

Accelerated lipid peroxidation in a rat model of gentamicin nephrotoxicity

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Received July 2, 2021; Accepted August 3, 2021

DOI: 10.3892/etm.2021.10652

Abstract. Kidney disease represents a burden for the health care system worldwide. As the prevalence continues to rise, discovering new biomarkers of early kidney damage has become crucial. Oxidative stress (OS) represents one of the main factors involved in the early stages of many syndromes leading to kidney damage. Therefore, it must be studied in detail. To date, many studies have focused on OS in advanced stages of acute kidney injury (AKI), with great success. The aim of the present study was to ascertain whether even mild renal function impairment can be linked to specific systemic markers of OS and systemic antioxidants in order to pinpoint certain biomarkers for early kidney damage. We used male rats (*Rattus norvegicus*) in which we induced kidney damage by injecting gentamicin for 7 days. Blood was collected 24 h after the last dose of gentamicin. Urea, creatinine, 3-nitrotyrosine (3-NT), nitric oxide (NO), malondialdehyde (MDA), thiols (TS), total oxidative stress (TOS), and interferon- γ (IFN- γ) were determined. In addition, for the antioxidant status we measured total antioxidant capacity (TAC) and interleukin-10 (IL-10). Our results demonstrated that the rats had mild renal impairment consistent with a pre-AKI stage due to the nephrotoxic effect of gentamicin. However, TOS, MDA and NO were significantly higher in the gentamicin group compared to the control group. In addition, TAC was higher in the control group. Hence, OS

markers reach higher levels and may potentially be used as markers of kidney damage even in cases of mild renal function impairment.

Introduction

Acute deterioration of renal function translates into a syndrome known as acute kidney injury (AKI). The sudden drop in the glomerular filtration rate leads to the accumulation of the end products of creatinine and nitrogen metabolism in the blood. Diagnosing AKI relies greatly on determining the serum levels of these products, which causes a delay in emphasizing the kidney damage (1,2).

Therefore, the need for earlier detection and better monitoring of these patients is vital. Oxidative stress (OS) is represented by reactive oxygen and nitrogen species (ROS, RNS) and free radicals that reach a level that exceeds the endogenous antioxidant capacity. OS is an important topic in research studies, and to date it has been declared a pathogenic factor in many diseases (3-6). In the early stages of AKI there are changes and alterations in the structure and function of the mitochondria leading to ATP depletion and dysfunction in the energetic metabolism. Mitochondria are a major source of ROS, but they are also a source of antioxidant molecules (7-10). Despite the numerous studies that have focused on this topic, the precise mechanisms that generate reactive species (RS) in AKI continue to be unknown. Homeostasis is maintained by counteracting the physiological production of RS with a continuous endogenous production of antioxidants. Antioxidants usually intervene early in the development of AKI in order to scavenge RS, to downregulate OS and subsequently to reduce lipid peroxidation and total oxidative damage (3,5,11).

The main purpose of the present study was to experimentally define the relationship between mild renal impairment (pre-AKI) and both the systemic OS and antioxidant status. In addition, we analyzed multiple markers in order to identify specific ones that could be further used to discover kidney injury at earlier stages.

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Key words: oxidative stress, antioxidants, acute kidney injury, gentamicin, nephrotoxicity

Materials and methods

Animals. A total of 14 adult male *Rattus norvegicus* rats, obtained from the Animal Facility of 'Iuliu Hatieganu' University of Medicine and Pharmacy, Cluj-Napoca, were used in this experiment (weighing, 400.07 ± 56.04 g). The animals were housed in standard cages equipped with wood chip bedding, in a room with an ambient temperature of $22 \pm 1^\circ\text{C}$, a 12/12-h light/dark cycle, with 40-50% humidity. Throughout the experiment, all animals were given *ad libitum* access to tap water and standard chow for rodents.

Experimental procedure. Rats were arbitrarily divided equally into two groups: The control group ($n=7$) and the gentamicin group ($n=7$). Total experiment time was 10 days. All rats were given 2 days to acclimatize to the environment before starting the injections. Rats in the control group were injected intraperitoneally (i.p.) with physiological saline solution for 7 consecutive days. Rats in the gentamicin group were injected i.p. with gentamicin for 7 consecutive days (KRKA D.D. Novo mesto, Slovenia, 60 mg/kg/day). The volume of physiological saline volume was equivalent to the volume of the gentamicin solution. For the collection of urine, rats were placed in metabolic cages (with *ad libitum* access to tap water) for 24 h: 2 rats from each group between day 8 and 9, and also 2 rats from each group between day 9 and 10. Blood was collected 24 h after the last injection, by retro-orbital puncture. Animals were sacrificed by cervical dislocation. The blood was left undisturbed to coagulate for 1 h at 4°C , then it was centrifuged at $1,730 \times g$ for 15 min to obtain the serum. The serum was stored at -20°C until further analysis.

Biochemical analysis. Serum concentrations of urea and creatinine were measured in order to determine renal function. The quantitative determination of urea (serum, urine) was made based on the principle of enzymatically hydrolyzation of urea into ammonia and carbon dioxide. Ammonia ions further react with α -ketoglutarate and glutamate dehydrogenase (GLDH) with simultaneous oxidation of NADH to NAD^+ . The urea concentration in the sample was proportional to the decrease in concentration of NADH. The working reagent (WR) consisted of R1 buffer (Tris pH 7.8, α -ketoglutarate, urease) mixed with R2 enzymes (GLDH, NADH), 4:1 (Urea-LQ, Spinreact). The absorbance was read at 340 nm at 30 sec and 90 sec after mixing the working reagent with the sample, at room temperature. To determine the urinary urea (urine/24 h), we used a 1:50 dilution with distilled water and followed the same steps as previously described (12,13).

The quantitative determination of creatinine in serum and urine was determined utilizing an assay based on a reaction described by Jaffé in which creatinine reacts with sodium picrate, forming a red complex. The intensity of the color is proportional to the concentration of creatinine in the sample. The working reagent consisted of R1 picric acid and R2 sodium hydroxide, 1:1 (Creatinine-J, Spinreact). The reading was carried out at 492 nm, at 30 sec after the sample addition and again after 90 sec (at room temperature). To determine the urinary concentration of creatinine, the sample (urine/24 h) was diluted with distilled water, 1:50, followed by the steps as described previously (14,15).

OS analysis. Malondialdehyde (MDA), 3-nitrotyrosine (3-NT), interferon- γ (IFN- γ), nitric oxide (NO) and total oxidative stress (TOS) were measured to determine the level of OS.

The peroxidation of lipids was determined by reactive substances with thiobarbituric-reactive substances (TBARs), using an adapted procedure reported by Pasha and Sadasivadu (16). Thus, 0.1 ml of serum was mixed with 0.1 ml 40% TCO, which was further mixed with 0.2 ml 0.67% TBA. The mix was placed in a boiling water bath for 30 min, after which it was cooled in an ice water bath. After cooling, the mix was centrifuged for 5 min at $3,461 \times g$. Finally, 0.1 ml of the supernatant was removed and subjected to reading at 530 nm. TBAR values are expressed as MDA nmol/l.

3-NT was determined using the 3-NT ELISA Kit (Elabsience®). An amount 50 μl of the sample was added to each well, together with 50 μl of biotinylated detection Ab working solution, followed by incubation for 45 min at 37°C . Afterwards, the plate was aspirated and washed for 3 times. HRP conjugate working solution (100 μl) was then added, followed by incubation for 30 min at 37°C . The plate was then aspirated and washed 5 times. Substrate reagent (90 μl) was then added, followed by another incubation of 15 min at 37°C . Finally, 50 μl of stop solution was then added. The plate was immediately read at 450 nm.

Serum concentration of IFN- γ was measured using an ELISA Kit (Elabsience®). Serum (100 μl) was added to the wells, and then incubated for 90 min at 37°C . The liquid was discarded, followed immediately by the addition of 100 μl biotinylated detection Ab working solution to each well. Afterwards the plate was incubated for 60 min at 37°C . The plate was aspirated and washed 3 times. HRP conjugate working solution (100 μl) was then added, followed by incubation for 30 min at 37°C . The plate was then aspirated and washed 5 times. Substrate reagent (90 μl) was then added, followed by another incubation of 15 min at 37°C . Finally, 50 μl of stop solution was then added. The plate was immediately read at 450 nm.

The serum concentration of NO was measured by a standard nitrate reduction and detection by the VCl_3 /Griess assay (17). A nitrate standard solution (100 μl) was diluted from 200 to 1.6 μM , in duplicate, in a polystyrene microtiter plate with 96 flat-bottomed wells. The medium used for dilution was used as the standard blank. The 96-well plate was loaded with 100 μl samples, then VCl_3 was added to each well. Immediately after, Griess reagents, SULF (50 μl) and NEDD (50 μl) were added. To obtain sample blank values, the diluting medium was substituted for Griess reagent. After 30 min of incubation at room temperature using 5% H_3PO_4 (300 μl total volume), the absorbance at 540 nm was measured. Results are expressed in $\mu\text{mol/l}$.

For the measurement of TOS, an assay calibrated with hydrogen peroxide was used, with results expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2 \text{ Eq/l}$) (18). The preparation of R1 consisted in dissolving 114 mg of xylenol orange and 8.18 g of NaCl in 900 ml of H_2SO_4 solution, 25 mM. Glycerol was then added to the solution, 100 ml. The reagent had a pH value of 1.75. For R2 1.96 g of ferrous ammonium sulfate and 3.17 g of *o*-dianisidine dihydrochloride were dissolved in 1,000 ml of H_2SO_4 solution,

Table I. Characteristics of the control group (group 1, physiological saline) and the study group (group 2, gentamicin).

Characteristics	Group 1 (n=7) Control group	Group 2 (n=7) Gentamicin group	P-value
Initial body weight (g)	383.57±44.86	416.57±64.47	NS
Final body weight (g)	388.57±47.60	392.42±66.76	NS
Urine volume (ml/24 h)	21.5±0.70 ^a	11±1.58 ^b	0.001

^an=2; ^bn=4. NS, not significant. Significant P-values are indicated in bold print.

25 mM. R1 (225 μ l) was added to 35 μ l of serum. Then, 11 μ l of R2 was then added. The measurement was made at a 560 nm wavelength. The first absorbance was taken before the mixing of R1 and R2 (sample blank). The last absorbance was taken after the reaction trace drew a plateau line (3-4 min after mixing).

For the measurement of the serum levels of protein thiol groups (-SH) we used Ellman's reagent (19). Serum (50 μ l) was mixed with 1 ml Tris (0.25M)-EDTA (20 mM), pH 8.2. Afterwards, 20 μ l of DTNB (10 mM) was added. After 15 min of incubation at room temperature, the measurement was made at 412 nm. Results are expressed in μ mol/l.

Antioxidant status analysis. The serum levels of interleukin-10 (IL-10) and total antioxidant capacity (TAC) were measured in order to evaluate the antioxidant capacity.

Serum IL-10 was determined using an ELISA Kit (Elabscience®). Serum 100 μ l was added to the wells and incubated at 37°C for 90 min. The liquid was then discarded, and then 100 μ l of biotinylated detection Ab working solution was added to each well, followed by 60 min of incubation at 37°C. The plate was then aspirated and washed for 3 times. HRP conjugate working solution (100 μ l) was then added to each well, followed by another incubation at 37°C for 30 min. The plate was aspirated and washed for 5 times. Substrate reagent was added, 90 μ l, followed by incubation (15 min, 37°C), and then by stop solution, 50 μ l. The plate was immediately read at 450 nm.

Serum TAC was measured using an automated measurement method, developed by Erel (20). R1 consisted of *o*-dianisidine (10 mM) and ferrous ammonium sulfate (45 μ M) that were added in KCl/HCl solution (75 mM, pH 1.8). R2 consisted of H₂O₂ (7.5 mM). Serum (5 μ l) was mixed with 200 μ l of R1 and 10 μ l of R2. The measurement was made at 444 nm. The first absorbance was taken before the mixing of R1 and R2 (sample blank). The last absorbance was taken at 3-4 min after the mixing with the serum. The results are expressed in mmol Trolox equivalent/l.

Statistical analysis. All results are presented as means \pm standard error of the mean value. Student's paired t-test was used for the analysis of experimental data with a Gaussian distribution. Data with non-Gaussian distribution were compared using the unpaired t-test. P-values <0.05 were considered statistically significant. Statistical analyses were performed using Excel and QI Macros package Windows® 2020.

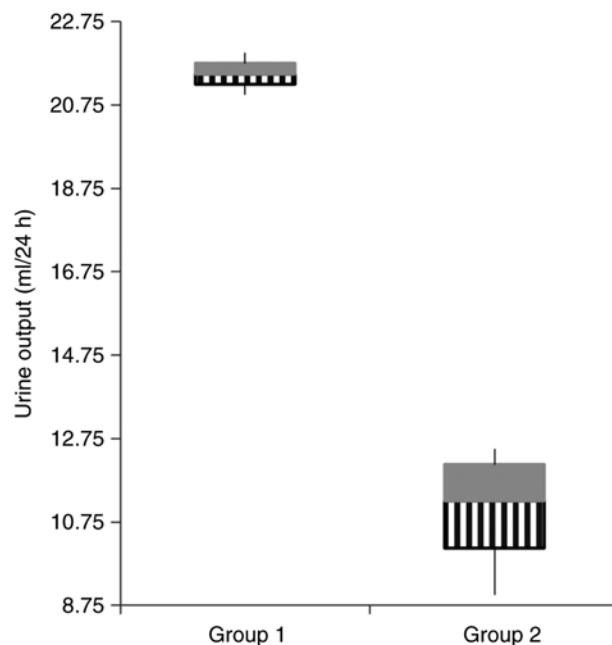


Figure 1. Box-plot showing the difference in the 24-h urine output between the control group (Group 1) and gentamicin group (Group 2) P=0.001).

Results

The survival rate of the rats was 100% during the experimental procedures. The characteristics of our study groups are summarized in Table I. As Table I shows, there was no statistical difference between the two groups concerning body weight before and after the injection period, but a significant difference was observed in the 24 h urine output between the two groups (Fig. 1, P=0.001).

There were significant differences between the two groups concerning the renal function parameters. The gentamicin group presented markedly elevated urea values in both blood and urine compared to the values of creatinine (Table II, Figs. 2 and 3).

OS parameters and antioxidant status were assessed in the two study groups and are summarized in Table III. Concerning the OS parameters, we observed a significant difference between the two groups for NO (Fig. 4), MDA (Fig. 5), but no statistical difference was obtained for 3-NT, thiols (TS) and IFN- γ .

In the gentamicin group TOS was found to be significantly higher compared to the control group (Fig. 6, P=0.03).

Table II. Renal function parameters (serum and urinary) in the study groups.

Parameters	Group 1 (n=7) Control group	Group 2 (n=7) Gentamicin group	P-value
Serum urea (mg/dl)	44.57±6.84	56.25±5.67	<0.0001
Serum creatinine (mg/dl)	0.77±0.001	0.88±0.01	0.020
Urinary urea (mg/dl)	346.66±2,403.55	632.66±4,306.37	0.013
Urinary creatinine (mg/dl)	35.01±2.47	47.09±130.19	NS

NS, not significant. Significant P-values are indicated in bold print.

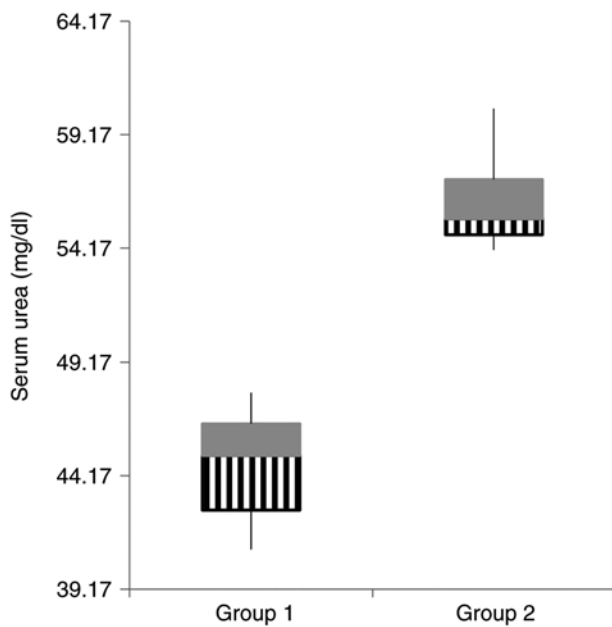
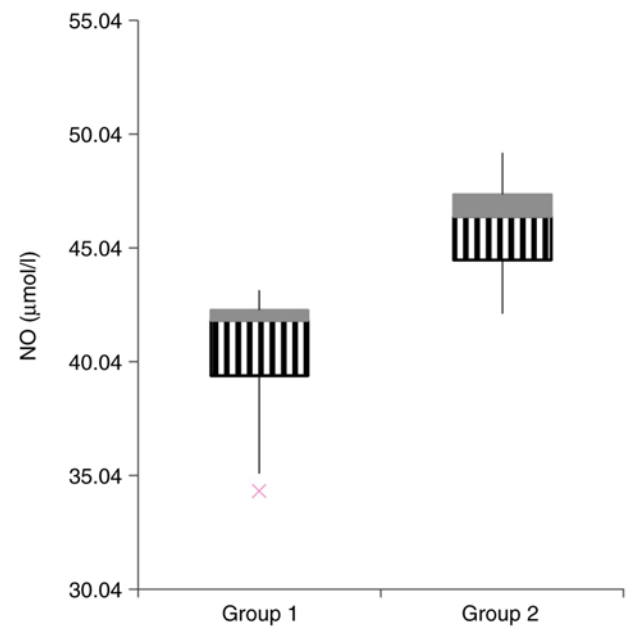
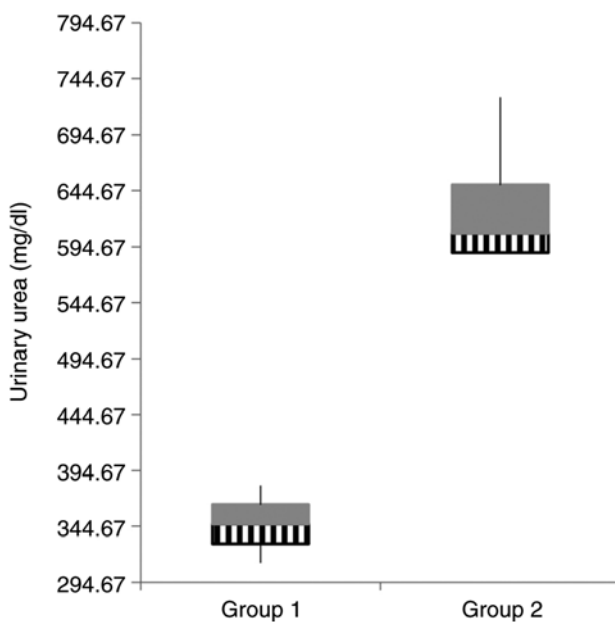
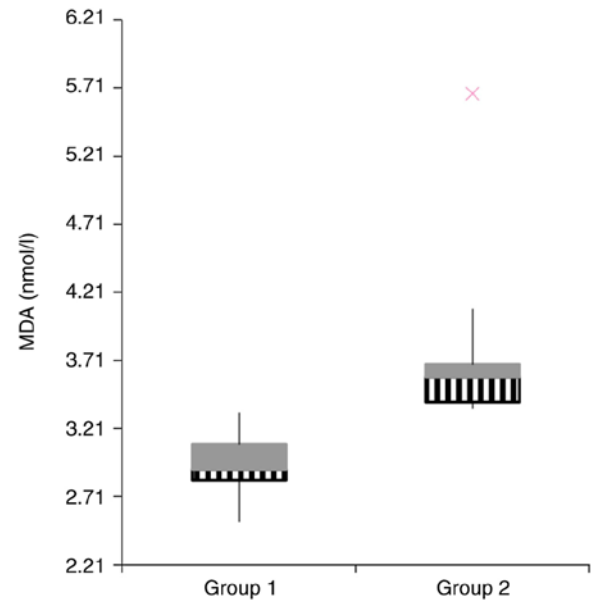
Figure 2. Box-plot showing the difference between the values of serum urea in the control group (Group 1) and gentamicin group (Group 2) ($P<0.05$).Figure 4. Box-plot showing the difference between the values of serum nitric oxide (NO) in the control group (Group 1) and gentamicin group (Group 2) ($P=0.01$).Figure 3. Box-plot showing the difference between the values of urinary urea in the control group (Group 1) and gentamicin group (Group 2) ($P=0.013$).Figure 5. Box-plot showing the difference between the values of serum malondialdehyde (MDA) in the control group (Group 1) and gentamicin group (Group 2) ($P=0.01$).

Table III. Oxidative stress parameters and antioxidant status in the study groups.

Parameters	Group 1 (n=7) Control group	Group 2 (n=7) Gentamicin group	P-value
3-NT (ng/ml)	19.53±32.67	28.87±701.46	NS
NO (μ mol/l)	40.40±9.30	45.94±5.93	0.01
MDA (nmol/l)	2.94±0.068	3.82±0.67	0.01
Thiols (μ mol/l)	394.14±3334.47	322.42±267.61	NS
IFN- γ (pg/ml)	24.27±45.71	19.97±8.30	NS
TOS (μ mol H ₂ O ₂ Eq/l)	21.98±5.91	25.05±5.04	0.03
IL-10 (pg/ml)	15.81±16.12	13.57±0.44	NS
TAC (mmol Trolox Eq/l)	1.09±1.04	1.09±4.81	0.001
OSI (TOS/TAC)	20.05±4.91	22.90±4.20	0.014

3-NT, 3-nitrotyrosine; NO, nitric oxide; MDA, malondialdehyde; IFN- γ , interferon- γ ; TOS, total oxidative stress; IL, interleukin; TAC, total antioxidant capacity; OSI, oxidative stress index. NS, not significant. Significant P-values are indicated in bold print.

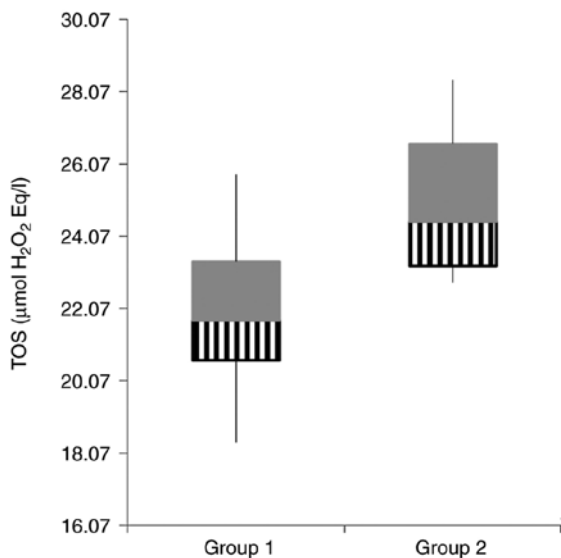


Figure 6. Box-plot showing the difference between the values of serum total oxidative stress (TOS) in the control group (Group 1) and gentamicin group (Group 2) (P=0.03).

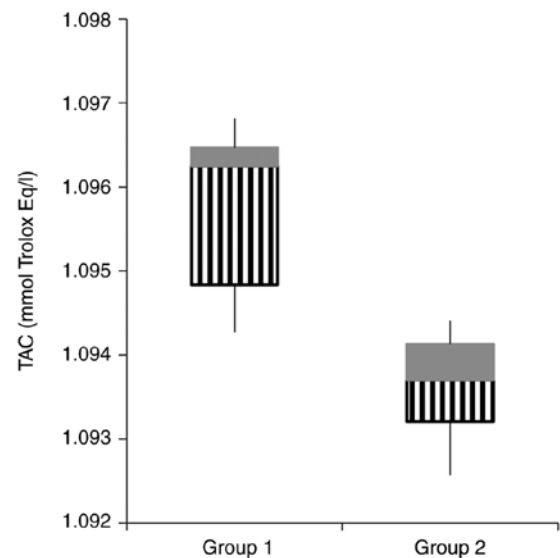


Figure 7. Box-plot showing the difference between the values of serum total antioxidant capacity (TAC) in the control group (Group 1) and gentamicin group (Group 2) (P=0.001).

The antioxidant status was evaluated using IL-10 and TAC, with TAC being statistically higher in the control group (Fig. 7). Oxidative stress index (OSI) was significantly higher in the gentamicin group compared to the control group (P=0.014).

Discussion

In the present study, we were able to induce a mild form of renal function impairment by injecting gentamicin (60 mg/kg/day, i.p.) for 7 consecutive days, as shown by the reduced urine output in the gentamicin group, correlated with increased serum values of both urea and creatinine. In addition, even though it was not statistically significant, a decrease in the weight of the rats in the gentamicin group was observed. These findings are in line with previous studies conducted on rats with kidney injury induced by gentamicin (21-23).

However, due to the fact that our animals received a smaller dose of gentamicin compared to the rats in the previously mentioned studies who received 100 mg/kg/day, the creatinine level was increased only 1.14-fold compared to the control group, showing that our animals did not achieve stage I AKI (a 1.5-fold increase was needed). However, our results showed a greater increase in the urea values compared to the values of creatinine, both in serum (1.26-fold vs. 1.14-fold) and in urine (1.82-fold vs. 1.34-fold). Increased serum levels of urea might also be due to dehydration, heart failure, gastrointestinal bleeding, high-protein diet or catabolic state, but in our case these criteria were not met, since the only difference between our groups was the administration of gentamicin (24). Therefore, in our study, the differences in the urea values were due to an impairment in renal function, secondary to the nephrotoxic effect of gentamicin. Our result confirmed that urea reaches higher concentrations faster than creatinine. The

accumulation of these end products in the blood demonstrated that gentamicin exerted a nephrotoxic effect on our rats, with mild renal function impairment, consistent with a pre-AKI stage.

With regard to TOS, it proved to be higher in the gentamicin group compared to the control group, which was correlated with an OSI which was also higher in the same group. Furthermore, we were able to identify two OS markers that were significantly higher in the group receiving gentamicin: NO and MDA.

NO production takes place in reaction to an inflammatory stimulus and can be triggered by increased levels of IFN- γ , tumor necrosis factor (TNF)- α , and IL-1 β (25). Larger levels of NO produce NO-derived reactive species that can further nitrosate TS. Previous studies have shown that NO represents a marker of OS that is upregulated in AKI (26-29). Pathak and Mayeux used an animal model of sepsis-induced kidney injury and showed that the generation of NO significantly increased compared to the control group (26). In accordance with these results, in our case, NO reached significantly higher serum levels after gentamicin administration compared to the control group, in spite of the rats in this group not having fully achieved stage I of AKI. This confirms that NO may play a crucial role in the early diagnosis of kidney damage or it may be used as a marker of nephrotoxicity.

MDA has been accepted as a reliable marker of OS, as it represents one of the lipid peroxidation products (30). In our study, MDA was 1.29-fold higher in rats with exposure to gentamicin compared to the control. In comparison, Su *et al* confirmed that *Panx1*-knockout mice had decreased MDA levels in kidney tissues when subjected to ischemic AKI compared to wild-type mice, which further demonstrates that MDA plays a pathogenetic role in AKI (31). Awodele *et al* conducted a study demonstrating that MDA is significantly increased in rats receiving gentamicin compared to the controls. They concluded that the mechanism of toxicity caused by gentamicin was via OS and subsequent lipid peroxidation (32). Lipid peroxidation was also demonstrated in an animal model of kidney injury induced by cisplatin (33). Kovalčíková *et al* showed that AKI leads to increased systemic OS, but they also demonstrated increased lipid oxidation markers in the frontal cortex that may explain uremic encephalopathy (34). These data show that lipid peroxidation is consistent in kidney damage, and MDA can be used as a marker in AKI.

However, 3-NT, TS and IFN- γ were confirmed to have similar concentrations in the serum of the two groups. In contrast, there are experimental and clinical studies that show a higher concentration of these markers in subjects with AKI compared to the control. One study included 158 patients with AKI and found that 3-NT was significantly higher in those subjects compared to healthy controls and to critically ill subjects without AKI ($P < 0.001$), concluding that 3-NT levels are associated with mortality of patients with AKI, independent of the gravity of the disease (35). TS were also previously associated with AKI, as Boekhoud *et al* confirmed in their study conducted on 301 critically ill patients (36). Burks *et al* extensively investigated the effects of pulsed focused ultrasound (pFUS) on mesenchymal stem cells (MSCs) in mice with cisplatin-induced AKI. They demonstrated that following the infusion of cisplatin and subsequent AKI, pFUS was able to upregulate renal IFN- γ which further stimulated MSCs that

were subsequently infused to upregulate IL-10 and therefore to promote healing (37). Even though IFN- γ represents an OS marker, they showed that the IFN- γ /IL-10 cytokine axis plays an essential role in the outcome of AKI (38).

In the present study, IL-10 had similar concentrations in the serums of the two groups. In contrast, IL-10 proved to reduce injury in several models of AKI (39-44). Our results may be due to the fact that our rats did not achieve stage I of AKI. It is to be mentioned that IL-10 was 1.16-fold higher in the control group compared to gentamicin group, with a P -value of 0.09.

TAC was significantly higher in the control group compared to the pre-AKI group, which further demonstrates that the nephrotoxic effect of gentamicin impairs the oxidant-antioxidant balance, in favour of OS.

One limitation of our study is represented by the fact that we could not verify the renal parameters of the gentamicin group before injecting the nephrotoxic drug. In addition, another limitation is given by the fact that we were not able to collect urine from all of our animals due to a limited number of metabolic cages available; therefore, we could not calculate the precise glomerular filtration rate of the animals.

In conclusion, our study demonstrated that the oxidant-antioxidant balance is impaired in favour of OS in renal damage induced by gentamicin, a nephrotoxic drug. In addition, it provides strong evidence that lipid peroxidation plays a crucial role in gentamicin nephrotoxicity. Moreover, MDA and NO may be used as markers of early kidney damage when changes in serum creatinine are not yet fully relevant. The novelty brought by this study is represented by the fact that our animals suffered only mild renal impairment, in contrast with other experimental studies where the animals achieved different stages of AKI. Even so, we were able to pinpoint certain OS markers that were significantly modified in the gentamicin group compared to the control group. More studies are needed in order to validate these markers in other types of AKI (ischemic, sepsis-induced).

Acknowledgements

We would like to acknowledge Mr. Mirel Molnar (Department of Pathophysiology) for his essential work as a laboratory technician.

Funding

Anamaria Magdalena Tomșa is the recipient of an internal grant from 'Iuliu Hatieganu' University of Medicine and Pharmacy, Cluj-Napoca, Romania. This study was funded by this grant.

Availability of data and materials

All data analyzed during this study are included in this published article.

Authors' contributions

AMT, ALR, LMJ and AEP conceived the experimental protocol. AMT performed the *in vivo* experiment. SLP,

AB and AU performed the measurements. AMT and ALR analyzed the data and prepared the manuscript. LMJ and AEP performed the critical revision of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the 'Iuliu Hatieganu' University of Medicine and Pharmacy, Cluj-Napoca, Romania (authorization no. 193/18.05.2020). This study was also approved by The National Sanitary Veterinary and Food Safety Authority (authorization no. 225/22.06.2020). This study was carried out according to relevant national legislation.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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