of zinc-dependent angiotensin-converting enzyme activity, with a consecutive enhancement of angiotensin II levels, thus determines the renal blood vessels constriction, and the aggravation of kidney insufficiency (4). These constitute an argument on the role of zinc to preserve the renal function and to diminish the kidney damage induced by potential toxic agents. Zinc deficiency may be also associated with increased oxidative stress, immune defense capacity impairment and neurotransmission disturbances (5).

Different drugs used in the therapy (for example: antibacterial chemotherapeutic or immunosuppressive agents) may induce acute or chronic renal failure, with severe impact on patient health (6, 7). Acute kidney injury, responsible for a high incidence of long term mortality, has lately registered an increased prevalence. Experimental models are important for a better understanding of the mechanisms involved in renal injury and also for developing optimal management of this pathological condition (8-10).

Aminoglycosides are antibiotics widely used due to their chemical stability, rapid bactericidal effect, synergistic effect in association with beta-lactam antibiotics and low risk of resistance (11). Despite all these properties, aminoglycosides, particularly gentamicin (Ge), exert important toxic effects. Nephrotoxicity is one of the most important adverse effects,

occurring in 10 – 25% cases treated with aminoglycosides, even after rigorous treatment monitoring (12). It has been shown that oxidative stress plays an important role in gentamicin nephrotoxicity (7). Gentamicin induces the production of high reactive oxygen species that can activate some pro-inflammatory and pro-apoptotic mediators, leading to renal injuries (13).

Zinc (Zn) is an important trace element found in all body tissues, especially in the brain, muscles, bones, kidneys and liver. It plays an essential role in normal growth and development, protein synthesis, cellular division (14) and modulation of the affective behavior (5). Zn is involved in many biochemical functions. Over 300 enzymes are zinc-dependent for their activation and nearly 2,000 transcription factors require zinc for gene expression (15). Zn is a molecular signal for immune cells and many transcription factors involved in inflammatory cytokines are regulated by Zn (16). It also has antioxidant and anti-inflammatory properties, contributing to maintaining the antioxidant system homeostasis (17). Zn induces the decrease of reactive oxygen species and provides cellular membrane stability, showing a high protective effect against free radicals (18).

In our study, we investigated the effect of Zn in acute renal failure induced by gentamicin.

MATERIAL AND METHODS

Experimental procedure

The study protocol was approved by the Ethic Committee on Research of 'Grigore T. Popa' University of Medicine and Pharmacy, Iasi, Romania, in accordance with EU Directive 2010/63/EU for animal experiments.

We used gentamicin, ampoules with 80 mg/2 ml solution for injection, (commercial pharmaceutical form produced by KRKA Company) and $ZnCl_2$ (produced by Sigma Aldrich). $ZnCl_2$ was dissolved in distilled water 1mg/ml. The experiment was carried out on adult male Wistar rats (body weight 210 – 320 g). During the experiment the animals were housed in plastic cages, at a temperature of 22 – 24°C, with a constant 12-hour light/12-hour dark cycle, with free access to water and standard granulated food. After a quarantine period of 7 days, the rats were randomly divided into 4 groups of 12 animals each, treated intraperitoneally (i.p.), as follows: Group I (Control): distilled water 0.5 ml/100 g body weight/day for 17 days, Group II (Ge): distilled water 0.5 ml/100 g body weight/day, for the first 10 days and Ge 80 mg/kg body weight/day, for the next 7 days; Group III (Ge + Zn): $ZnCl_2$ 5 mg/kg body weight/day, for first 10 days and Ge 80 mg/kg body weight/day + $ZnCl_2$ 5 mg/kg body weight/day, for the next 7 days. The two substances were administered separately within two hours; Group IV (Zn): $ZnCl_2$ 5 mg/kg body weight/day, for 17 days.

Biochemical analysis

The animals were housed in metabolic cages in order to collect urine samples. The rats were anesthetized and blood samples were collected from the retro-orbital plexus.

Serum levels of urea, creatinine, total antioxidant status (TAS), superoxide dismutase (SOD), glutathione peroxidase (GPx) and urinary proteins were determined using Rx Daytona Chemistry Analyzer (RANDOX) and diagnostic kits RANDOX. The measurements were made before the nephrotoxicity induction (baseline - I_0) and 3, 7 and 10 days after the first Ge administration.

Histopathological evaluation

For histopathological evaluation at 3, 7 and 10 days after the first Ge administration, three rats of each group were euthanized and both kidneys were removed. All specimens were fixed in 10% buffered formalin and embedded in paraffin with a tissue processor Leica TP1020 (Leica Microsystems GmbH, Germany). Sections of 5 µm thickness were obtained with a Microtome SLEE CUT 6062 (SLEE Medical GmbH, Germany), deparaffinized and stained by the hematoxylin-eosin staining protocol. The qualitative histology was performed from stained sections using a light microscope Leica DM 750 with an attached digital camera Leica ICC50 HD (Leica Microsystems GmbH, Germany). The photographs were taken with Leica Application Suit Software (LAS) version 4.2.

Histological changes of renal tubules were evaluated after examining 50 tubules from proximal region (renal cortical and medulla). Morphological examination was made according to the published best practices (19). The following changes were quantified: degeneration and necrosis of renal tubules, interstitial inflammation and regeneration. Quantification of inflammation

Table 1. Variation of serum urea (mg/dl), serum creatinine (mg/dl) and urinary proteins (mg/dl) in Ge, Ge+Zn, Zn and control groups (mean±SD).

Parameter	n	Time	**Ge group** (mean ± S.D.)	**Ge + Zn group** (mean ± S.D.)	**Zn group** (mean ± S.D.)	**Control group** (mean ± S.D.)
Urea (mg/dl)	6	Io	33.42 ± 3.13	32 ± 2.91	32.34 ± 2.71	32.26 ± 2.63
	6	3 days	35.5 ± 2.08	33.4 ± 3.04	31.62 ± 4.65	33.46 ± 3.59
	6	7 days	203 ± 33.14*	61.8 ± 18.47**	32.54 ± 4.36	32.86 ± 4.62
	6	10 days	46 ± 6.73	44.8 ± 5.26	31.06 ± 3.34	32.70 ± 4.01
Creatinine (mg/dl)	6	Io	0.39 ± 0.03	0.38 ± 0.02	0.48 ± 0.08	0.61 ± 0.02
	6	3 days	0.41 ± 0.05	0.36 ± 0.02	0.56 ± 0.11	0.59 ± 0.04
	6	7 days	2.95 ± 0.61*	0.64 ± 0.14**	0.48 ± 0.08	0.59 ± 0.04
	6	10 days	0.59 ± 0.15	0.44 ± 0.11	0.52 ± 0.14	0.62 ± 0.04
Urinary proteins (mg/dl)	6	Io	0.43 ± 0.04	0.45 ± 0.05	0.44 ± 0.05	0.41 ± 0.03
	6	3 days	2.10 ± 0.15	1.34 ± 0.32	0.43 ± 0.07	0.43 ± 0.07
	6	7 days	4.59 ± 1.08*	2.2 ± 0.24**	0.48 ± 0.06	0.44 ± 0.08
	6	10 days	1.91 ± 0.12	1.66 ± 0.59	0.45 ± 0.04	0.42 ± 0.09

*P < 0.001 versus Io; **P < 0.001 versus Ge; n = number of samples.

was assessed by examining the entire section, in order to establish a qualitative and quantitative estimation of inflammatory cell infiltrate. Depending on the extension of the process, 5 degrees of severity were established for tubular necrosis, degeneration and epithelial regeneration: 0 - no pathological changes; 1 - mild damage (< 25%); 2 - moderate damage (25 – 50%); 3 - moderately severe damage (50 – 75%); 4 - severe damage (> 75%). For interstitial inflammation 3 degrees of severity were established: 0 - absence of inflammatory cells; 1 - moderate inflammation (rare lymphocytes, macrophages, histiocytes); 2 - pronounced inflammation (numerous lymphocytes and histiocytes) (20).

Statistical analysis

All data are presented as mean values ± standard deviation (S.D.). The obtained biochemical data was statistically analyzed using one-way ANOVA and Tukey post-hoc test. Histopathological data was analyzed using Kruskal-Wallis test. The values $P < 0.05$ were considered statistically significant.

RESULTS

The results showed that in Ge group serum urea and creatinine levels increased significantly 7 days after Ge administration, compared to the initial moment I_0 ($P < 0.001$).

An increase of urea and creatinine was also observed in Ge + Zn group at 7 days compared to I_0, but the values were significantly lower compared to Ge group at 7 days ($P < 0.0001$) (*Table 1*). The urine analysis showed that in both Ge and Ge + Zn groups urinary proteins values augmented significantly compared to I_0 ($P < 0.001$). The administration of Zn resulted in a significant reduction of proteinuria in Ge + Zn group compared to Ge group at 7 days ($P < 0.01$). In Zn group no significant variation of urinary proteins level was observed during the experiment.

Urinary volume showed a significant increase at 7 and 10 days after Ge administration, compared to I_0. In Ge + Zn group, rats had an incresed diuresis at 7 and 10 days compared to I_0 and control values. Though, compared to rats treated with Ge, the urinary volume was reduced at 10 days in Ge + Zn group (*Fig. 1*).

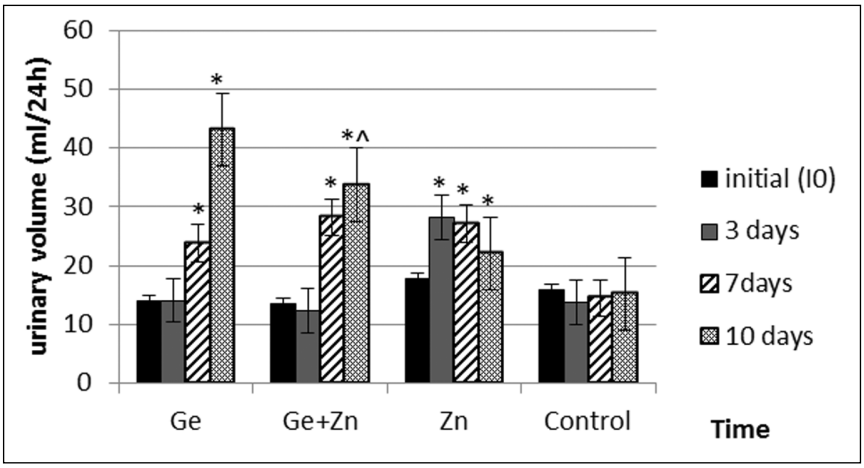

Fig. 1. Variation of urinary volume (ml/24 hours) in Ge, Ge + Zn, Zn and control groups *P < 0.05 versus I_0; ^P < 0.05 versus Ge.

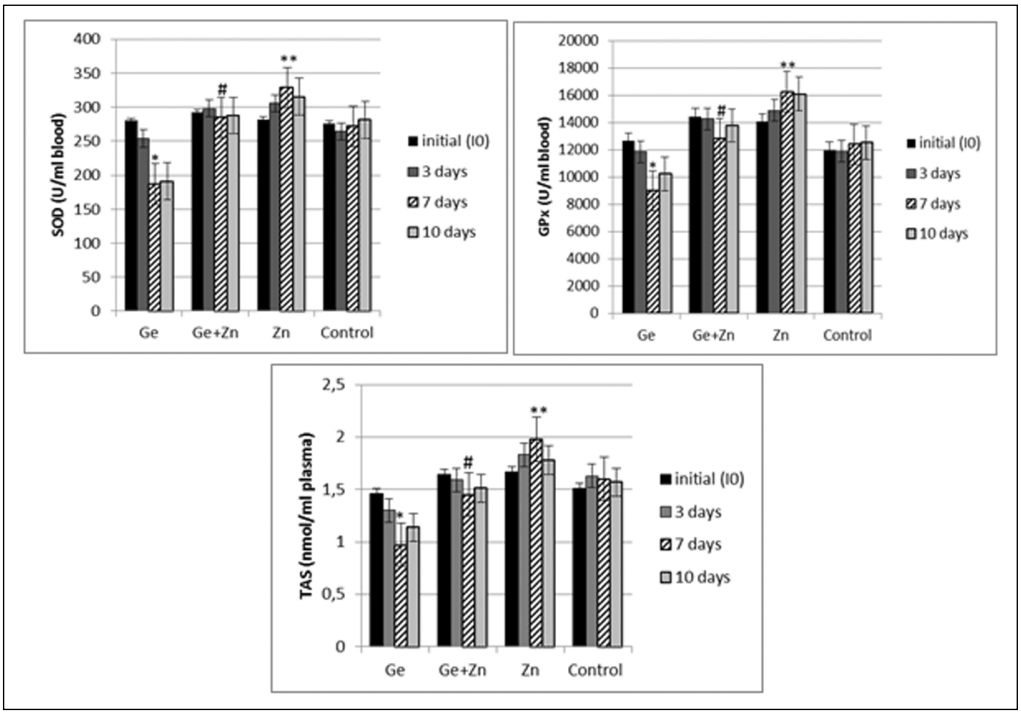

Fig. 2. Variation of TAS, SOD and GPx in Ge, Ge + Zn, Zn and control groups (mean ± S.D.), *P < 0.0001 versus I_0; **P < 0.001 versus I_0; #P < 0.0001 versus Ge.

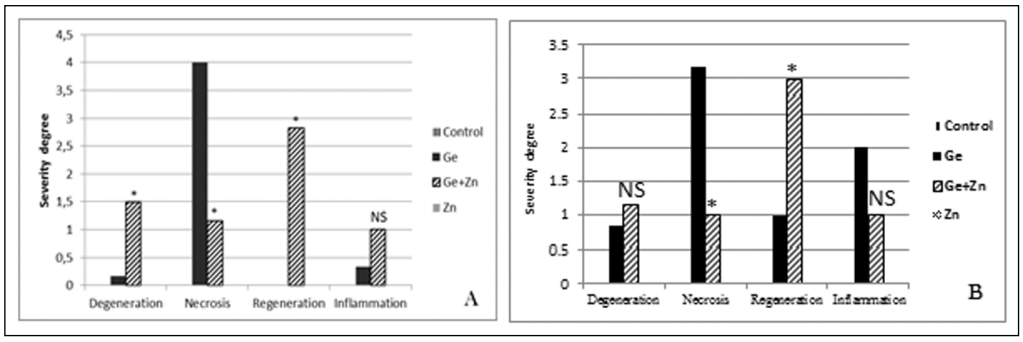

Fig. 3. Lesions score resulted from semi-quantitative analysis of histopathological changes at 7 days (A), respectively 10 days (B) in Ge, Ge + Zn, Zn and control groups *P < 0.05 versus Ge; NS, non-significant.

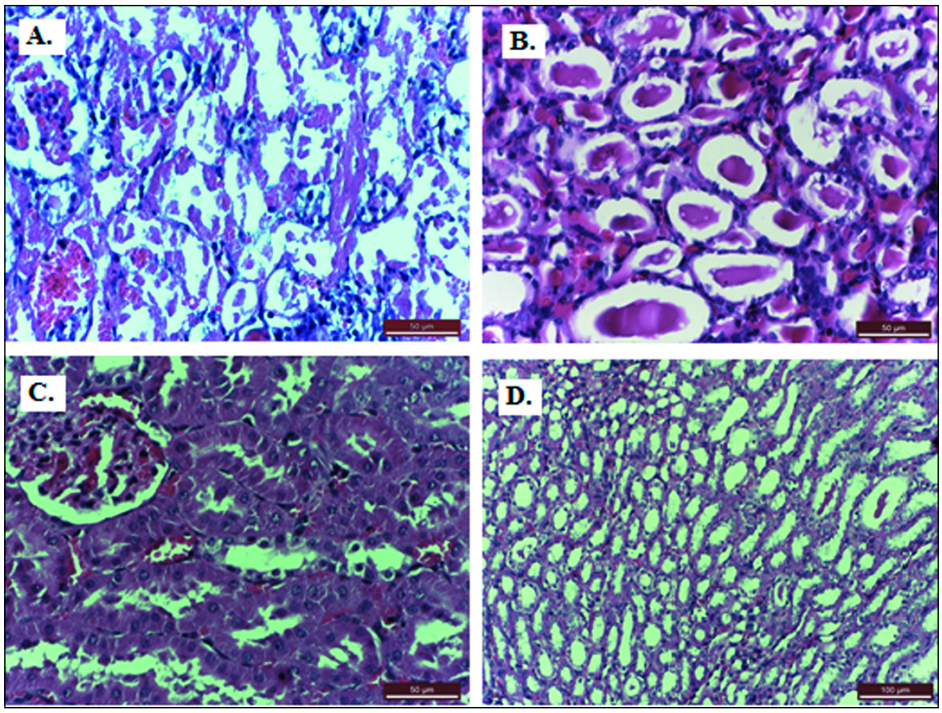

Fig. 4. Histopathological changes in Ge and Ge + Zn groups at 7 days: (A) renal cortex, Ge group: severe necrosis, mild degeneration, moderate inflammation, col.HE, × 400; (B) renal medulla, Ge group: hyaline cylinders obliterating renal tubules, col.HE, × 400; (C) renal cortex, Ge + Zn group: mild necrosis, mild degeneration, 3rd degree regeneration, moderate inflammation, col.HE, × 400; (D) renal medulla, Ge + Zn group: a few hyaline casts, col.HE, × 200.

The evaluation of antioxidant parameters SOD, GPx and TAS revealed decreased values in Ge group compared to I_0, with the most significant variation ($P < 0.0001$) after 7 days. Zn supplementation was associated with an increase of SOD, GPx and TAS in Ge + Zn group compared to Ge group at 7 days ($P < 0.0001$). In rats treated with Zn, the parameters registered the highest level at 7 days compared to I_0 (*Fig. 2*).

The histopathological examination of the kidneys revealed a normal renal morphology in both control and Zn groups. Semi-quantitative analysis of histopathological changes (degeneration, necrosis, regeneration and inflammation) is summarized in *Fig. 3.*

All examined sections of Ge group at 7 days exhibited severe tubular necrosis which affected more than 90% of proximal tubules and basement membrane lysis. Only one case was observed to exhibit a degeneration process (1st degree) with necrotic debris in the intact basement membrane.

The renal cortical tubules were dilated, with discontinuous membranes, containing cytoplasm blocks and free pyknotic nuclei. The glomeruli did not present significant changes. Epithelial regeneration was absent. There was a low degree of inflammation as observed in the rare mononuclear cells in the renal interstitium (*Fig. 4A*). In the renal medulla, more than 90% of the distal and collecting tubules were obliterated with hyaline cylinders (*Fig. 4B*). In Ge + Zn group at 7 days the tubular necrosis registered a reduced severity grade, affecting < 25% tubules. The degenerative process, represented by swollen renal cells, narrow tubular lumens, dense and abundant cytoplasm, intensely eosinophilic, rare intracytoplasmic vacuolation, was predominant in the proximal region. The basement membranes were normal. Third degree tubular regeneration was present in all rats. The inflammation was reduced, with rare mononuclear cells in the renal interstitium (*Fig. 4C*). In the renal medulla, a few renal tubules presented hyaline casts (*Fig. 4D*).

Ge group at 10 days exhibited extensive 3rd and 4th degree necrosis and accentuated 2nd degree inflammation. The necrosis process was more significant than the epithelial degeneration, dominating the renal cortex. The inflammation located in the renal interstitium was characterized by a predominantly lymphocytic infiltrate. Epithelial regeneration was reduced, being directly proportional to the number of tubules with normal basement membrane (*Fig. 5A*). In the renal medulla we observed the persistence of hyaline casts in the renal tubules, albeit in a reduced number (*Fig. 5B*). In rats treated with Ge + Zn at 10 days, 3rd degree regeneration processes were predominant, with young renal cells redistributed on the basement membrane. Interstitial inflammation was reduced in the majority of rats (1st degree). The degeneration processes were in the 1st degree of severity, with intracytoplasmic vacuolation, which is a distinctive aspect of hydropic dystrophy (*Fig. 5C*). The majority of renal tubules from the renal medulla had free lumens. Rare hyaline casts were observed intraluminally (*Fig. 5D*).

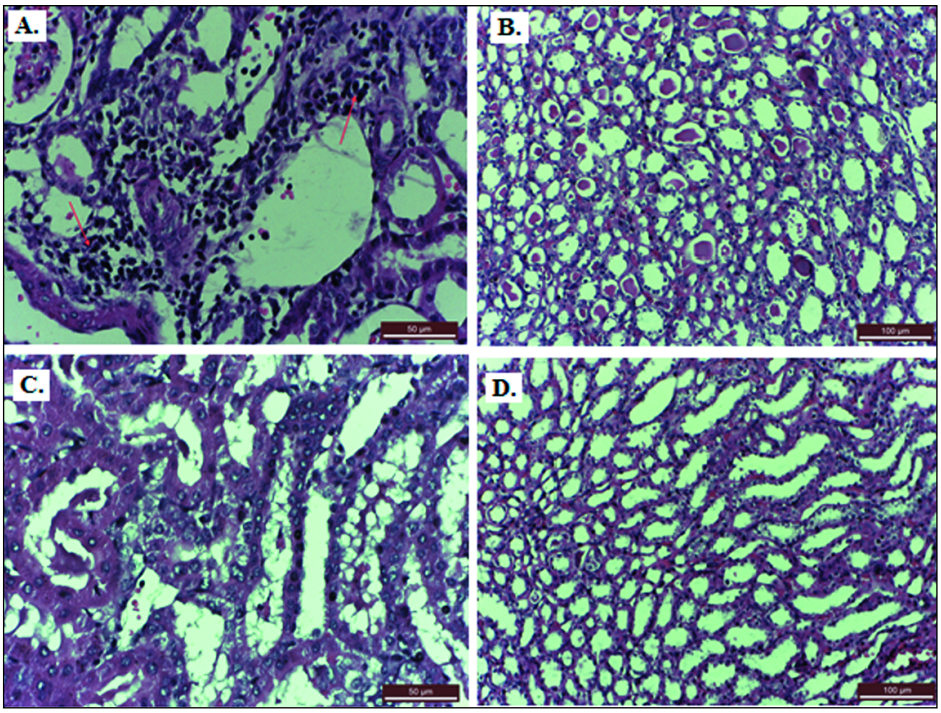

Fig. 5. Histopathological changes in Ge and Ge + Zn groups at 10 days: (A) renal cortex, Ge group: moderately severe necrosis, pronounced inflammation, col. HE, × 400; (B) renal medulla, Ge group: a few hyaline casts, col. HE, × 200; (C) Ge + Zn group: mild necrosis and degeneration, moderate inflammation, 3rd degree regeneration, col. HE, × 400; (D) renal medulla, Ge + Zn group: a few hyaline casts, col. HE, × 200. *Table 1*. Variation of serum urea (mg/dl), serum creatinine (mg/dl) and urinary proteins (mg/dl) in Ge, Ge+Zn, Zn and control groups (mean±SD).

DISCUSSIONS

Aminoglycosides, including Ge, are widely used in the treatment of gram-negative infections due to their efficacy and low cost. One of the mechanisms involved in Ge nephrotoxicity consists in its preferential accumulation in the proximal tubules. Having a cationic charge, Ge binds to anion phospholipids within plasma membrane of the proximal tubules cells (21). Accumulation of Ge in lysosomes induces morphological alterations, resulting in cell death (22). Aminoglycosides alter glomerular filtration. Some factors incriminated for this effect are the release of vasoconstrictive hormones and platelet aggregation factor, as well as nephron obstruction with proteic deposits or the alteration of glomerular permeability (23). Gentamycin causes a reduction in renal blood flow associated to tubular injury (24). Hypoxia activates inducible nitric oxide synthase (iNOS) expression, which induces oxidative stress, inhibiting ATP synthesis and generating apoptosis through mitochondrial pathway (25). These aspects suggest the involvement of nitric oxide, in the mediation of the glomerular endothelial cells damage in drug-induced kidney toxicity (10).

Gentamycin nephrotoxicity consists in a dysfunction in renal excretion associated with high levels of urea and creatinine, proteinuria, aminoaciduria and electrolytic disorders (23) and has been shown to cause severe histological damage, in particular to renal proximal convoluted tubules resulting in swelling, vacuolization and necrosis of epithelial cells (26).

Zn is considered a part of the cell/tissue antioxidant defense network. Oxidative stress associated with zinc deficiency and protective effect of zinc supplementation on the oxidative damage has been reported by other studies (27). Experimental data from the present study showed that 80 mg/kg b.w./day of Ge administered intraperitoneally resulted in the elevation of serum urea and creatinine levels after 7 days compared to I_0. Our results were consistent with data from related studies (3). In Ge + Zn group the values of urea and creatinine also increased compared to I_0, but they were significantly lower compared to Ge group at 7 days. Zn supplementation significantly reduced the alteration of renal parameters in Ge + Zn group compared to Ge group ($P < 0.01$). The data we obtained correlate with the results communicated by Yonova who reported a decreased plasmatic zinc level in patients suffering from renal failure (28).

Oxidative stress also plays an important role in Ge nephrotoxicity (29). Ge induces an increase in the mitochondrial production of reactive oxygen species, being responsible for cellular death, mesangial and vascular constriction and inflammation (30). Moreover, an excess of superoxide results in a reduced biological effect of nitric oxide (NO), an endogenous vasodilator involved in regulation of renal microcirculation and tubule function. The decrease of NO was associated with renal damage in experimental models of kidney failure due to renal lipid peroxidation (10). Our data revealed that in rats treated with Ge there was an accelerated lipid peroxidation, reflected by the decreased level of serum TAS, and erythrocyte SOD and GPx activity at 7 days compared to I_0. Zn is considered to be an antioxidant agent, being involved in inducing the expression of metallothioneins, glutathione metabolism regulation and redox signaling (5, 27). Zn is a component of superoxide dismutase (SOD), an antioxidant enzyme, which transforms superoxide ions into hydrogen peroxide. Zn acts competitively with iron and copper, redox active metals, which catalyzes free radical production through lipid peroxidation. The replacement of these metal ions with Zn may prevent the formation of reactive oxygen species (31). In the present study, Zn administration 10 days before Ge and 7 days simultaneously with Ge determined an increase of TAS, SOD and GPx in Ge + Zn group compared to Ge group.

Gentamycin nephrotoxicity involves an inflammatory response, with cellular infiltration, increased capillary permeability and cytokine production (32, 33). At glomerular level, Ge induces mesangial constriction and loss of glomerular filtration selectivity, due to the neutralization of negative charges, leading to proteinuria (34). Zn is known to influence

cytokine production and to stabilize the cellular membrane, thus preventing inflammatory lesions (35). Inflammatory cytokines such as TNF-α, IL-1β, known to generate reactive oxygen species, are inhibited by Zn. Plasma zinc levels are negatively correlated to TNF-α (36-39).

In our study, the proteinuria induced by gentamicin observed in Ge group was significantly reduced in Ge + Zn group after Zn administration at 7 days.

The histopathological examination showed important structural changes in the kidney, such as hyaline casts, necrosis and degeneration in rats treated with Ge. Similar changes were reported by other studies in experimentally-induced acute renal failure in laboratory animals (40, 41). These morphopathological changes can be caused by an increased reactive oxygen species production induced by Ge administration. Oxidative stress occurred during Ge treatment mediates the inflammatory response in experimental animals (42, 43), and may be implicated in tubular injury and contribute to renal damage (44). Our results showed that renal injuries induced by Ge are significantly reduced by Zn supplementation. In the group treated with Ge + Zn the examination of renal tissue revealed less intense injuries compared to Ge group, with reduced degenerative signs and minor alteration of renal epithelium (1st versus 3rd degree). Tubular necrosis had a milder degree compared to Ge group. We noticed a moderate desquamation of basement membrane cells, the majority of cells preserving the cellular membrane. The tubular regeneration was present with a medium intensity, and the inflammatory processes were in the 1st degree of severity.

The improvement of renal impairment parameters investigated in experimentally-induced renal failure after Zn administration is consistent with the data showing the implication of Zn deficiency in the pathogenesis of renal failure in humans (45).

The novelty of our work consists of *in vivo* evaluation of the effects of zinc chloride on the systemic oxidative stress and the histological kidney modifications in rats with experimental induced acute nephrotoxicity with Ge.

Our obtained data suggest that Zn supplementation might be beneficial to protect the patient against the eventual nephrotoxic effect of Ge administration during the treatment, especially in persons with Zn deficiency.

Conclusion

Zn administration proved a protective role in experimental gentamicin-induced acute renal failure. The treatment with Zn significantly diminished the alteration of renal function, with an evident improvement of serum urea and creatinine levels and also of the urinary protein values. Zn supplementation also decreased lipid peroxidation, TAS levels being significantly higher after Zn administration. The treatment with Zn attenuated the severity of renal histopathologic lesions induced by Ge.

Conflict of interests: None declared.

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