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Potential protective effect of alpha lipoic acid against testicular oxidative stress and altered gene expression induced by gentamicin treatment

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Abstract

Background: Gentamicin induces testicular damage in association with oxidative stress explained by different mechanisms, including gene expression suppression of some antioxidant enzymes. **The aim of the study:** To investigate the potential protective effects of α-lipoic acid against gentamicin-induced testicular oxidative stress in terms of altered gene expression. **Materials and methods:** A parallel experimental study was conducted including fifty adult male albino rats. The animals were grouped into five. The control group received intraperitoneal NaCl 0.9%, while the other groups received 36.5mg/kg/day intraperitoneal gentamicin. Group-1 received gentamicin only, group-2 received gentamicin + intraperitoneal α-lipoic acid 100mg/kg/day, group-3 received gentamicin + intraperitoneal α-lipoic acid 200mg/kg/day, and group-4 received gentamicin + oral vitamin E 100mg/kg/day. All treatments were given for 14 days. The animals were euthanized in two halves on the 15th and 60th days. Testes were immediately removed, frozen, and evaluated for oxidative stress biomarkers and gene expression of antioxidant enzymes. **Results:** Gentamicin increased malondialdehyde by mean difference ± standard error of 115.57 ± 2.18, decreased total antioxidant capacity by -9.23 ± 0.27, and decreased catalase and superoxide dismutase activities by -1.89 ± 0.45 and -11.77 ± 2.86, respectively, compared to the control. Additionally, gentamicin downregulated gene expression of catalase, glutathione peroxidase, and superoxide dismutase. However, adding vitamin E or α-lipoic acid cured the oxidative stress and partially to completely upregulated the gene expression. **Conclusion:** Reversal of testicular oxidative and gene suppression associated with gentamicin could be achieved with α-lipoic acid co-treatment, which might be applied in human studies.

Keywords: Alpha lipoic acid; Aminoglycosides; Antioxidant genes; Testis
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Introduction. Gentamicin, a widely used antibiotic to treat many infectious diseases, is a bactericidal broad-spectrum aminoglycoside [1, 2, 3]. It has been demonstrated that gentamicin can cause cellular damage in certain vulnerable organs tissue such as the kidney, inner ear, and testis [4, 5, 6], which is influenced by the high hydrophilic nature of gentamicin that limits its volume of distribution and makes the molecule highly selective to specific organs [7]. Oxidative stress has been accused as one of the main mechanistic approaches to such damage [8]. Numerous mechanisms underlying gentamicin-induced oxidative stress have been reported with *in vitro* or *in vivo* evidence. One mechanism is mitochondrial pathway-dependent oxidative stress through increasing the production of mitochondrial reactive oxygen species (ROS) from the respiratory chain [9]. Another mechanism is the peroxidation of phosphoinositide, a membrane lipid rich in arachidonic acid, by gentamicin-iron complex, accompanied by arachidonic acid release [10]. The released arachidonic acid also forms an arachidonic acid-Fe^{2+}-gentamicin ternary complex that reacts with molecular oxygen or lipid peroxides, leading to the propagation of arachidonic acid peroxidation [10]. Other mechanisms include reduced cellular concentrations of glutathione, glutathione peroxidase (GPx), superoxide dismutase enzyme (SOD), and catalase enzyme [11]. The reduced cellular antioxidant enzymes have been investigated in the context of altering their gene expressions, and it has been reported that gentamicin reduced the gene expression of some antioxidant enzymes including SOD [11], catalase, and GPx [12]. On the other hand, gentamicin-induced cellular damage can be also explained by induced endoplasmic reticulum stress and hence apoptosis [13]. In the testicular tissues, germ cells are very rich in polyunsaturated fatty acid and thus become vulnerable to oxidative stress-related damage [14]. Additionally, endoplasmic reticulum stress is correlated with male infertility [15]. Gentamicin treatment was associated with reduced reproductive organs' weight, altered sperm parameters, disturbed sexual hormone levels, and abnormal histopathological findings, accompanied by induced testicular oxidative stress [16]. However, many attempts tried to mitigate these effects using different antioxidants including ginger [17], lycopene [6], carrot seed [18], melatonin [19], dandelion [20], α-lipoic acid [21], curcumin and propolis [22], omega-3-fatty acids [23], vitamin C and E [24], and selenium [25]. On the other hand, α-lipoic acid is a powerful exogenous antioxidant that serves as a coenzyme of the mitochondrial pyruvate dehydrogenase complex and as a component of the mitochondrial glycine cleavage system [26]. It could prevent testicular oxidative stress induced by oxidant agents such as carbimazole [27]. Additionally, α-lipoic acid showed promising results in protecting testicular structure, preserving sperm parameters, and normalizing reproductive hormone levels when co-administered with gentamicin [21, 28]. Moreover, α-lipoic acid showed upregulation of gene expression of antioxidant enzymes in diabetic rat kidneys in previous studies [29, 30]. It was hypothesized that the protective effect of α-lipoic acid against gentamicin-induced testicular damage is mediated by the antioxidant effects of α-lipoic acid including direct effect and antioxidant enzymes upregulation. However, the potential protective effect of alpha-lipoic against altered gene expression of antioxidant enzymes induced by gentamicin has been poorly studied.

The aim of the study. To evaluate the potential protection of α-lipoic acid against gentamicin-induced testicular oxidative stress.

Materials and Methods

Drug preparation

Gentamicin was obtained as Garamycin® 80 mg/2 ml ampoules manufactured by Schering-Plough Corporation, Egypt. α-lipoic acid was obtained as Thiotacid® 300 mg/ 10 ml ampoules manufactured by EVA Pharmaceutical Industries, Egypt. Both drugs were

given intraperitoneally with a 25-gauge needle. Vitamin E was obtained under the generic name of vitamin E 1000 mg®, Pharco Pharmaceuticals. The capsules were evacuated, diluted with sunflower oil to a final volume of 1 ml per rat, and administered orally using 18-gauge soft gavage tubes.

Drug dosing

The double average dose of gentamicin was used in this study which was calculated as the following: The therapeutic human dose of 5 mg/kg/day was converted to the equivalent dose for rats based on the body surface area ratio according to Paget and Barnes method to be 31.5 mg/kg daily [31]. The rat dose was then calculated as the average dose of 31.5 mg/kg/day (based on body surface area) and 5 mg/kg/day (based on body weight) to be 18.25 mg/kg/day. The double average dose is 36.5 mg/kg/day. Alpha lipoic acid was given in two different doses; 100 and 200 mg/kg/day [32, 33], while vitamin E was administered as 100 mg/kg/day [34]. All treatments were given for 14 days [35].

Animals

Sixty male Wistar rats aged 8 weeks and weighing 200 ± 20 g were obtained from the Animal House of the Faculty of Veterinary Medicine, Benha University. The rats were housed at normal room temperature (25 - 30°C), humidity (40-60%), and 12h/12h light/ dark cycle before use in the experimental protocols. The animals were fed laboratory formula and tap water *ad libitum* [36].

Study design

A parallel experimental study was conducted at the Departments of Pharmacology and Theriogenology, Faculty of Veterinary Medicine, Benha University. The study was approved by the ethics committee of the faculty with an ethical approval number of BUFVTM-080422. After two weeks of acclimatization to the diet and house conditions, ten rats with abnormal testicle size were excluded based on ultrasound screening. Fifty rats were randomly assigned to five groups of ten. Rats in group 1 received gentamicin once daily with 36.5 mg/kg. Rats in groups 2, 3, and 4 received the same gentamicin dose plus 100 mg/kg α-lipoic acid (group 2), 200 mg/Kg α-lipoic acid /day (group 3), and100 mg/kg vitamin E (group 4). Group 5 served as a control and received an intraperitoneal 0.5 ml NaCl 0.9% once daily. All treatments were administered from day one to day fourteen. Half of the animals in each group were euthanized under anesthesia using isoflurane anesthesia [37] on the 15^{th} day, while the second half was euthanized on the 60^{th} day of the treatment.

Testis sampling

Immediately after euthanasia, one testis from each animal was dissected, weighed, and divided into two parts. To assay testicular oxidative stress biomarkers, the first part of the testis was homogenized with a phosphate buffer solution at pH 7.4 and centrifuged at 1500 xg for 5 minutes at 4 ºC [38]. The supernatant was removed and kept at -20 C till used in the analysis of oxidative stress biomarkers. The second part of the testis was immediately kept at -80 ºC to quantify testicular gene expression of antioxidant enzymes.

Oxidative stress assessment

The separated testicular homogenate was used to assay the oxidative stress parameters including malondialdehyde (MDA) concentration, SOD and Catalase activities, and total antioxidant capacity (TAC) by using commercial kits from Biodiagnostic, Dokki, Giza, (Catalog No. TA2513 for TAC, Catalog No. TA2529 for MDA, Catalog No. TA2521 for SOD, Catalog No. TA2517 for catalase). All procedures were carried out according to the manufacturer's instructions [39], in the Central Laboratory of the faculty.

Gene expression assessment

Total RNA was extracted from the frozen samples using RNeasy® Mini kit (Qiagen) by adding easy red TRIzol solution to the tissue and following the manufacturer's protocol. Single-stranded complementary DNA (cDNA) was synthesized from 1000 ng of total RNA according to the manufacturer’s protocol of high-Capacity cDNA Reverse Transcription Kits (Applied Biosystems®). The RNA quantity was determined using SPECTROstar Nano (BMG LABTECH®). Real-time polymerase chain reactions (RT-PCR) were conducted for each gene of catalase, SOD, and GPx enzymes. Each PCR reaction consisted of 1.5 µl of

1µg/µl cDNA, 10 µl SYBR Green PCR Master Mix (QuantiTect SYBR® Green RT-PCR Kit, Qiagen), 1 µM of each forward and reverse primer for each gene [40] **(**Table 1**)**, and nuclease-free water to a final volume of 20 µl. Reactions were then analyzed on an Applied Biosystem® 7500 Fast RT-PCR Detection system under the following conditions: 95°C for 10 minutes (holding stage) and 40 cycles of 95°C for 15 seconds (denaturation stage) followed by 60°C for 1 minute (annealing and extension stage). Changes in gene expression were calculated from the obtained cycle threshold (Ct) values provided by real-time PCR instrumentation using the comparative CT method to a reference (housekeeping) gene (β-actin). Gene expression is expressed as relative mRNA expression of catalase, SOD, and GPx to β-actin mRNA.

Table 1

Forward and reverse sequences of primers used in gene expression testing

Primer	Forward sequence	Reverse sequence
B-actin	AGAAGAGCTATGAGCTGCCTGACG	CTTCTGCATCCTGTCAGCGATGC
Catalase	ACACTTTGACAGAGAGCGGA	TTTCACTGCAAACCCACGAG
GPx	GACCGACCCCAAGTACATCA	GCAGGGCTTCTATATCGGGT
SOD	GCGTCATTCACTTCGAGCAG	GGTCTCCAACATGCCTCTCT

Note: GPx – glutathione peroxidase; SOD – superoxide dismutase.

Statistical methods

Values are expressed as mean ± standard error (SE), and for effect size as mean difference (MD) ± SE. A one-way analysis of variance (ANOVA) test was conducted among the study groups at 0.05 level of significance followed by, for a significant F-statistic value, post hoc Tukey's test at a Bonferroni adjusted level of significance. Correlations between enzyme activities and gene expression were assessed by Pearson correlation test using correlation coefficient Rho (r) to indicate correlation direction and magnitude at 0.05 level of significance. Regression-based adjusted R squared (R^2) was used to show how much enzyme activity variation could be explained by its gene expression, and regression coefficient (beta) was used to quantify the relationship. Statistical Package for the Social Sciences (SPSS) v.26 software was used for the required analysis [41].

Results

Oxidative stress biomarkers

As shown in Table 2, on day 15, rats treated with gentamicin showed higher MDA concentration than the control group (MD ± SE = 115.57 ± 2.18). Despite the reduction in MDA concentration amongst testes cotreated with 100 mg/Kg α-lipoic acid compared to gentamicin alone (MD ± SE = -94.29 ± 2.18), it was still higher than the control group ((MD ± SE = 21.29 ± 2.18). However, co-treatment with gentamicin plus 200 mg/Kg α-lipoic acid normalized MDA concentration compared to the control group (MD ± SE = -2.05 ± 2.18). Additionally, the group cotreated with gentamicin plus vitamin E could normalize MDA concentration compared to the control group (MD ± SE = -5.03 ± 2.18). TAC was reduced with gentamicin treatment compared to the control group (MD ± SE = -9.23 ± 0.27). However, adding 100 or 200 mg/kg α-lipoic acid, or vitamin E to gentamicin treatment could restore TAC compared to the control (MD ± SE = -0.57 ± 0.27, 0.28 ± 0.27, 0.002 ± 0.27, respectively). Catalase and SOD enzyme activities were also reduced in rats treated with gentamicin compared to the control group (MD ± SE = -1.89 ± 0.45 and -11.77 ± 2.86, respectively). However, coadministration of 100 mg/Kg α-lipoic acid with gentamicin could normalize the enzyme activities as compared to the control (MD ± SE = 0.19 ± 0.45, 2.23 ± 2.86, respectively). Moreover, coadministration of either 200 mg/Kg α-lipoic acid or vitamin E normalized catalase and increased SOD enzyme activities compared to the control (MD ± SE = 1.14 ± 0.45, 20.23 ± 2.86, respectively for 200 mg α-lipoic acid; MD ± SE = 0.07 ± 0.45, 13.23 ± 2.86, respectively for vitamin E). On day sixty, no statistically significant differences were observed among the study groups.

Table 2

Effect of gentamicin with/without alpha-lipoic acid or vitamin E on oxidative stress biomarkers in rats' testis

Day	Group	MDA (nmol/0.1g.tissue)		TAC (mM/L)		SOD (U/ml tissue)		Catalase (U/g tissue)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
15	Control	46.61	0.64[a]	16.90	0.22[a]	320.0	2.90[a]	4.34	0.19[a]
	1	162.19	0.80[b]	7.67	0.14[b]	308.2	0.71[b]	2.46	0.02[b]
	2	67.90	2.55[c]	16.33	0.20[a]	323.2	0.71[a]	4.52	0.24[a]
	3	44.56	1.99[a]	17.18	0.21[a]	340.2	3.24[d]	5.47	0.64[a]
	4	41.58	0.55[a]	16.90	0.18[a]	333.2	0.71[d]	4.41	0.06[a]
60	Control	65.07	0.81	15.46	0.18	537.86	18.47[a]	7.18	1.32
	1	54.09	3.49	15.81	0.28	479.83	43.85	7.01	1.12
	2	56.31	2.31	13.82	0.94	439.72	37.23	7.08	1.00
	3	52.40	3.28	15.42	0.11	424.29	16.13	6.01	1.05
	4	52.68	4.11	15.14	0.54	413.76	8.62	6.94	1.23

Note: MDA – malondialdehyde; SE – standard error; SOD – superoxide dismutase; TAC – total antioxidant capacity; group 1 refers to gentamicin alone; group 2 refers to gentamicin + 100mg/kg α-lipoic acid; group 3 refers to gentamicin + 200 mg/kg α-lipoic acid; group 4 refers to gentamicin + vitamin E. Values bearing different superscript letters in the same column differ significantly between groups.

Gene expression

As shown in Table 3, on day 15, relative catalase and SOD gene expression was reduced among rats treated with gentamicin compared to the control rats (MD ± SE = -0.84 ± 0.09 and -0.66 ± 0.07, respectively). However, co-treatment with 100 mg/kg α-lipoic acid provided a partial increase in the catalase and SOD gene expression compared to gentamicin alone (MD ± SE = 0.43 ± 0.09 and 0.26 ± 0.07, respectively), but less than the control (MD ± SE = -0.41 ± 0.09 and -0.42 ± 0.07, respectively). Additionally, co-treatment with 200 mg/kg α-lipoic acid fortified the increase in the catalase and SOD gene expression compared to the control (MD ± SE = 0.29 ± 0.09 and 0.46 ± 0.07, respectively). On the other hand, co-treatment with vitamin E partially upregulated catalase and SOD gene expression compared to treatment with gentamicin alone and the control group, which approximated α-lipoic acid 100 mg/kg effect (p = 0.204 and 0.326). Relative GPx gene expression was also reduced with gentamicin treatment compared to the control (MD ± SE = -0.49 ± 0.06) that was mitigated with 100 mg/kg α-lipoic acid (MD ± SE =0.23 ± 0.06) or normalized with 200 mg/kg α-lipoic acid (MD ± SE = 0.003 ± 0.06) compared to gentamicin alone. On the other hand, co-treatment with vitamin E partially upregulated GPx gene expression compared to treatment with gentamicin alone and the control group, which approximated α-lipoic acid 100 mg/kg effect (p = 0.992). On day sixty, the difference among the groups in the relative genes' expression was not significant.

Relationship between oxidative stress and gene expression

Catalase enzyme activity was significantly correlated with catalase gene expression (r = 0.69, p < 0.001). The univariate regression model of catalase enzyme activity showed that R^2 = 0.457, catalase gene expression beta = 1.905, and p < 0.001, concluding that about 46% of the reduced catalase activity could be explained by the suppressed gene expression. SOD enzyme activity was significantly correlated with SOD gene expression (r = 0.535, p = 0.006). The univariate regression model of SOD enzyme activity showed that R^2 = 0.275, SOD gene expression beta = 15.759, and p = 0.004, concluding that about 28% of the reduced SOD activity could be explained by the suppressed gene expression.

Table 3

Gene expression of antioxidant enzymes obtained from rats' testis treated with gentamicin with/without alpha-lipoic acid or vitamin E

Day	Group	Cat. gene expression (Fold change)		SOD gene expression (Fold change)		GPx gene expression (Fold change)	
		Mean	SE	Mean	SE	Mean	SE
15	Control	1.18	0.07[a]	1.28	0.08[a]	1.11	0.07[a]
	1	0.34	0.02[b]	0.63	0.04[b]	0.62	0.03[b]
	2	0.77	0.05[d]	0.86	0.03[c]	0.85	0.03[c]
	3	1.46	0.11[c]	1.74	0.06[d]	1.12	0.04[a]
	4	0.97	0.04[ad]	1.01	0.05[c]	0.88`	0.05[c]
60	Control	0.98	0.13	1.11	0.09	0.97	0.12
	1	0.92	0.09	1.20	0.07	0.92	0.08
	2	1.05	0.14	1.16	0.10	1.02	0.09
	3	0.91	0.10	1.17	0.09	0.99	0.11
	4	1.12	0.08	1.04	0.12	0.94	0.13

Note: Cat. – Catalase; GPx – glutathione peroxidase; SOD – superoxide dismutase; group 1 refers to gentamicin alone; group2 refers to gentamicin + 100mg/kg α-lipoic acid; group 3 refers to gentamicin + 200 mg/kg α-lipoic acid; group 4 refers to gentamicin + vitamin E. Values bearing different superscript letters in the same column differ significantly between groups.

Discussion. The complexity of gentamicin-induced testicular oxidative stress promotes investigating one of the underlying potential mechanisms which is gene expression. The oxidative stress is not only limited to testicular tissue, but also extends to renal tubules [42], and vestibular tissues [43]. Testicular oxidative stress as a mechanism of gentamicin-induced gonadotoxicity has been studied and demonstrated in rat animal models [16]. Suppression of antioxidant enzymes gene expression as a mechanism of gentamicin-induced testicular oxidative stress has also been reported in a recent study [11]. Furthermore, gene expression downregulation associated with gentamicin treatment in other organs such as the kidney has been also reported [12]. The present study found an increased testicular MDA and a decreased TAC and decreased catalase and SOD enzyme activities in association with decreased gene expression of catalase, SOD, and GPx enzymes among animals treated with gentamicin compared to control, indicating testicular oxidative. In agreement with our findings, a study treated rats with 3 doses of gentamicin for 10 days and found a dose-dependent reduction in testicular catalase and SOD enzyme activities and an increase in lipid peroxidation [44]. Consistently, a 6-day treatment course with 100 mg/kg gentamicin increased testicular MDA and reduced antioxidant enzyme activities including catalase and glutathione reductase [19]. Another study reported a reduction in testicular catalase and SOD enzyme activities after treating rats with 5 mg/kg gentamicin for 10 days [45]. One more study reported a dose-dependent reduction in catalase and SOD enzyme activities on day 1 after treating animals with 3 and 5 mg/kg gentamicin for 10 days, which was restored on day 35 [46].

Alteration of testicular gene expression of antioxidant enzymes concerning gentamicin treatment was studied to a lesser extent. A study administered 100 mg/kg gentamicin for 6 days to rats and found testicular gene expression suppression of SOD by about 75% compared to control, but not catalase gene expression [11], which agreed with the current findings in SOD expression only. Another study examined the effect of 100 mg/kg gentamicin for 12 days on renal gene expression of SOD and GPx enzymes and reported great suppression of the two enzymes to about 90% [12], which is consistent with the present study results.

We added α-lipoic acid in two doses to gentamicin during the gentamicin-treatment

course and recorded a dose-dependent recovery from oxidative stress and restoration of gene expression compared to gentamicin alone. Consistently, α-lipoic acid as a protective agent against gentamicin-related gonadotoxicity was recently investigated. It was reported that 600 mg/kg α-lipoic acid protected against 80mg/kg gentamicin adverse effects when co-administered for 4 weeks [21]. However, to the best of our knowledge, the potential protective effect of α-lipoic acid against gentamicin-induced oxidative stress and altered gene expression has not been studied. Noteworthy, α-lipoic acid protected against gentamicin-induced nephrotoxicity [47] and ototoxicity [48], and the associated oxidative stress among animals treated with gentamicin and alpha lipoic 50-200 mg/kg. Upregulation of antioxidant enzymes gene expression by α-lipoic acid in diabetic rat kidneys has been reported in a study that treated rats with 10 mg/kg α-lipoic acid for 4 weeks. The relative mRNA expression of catalase and SOD to β-actin in diabetic rats was increased compared to normal rats [29]. Similarly, another study reported upregulation of relative gene expression of SOD, GPx, and catalase among α-lipoic acid-treated diabetic rat kidneys [30]. The gene expression upregulation by α-lipoic acid was proposed to be mediated by O-GlcNAc (O-linked β-N-acetylglucosamine)-dependent mechanism [29], besides its direct antioxidant activity through the oxidized and reduced forms [49]. It also has indirect antioxidant activity through metal chelation of Mn^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} [50] via electrophilic mechanisms, and LA-induced heme-oxygenase-1 (HO-1) expression and cellular protection [51].

In the present study, rats cotreated with vitamin E showed that the gentamicin-induced testicular oxidative stress was ameliorated, and the gene expression of antioxidant enzymes was partially upregulated which approximated the protective action of α-lipoic acid. Consistently, rats treated with 80 mg/kg gentamicin + vitamin E 50 mg/kg for 8 days showed a reduced renal MDA and an increased GSH concentration [52]. In another study, 100 mg/kg of vitamin E could partially restore GSH concentration in liver cells among rats treated with gentamicin [53]. The possible underlying mechanism is that vitamin E has a direct free radical scavenger action by donating its phenolic hydrogen atom to lipid peroxyl radical forming lipid hydroperoxide and α-tocopheroxyl radical [54]. The latter reacts with lipid peroxyl radical, lipid alkoxyl radical, or carbon-centered lipid radical forming inactive nonradical product, or reacts with another α-tocopheroxyl radical forming nonradical dimer [54]. On the other hand, vitamin E was reported to upregulate, but not completely restore, catalase and SOD gene expression in mice liver cells that were treated with an oxidative stress inducer [55].

Conclusion. Gentamicin downregulates gene expression of catalase, SOD, and GPx enzymes, supplying an explanation of gentamicin-induced testicular oxidative stress in rat testicular tissue. Adding α-lipoic acid or vitamin E to gentamicin treatment provides significant protection against oxidative stress that could be applied in human studies.

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Conflict of interests

The authors have no conflict of interest to declare.

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