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# Effect of normobaric hyperoxia on gentamicin-induced nephrotoxicity in rats

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ABSTRACT

***Objective(s):*** Gentamicin sulphate (GS) nephrotoxicity seems to be related to the generation of reactive oxygen species. There is evidence that oxygen preconditioning increases the activity of antioxidant enzymes.

***Materials and Methods:*** Forty eight female rats were divided into 6 groups (n=8) as follows: group 1 was the control, group 2 received daily GS, groups 3,4 and 5 received oxygen 2 hr/day for 2 days, 4 hr/day for 2 days, 4 hr/day for 4 days, recpectively and then received daily GS, group 6 received oxygen 2 hr/day for 2 days and then received 2 hr oxygen before daily GS injection. Oxygen (with 90% purity) used at the flow rate of 4 l/min. GS administred for 8 days (100 mg/kg, IP). Tissue sections prepared from the left kidney, stained with PAS method and then studied hisopathologically and stereologically. The right kidneys were homogenized and the supernatants were prepared. Serum MDA, creatinine and urea, renal MDA, gluthatione and catalase activity were measured. The data were analyzed by Mann-Whitney U test at the significant level of $P<0.05$.

***Results:*** Oxygen therapy significantly improves serum creatinine and urea, preserve tubular volume density, reduce tubular necrosis in groups 4 and 6 compared to group 2. Oxygen therapy significantly increases renal catalase in groups 4 and 6 compared to group 2.

***Conclusion:*** Pretreatment with normobaric hyperoxia and daily oxygen therapy improved gentamicin nephrotoxicity possibly via inhibition of lipid peroxidation and increasing the renal catalase activity but could not restore any parameter at the same levels as control group.

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## Introduction

Gentamicin sulphate (GS) is commonly used for the treatment of Gram-negative bacterial infection. In many cases, it has been the only effective drug against bacterial strains which were resistant to other antibiotics, but its nephrotoxicity limits its administration (1). Gentamicin has been clinically used against Gram-negative bacterial infections caused by *Pseudomonas*, *Proteus*, and *Serratia* (2). Besides, GS-induced nephrotoxicity is an animal model to study the acute renal failure. The mechanisms of GS-induced nephrotoxicity are not completely understood. However, proposed pathological mechanisms are: induction of oxidative stress, apoptosis, necrosis, elevation of endothelin I and increase in monocyte/macrophages infiltration (3- 6).

GS-induced nephrotoxicity is characterized by increased serum creatinine and urea , decreased glomerular filtration rate (7, 8) and morphologically characterized by proximal convoluted tubule (PCT) cells desquamation, tubular cells necrosis, epithelial edema and glomerular hypertrophy (5, 9, 10). GS increases the generation of reactive oxygen species (ROS) such as superoxide anions (11-14), hydroxyl radicals, hydrogen peroxide and reactive nitrogen species in the kidney (2, 15). ROS also activate nuclear factor kappa β that plays a key role in the induction of inflammatory process (16).

GS induces renal damage via lipid peroxidation (17, 18) and protein oxidation in the renal cortex (19). GS reduces the activity of renal antioxidant enzymes like catalase, glutathione peroxidase (GPX) and the glutathione content (6, 12, 14, 20, 21).

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There is increasing evidence that hyperbaric oxygen preconditioning increases the activity of antioxidant enzymes, *in vivo* (22- 25). Treatment with normobaric hyperoxia (NH), reduced ischemia-reperfusion injuries significantly in rat brain via up regulation of glutamate transporter and tumor necrosis factor α (26), triggering tumor necrosis factor-alpha converting enzyme, nuclear factor-κβ (27), improvement of NO production (28), and increasing glutathione peroxidase, catalase and superoxide dismutase activity (29).

Some researchers showed that oxygen preconditioning improved renal ischemia-reperfusion injuries by inducing tissue antioxidant enzymes activity (22- 24). So far, no detailed study has been done on the efficacy of NH in the improvement of gentamicin-induced nephrotoxicity in experimental animals.

## Materials and Methods

Forty-eight male Sprague-Dawley rats (weighing 135-150 g) were purchased from Pasteur Institute, Tehran, Iran. They were kept at 22°C and a humidity of 50 ± 10% with 12 hr light/dark cycles. This study was approved by the Animal Ethics Committee of Lorestan University of Medical Sciences and was in accordance with the National Health and Medical Research Council guidelines.

After acclimatization, rats were randomly divided into 6 groups (n=8) as follows: group 1 as control, group 2 received daily GS (100 mg/kg, IP), group 3 received oxygen 2 hr/day for 2 days and then received daily GS, group 4 received oxygen 4 hr/day for 2 days and then received daily GS, group 5 received oxygen 4 hr/day for 4 days and then received daily GS and group 6 received oxygen 2 h/day for 2 days and then received 2 hr oxygen before daily GS injection**.** Oxygen (with 90% purity) was used at the flow rate of 4 l/min by super oxygen HMS-5 system and GS was administered for 8 days.

The weights of the animals were measured before and after the treatment (day 0 and day 8).

After the last injection of GS, animals were immediately placed in individual metabolic cages in order to collect 24 hr urine. Blood samples were obtained from animals hearts under anesthesia. Samples were allowed to clot for 20 min at room temperature and then centrifuged at $1\times10^4$ rpm for 10 min for serum separation. The animal kidneys were excised. The left kidney was fixed in 10% formaldehyde solution and the right kidney was used for homogenization. The fixed kidney was cut into the slices of approximately 1-mm thickness and after tissue processing, paraffin sections (5-µm thickness) were prepared and stained by periodic acid Schiff (PAS) method. The right kidney was homogenized in Tris-HCl buffer (0.05 mol/l Tris-HCl and 1.15% KCl, pH 7.4), using a homogenizer. The homogenate was centrifuged at $1.8\times10^4$ g (4°C) for 30 min (11). The supernatant was utilized for the biochemical analysis of the renal tissue.

### *Biochemical analysis*

#### *Serum and urine parameters*

Serum malondialdehyde (MDA), a marker of lipid peroxidation was assessed using thiobarbituric acid test (30). Serum creatinine and urea were determined using commercial kits (Randox, UK) according to the manufacturer guideline. All biochemical measures were done in duplicate.

#### *Renal tissue biochemical parameters*

Renal MDA, glutathione content and catalase activity were assessed in the kidney homogenate using commercial kits (Randox, UK) according to the manufacturer guideline.

#### *Histopathological studies*

Blind evaluation of the PCT necrosis in the kidney sections was done by an expert histologist. PCT necrosis was scored as follows: zero; no cell necrosis, 1; mild usually single-cell necrosis in sparse tubules, 2; moderate, more than one necrotic cell involved in sparse tubules, 3; marked tubules exhibiting total necrosis in almost every microscopical field and 4; massive total necrosis (31).

#### *Stereological study*

The volume density of PCT per cortex was estimated by the point counting rule. Sections of the kidney slices (one section from each slice) were used. Microscopical image from each section was projected on a point probe (frame 13×14 cm square with 360 **+** in it that was traced on paper) by video projector via a microscope equipped with Leica DFC camera attached to the computer. At the magnification of ×300, points that hit epithelium of proximal tubules (positive periodic acid Schiff brush border) were counted (32). The tubular profiles that fell inside the probe and did not cross the lower and left lines of the probe were selected for point counting (32). From each kidney, at least 60 microscopical fields were assessed. The volume density of PCT per cortex was estimated using the following equation (33):

$Vv\ (PCT/Cortex) = \Sigma Pp/\Sigma Pt$

Where Σ Pp is the sum of the points hitting PCT epithelium and Σ Pt is the sum of the points falling on reference space (frame). If 10 fields are assessed then Σ Pt is 10×360.

#### *Statistical analyses*

All values are expressed as mean±SEM. The data was compared between groups by nonparametric Mann-Whitney *U*-test. Differences between the animals'

**Table 1.** Effect of normobaric hyperoxia on animal body weight and % body weight changes in rats treated with gentamicin sulphate

| Experimental groups | Average body weight (g) | | % Body weight change |
|---|---|---|---|
| | Before treatment (day 0) | After treatment (day 8) | |
| 1 | 146.5±3.30 | 150.75±3.19 | 2.92±0.62 |
| 2 | 143.05±2.14 | 134±2.01 * | -6.61±0.6 * |
| 3 | 138.5±3.52 | 129.3±3.22 * | -6.59±0.83 * |
| 4 | 138.3±3.15 | 131.1±3.1 * | -5.18±0.76 * |
| 5 | 139.6±2.43 | 132.5±2.72 * | -5.13±0.88 * |
| 6 | 140.5±2.33 | 133.8±2.16 * | -4.61±0.32 * # |

Values represented as mean ±SEM. * Significant change in comparison with control at $P < 0.05$. # Significant change in comparison with gentamicin only treated at $P< 0.05$. group 1 as control; group 2 received daily GS (100 mg/kg Ip); group 3 received oxygen 2 hr daily for 2 days and then received daily GS; group 4 received oxygen 4 hr daily for 2 days and then received daily GS; group 5 received oxygen 4 hr daily for 4 days and then received daily GS and group 6 received oxygen 2 hr daily for 2 days and then during daily injection GS received 2 hr oxygen before injection

**Table 2.** Effect of oxygen therapy on serum malondialdehyde and renal function markers in gentamicin induced nephrotoxicity

| Experimental groups | Serum MDA (nmol/ml) | Serum creatinine (mg/dl) | Serum urea (mg/dl) | Urine creatinine (mg/dl) | Urine urea (mg/dl) |
|---|---|---|---|---|---|
| 1 | 0.23 ± 0.027 | 0.69± 0.04 | 56.8 ±5.14 | 2.04 ± 0.07 | 168.6 ± 13.04 |
| 2 | 0.701 ± 0.056 * | 2.98± 0.25 * | 198.42 ±16.58 * | 0.78 ± 0.07 * | 40 ± 6.56 * |
| 3 | 0.543 ± 0.045 * | 2.7± 0.40 * | 160.14± 13.21 * | 0.67 ± 0.16* | 44.7 ± 7.19 * |
| 4 | 0.504 ± 0.705 * | 1.60± 0.06 * # | 120.5 ± 5.25 * # | 1.301 ± 0.10 * # | 80.07 ± 5.43 * # |
| 5 | 0.505 ± 0.051 * | 2.77 ± 0.45 * | 192.3 ± 17.63 * | 0.55 ± 0.13 * | 30.66 ± 6.67 * |
| 6 | 0.414 ± 0.026 * # | 1.77 ± 0.17 * # | 117.66 ± 5.38 * # | 1.26 ± 0.05 * # | 74.25 ± 5.6 * # |

Values represented as mean ±SEM. * Significant change in comparison with control at $P < 0.05$. # Significant change in comparison with gentamicin only treated at $P< 0.05$. group 1 as control; group 2 received daily GS (100 mg/kg Ip); group 3 received oxygen 2 hr daily for 2 days and then received daily GS; group 4 received oxygen 4 hr daily for 2 days and then received daily GS; group 5 received oxygen 4 hr daily for 4 days and then received daily GS and group 6 received oxygen 2 hr daily for 2 days and then during daily injection GS received 2 hr oxygen before injection

weights before and after the treatment in each group, were analyzed by paired *t* test. Comparison of weights and percentage of body weight changes among experimental groups were analyzed through one-way ANOVA followed by Tukey test. Statistical analyses were performed using the SPSS13 software. *P* value less than 0.05 was considered significant.

## Results

### *Effect of NH on the changes in the percentage of body weights*

GS decreased the percentage of changes in body weights compared to the control group ($P<0.05$). Treatment with oxygen did not make significant difference in % body weight when compared to the GS only treated group ($P<0.05$). Only pretreatment with and daily use of oxygen during GS administration could inhibit the loss of % body weight in comparison with GS treated group but could not restore it to that of the control group (Table 1).

### *Effect of NH on serum MDA*

Malondialdehyde was significantly increased in sera of rats with GS-induced nephrotoxicity in comparison with the control group ($P<0.05$). Pretreatment with NH could not decrease serum MDA significantly when compared with GS treated group. In group 6, pretreatment with and daily use of oxygen during daily GS administration, could inhibit the increase in serum MDA compared to GS treated group but could not restore its level to the level of the control group (Table 2).

### *Effect of NH on renal function markers*

In the GS only treated group, the level of serum creatinine significantly increased compared to control group ($P<0.05$, Table 2). Upon oxygen therapy in groups 4 and 6, a significant decline ($P<0.05$) in the levels of serum creatinine was observed in comparison with the GS treated group (Table 2). There was no significant difference between the serum creatinine of groups 3 and 5 and group 2.

Serum urea concentrations in the experimental groups are presented in Table 2. Significant increase in blood urea was found in the GS only treated group compared to the control group ($P<0.05$). The increase in blood urea in groups 4 and 6 was significantly inhibited ($P<0.05$) in comparison with the GS only treated group. However, oxygen therapy could not maintain the level of blood urea at its level in the control group.

The level of urine creatinine and urea decreased in GS treated group significantly against control group. In group 4 and 6, oxygen therapy increased urine creatinine and urea in comparison with GS treated group but could not restore it back to the same level as control group (Table 2).

### *Effect of NH on proximal convoluted tubules*

Gentamicin treated animals showed a significant decrease in PCT volume density compare to control group ($P<0.05$). In groups 4 and 6, treatment with NH showed significant increase in PCT volume density when compared with GS only treated group ($P<0.05$). NH could not maintain the PCT volume density at its level in the control group (Table 3).

**Table 3.** Effect of oxygen therapy on tubular necrosis, volume density of proximal convoluted tubules and tubular cast in gentamicin induced nephrotoxicity

| Experimental groups | Tubular necrosis (score 0-4) | Volume density of PCT | Tubular cast (score 0-4) |
|---|---|---|---|
| 1 | 0.24±0.05 | 0.229±0.009 | 0 |
| 2 | 1.91±0.29 * | 0.093±0.011 * | 1.83±0.30 * |
| 3 | 1.38±0.24 * | 0.120±0.015 * | 1.66±0.21 * |
| 4 | 1.06±0.19 *# | 0.12±0.004 *# | 0.83±0.30 *# |
| 5 | 1.44±0.16 * | 0.107±0.013 * | 2.50±0.56 * |
| 6 | 0.91±0.20 *# | 0.16±0.015 *# | 0.84±0.23 *# |

Values represented as mean ±SEM. * Significant change in comparison with control at $P < 0.05$. # Significant change in comparison with gentamicin only treated at $P < 0.05$. group 1 as control; group 2 received daily GS (100 mg/kg Ip); group 3 received oxygen 2 hr daily for 2 days and then received daily GS; group 4 received oxygen 4 hr daily for 2 days and then received daily GS; group 5 received oxygen 4 hr daily for 4 days and then received daily GS and group 6 received oxygen 2 hr daily for 2 days and then during daily injection GS received 2 hr oxygen before injection

**Table 4.** Effect of normobaric hyperoxia on renal malondialdehyde, glutathione and catalase in gentamicin induced nephrotoxicity

| Experimental groups | Renal MDA (nmol/g protein) | Renal GSH (nmol/mg protein) | Renal CAT (u/mg protein) |
|---|---|---|---|
| Group 1 | 0.84 ± 0.202 | 0.681 ± 0.174 | 68.47 ± 14.58 |
| Group 2 | 3.65 ± 0.581 * | 0.293 ± 0.053 * | 11.99 ± 4.4 * |
| Group 3 | 2.628 ± 0.465 * | 0.274 ± 0.061 * | 18.25 ± 3.41 * |
| Group 4 | 2.185 ± 0.596 * | 0.203 ± 0.054 * | 34.88 ± 8.49 * # |
| Group 5 | 2.905 ± 0.419 * | 0.314 ± 0.084 * | 26.85 ± 6.15 * |
| Group 6 | 1.921 ± 0.305 * # | 0.261 ± 0.073 * | 53.81 ± 8.26 # |

Values represented as mean ±SEM. * Significant change in comparison with control At $P < 0.05$. # Significant change in comparison with gentamicin only treated at $P < 0.05$. group 1 as control; group 2 received daily GS (100 mg/kg Ip); group 3 received oxygen 2 hr daily for 2 days and then received daily GS; group 4 received oxygen 4 hr daily for 2 days and then received daily GS; group 5 received oxygen 4 hr daily for 4 days and then received daily GS and group 6 received oxygen 2 hr daily for 2 days and then during daily injection GS received 2 hr oxygen before injection

Tubular necrosis significantly increased after treatment with GS ($P<0.05$). Co-treatment with GS and NH in groups 4 and 6, significantly decreased the level of PCT necrosis when compared with GS only treated rats ($P<0.05$). NH therapy could not keep tubular necrosis at its level in the control group (Table 3).

### *Effect of NH on amount of tubular cast*

Tubular cast in GS treated group was significantly increased compared with control group. Oxygen therapy in groups 4 and 6 resulted in significant decrease in tubular cast against GS treated group but could not maintain tubular cast at its level in the control group ($P<0.05$, Table 3).

### *Effect of NH on renal MDA, glutathione and catalase*

Renal MDA increased significantly in GS treated group against control group. The increased renal MDA was only inhibited in group 6 when compared with GS treated group ($P<0.05$, Table 4).

Renal glutathione content decreased significantly in GS treated and oxygen treated groups compared with control group. Oxygen therapy did not show beneficial effect on the increase in glutathione content ($P<0.05$, Table 4).

In group 2, renal catalase decreased significantly compared to control group. In groups 4 and 6, oxygen therapy significantly increased the catalase activity in comparison with group 2. In group 6, oxygen therapy only restored renal catalase to the level in control group ($P<0.05$).

## Discussion

In our study, administration of GS (100 mg/kg/day, IP) led to significant increase in serum creatinine, urea, and MDA which is similar to the results that were previously reported (14, 31, 34). Although the increased serum creatinine and urea was significantly decreased in group 4 and 6, in comparison with group 2 (GS only treated group), but these variables are approximately two times greater than those of control group. In our study, the decrease in serum creatinine and urea reflects the amelioration of renal function following oxygen therapy. Because of the glomerular filtration and tubular secretion of creatinine, it can be speculated that amelioration of serum creatinine level by NH is due to the recovery of tubular and glomerular function in GS-induced nephrotoxicity. As an indicator of the initial phases of the kidney disease, serum creatinine concentration is more reliable than the urea. Furthermore, urea concentration begins to increase only after parenchymal injury (34).

The elevated level of serum MDA indicates increase of free radicals generation or lipid peroxidation in GS induced nephrotoxicity. The increase in serum MDA was significantly decreased only in groups 6. Decrease of serum MDA by using different antioxidants against GS nephrotoxicity was reported by others (14, 20, 21, 31, 32, 35-38). Amelioration of serum MDA following oxygen therapy against GS nephrotoxicity showed that oxygen therapy reduced oxidative stress. From these observations, it can be concluded that administration of NH prevents lipid peroxidation, which may, at

least partly, play an important role in the injury cascade of gentamicin-induced nephrotoxicity.

According to our findings, tubular necrosis was ameliorated in the animals of group 4 and 6 in comparison with GS only treated animals. Reduction of PCT necrosis was previously reported by other researchers using different agents (13, 20, 31, 39-41). Gentamicin causes PCT cell death via oxidative stress, apoptosis, inflammation, phospholipidosis and increasing intracellular sodium ions (34, 42). It has been suggested that ROS plays the main role in the mechanisms that lead to tubular necrosis (16). Improvement of tubular necrosis via oxygen therapy may be due to attenuation of mechanisms involved in PCT cells death. After tubular cells necrosis and cells desquamation, new tubular cells arise from differentiated mature tubular cells. In this way, some tubular cells dedifferentiate and then proliferated under effect of factors such as HGF, EGF, IGF-1, TGF-B and PDGF to produce new differentiated tubular cells (43). It may be postulated that reduction in PCT necrosis by oxygen therapy was due to reduction of tubular cells necrosis or activation of tubular cell regeneration.

Volume density of PCT decreased significantly in GS treated rats compared with control group. The reduction of PCT volume density was inhibited in groups 4 and 6 compared with GS only treated group. Despite this significant difference, PCT volume density in control group is about 1.5 times greater than that of groups 4 and 6. In the assessment of PCT, the tubules that had normal appearance and showed PAS positive brush border, were selected as PCT. Absence and disappearance of brush border are the results of GS nephrotoxicity and NH ameliorates the brush border as observed under light microscopy.

Although Sepehri *et al* reported the occurrence of glomerular atrophy following gentamicin injection (44), but via stereological rules we previously showed that GS did not significantly increase glomerular volume in comparison with control group (32, 38). Nevertheless, some researchers reported glomerular hypertrophy following GS administration by morphometric rule (5, 9, 10).

The significant and progressive weight losses in GS treated rats are remarkable. Only in group 6, % body weight change (after and before the experiment) is significantly different from that of GS only treated group. Oxygen therapy cannot maintain animal weight at the same level as the control group. Such results were also reported following the administration of aqueous leaf and seed extract of *Phyllanthus amarus* for prevention of weight loss induced by GS (40).

This study reveals that GS consumption leads to significant increase in renal MDA compared with the control group. The same results were reported by other researchers (13, 20, 39). Increased renal MDA was inhibited only in group 6 in comparison with the GS only treated animals.

According to our results, while GS significantly decreases renal glutathione, oxygen therapy had no effects on renal tissue glutathione content.

Catalase, the endogenous antioxidant enzyme detoxifies hydrogen peroxide to water and oxygen. According to our results, renal tissue catalase significantly declined in GS treated rats when compared to control group, which is similar to that reported by others (12, 14, 20, 21, 32, 38).Oxygen therapy increased catalase in groups 4, 5 and 6 when compared with group 2 ($P<0.05$).

Although some researchers showed that oxygen preconditioning improves renal ischemia-reperfusion injuries by inducing tissue antioxidant enzymes activity (22-24) or increasing catalase by NH in rats (29), but regarding the effect of oxygen therapy on GS-induced nephrotoxicity, there is no similar study to compare our results with.

Increase in renal catalase activity, decrease in renal and serum MDA, amelioration of serum creatinine, urea and histological changes in group 6 showed that NH (preconditioning and daily use of NH) ameliorates renal nephrotoxicity probability via inhibition of lipid peroxidation or activation of tissue antioxidant enzymes such as catalase.

Despite the increase in catalase activity in group 5, MDA is high and this treatment did not show any improvement in GS nephrotoxicity. Probably, when the dose of oxygen therapy is higher than that tolerated by animals, it may lead to high lipid peroxidation and oxidative stress. In other words, this dose of oxygen therapy is higher than rat tolerance.

Although, the decrease in MDA in group 4, was not significant but the increase in renal catalase activity could ameliorate GS nephrotoxicity. It may be concluded that oxygen therapy (preconditioning 4 h/day for 2 days) ameliorates renal GS nephrotoxicity via increase of renal tissue catalase activity.

Although oxygen therapy ameliorates GS nephrotoxicity but treatments could not preserve tissue changes and renal functional factors at the same level with that of the control group. Based on our results, the molecular mechanisms of the protective effects of oxygen therapy cannot be fully explained but the authors believe that the obtained histological and biochemical results are satisfactory.

## Conclusion

NH ameliorates GS-induced nephrotoxicity possibly via inhibition of lipid peroxidation and increasing renal catalase activity. Our findings suggest the potential therapeutic use of NH as a new nephroprotective agent against acute renal failure induced by nephrotoxins like GS. Further studies with larger sample sizes, different doses of NH and

different duration of preconditioning are required to confirm these results.

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## Conflict of Interests

All authors declare that they have no conflicts of interest.

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