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DRUG BIOCHEMISTRY

GENTAMICIN INDUCED LIPID PEROXIDATION AND ITS CONTROL WITH ASCORBIC ACID

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Abstract: Lipid peroxidation is the oxidative deterioration of polyunsaturated fatty acids (PUFAs), which is a free radical related process. Studies showed that reactive oxygen species (ROS) are involved in a diversity of biological phenomena including atherosclerosis, neurodegenerative diseases, carcinogenesis etc. ROS and other pro-oxidant agents have the capacity to exhibit oxidative decomposition of PUFAs of membrane phospholipids leading to the formation of toxic end products, including malonyldialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) etc. Drug-induced lipid peroxidation contributes to significant toxicity. Antioxidants have the ability to reduce generation of free radicals in the body by inhibiting lipid peroxidation process. In view of this concept, the present *in vivo* study is carried out to evaluate the inhibitory effect of ascorbic acid on lipid peroxidation induced by gentamicin, an aminoglycoside antibiotic. Levels of MDA, HNE, reduced glutathione (GSH) and nitric oxide (NO) are used as markers of lipid peroxidation. Rabbits divided into different experimental groups are treated with drug and co-administered with ascorbic acid. The levels of MDA, HNE, GSH and NO in the blood are estimated and compared with the control. The results revealed that gentamicin significantly increases MDA and HNE contents, but decreases GSH and NO levels; and ascorbic acid on co-administration has capability to inhibit drug-induced lipid peroxidation.

Keywords: lipid peroxidation, drug toxicity, gentamicin, ascorbic acid

Polyunsaturated fatty acids (PUFAs), important components of biomembranes, are susceptible to lipid peroxidation, which is a free radical related process and occurs in the biological system under enzymatic control or nonenzymatically (1-3). Lipid peroxidation is a measure of the membrane damage. It is a highly destructive process that induces a wide variety of alterations in the structure and function of cellular membranes (4) and is an important cause of certain diseases or disorders including aging (5), atherosclerosis (6), diabetes mellitus, gastric ulcer, neurodegenerative diseases (7) and many more. Drug-induced lipid peroxidation contributes to significant toxicity as exemplified by doxorubicin induced cardiotoxicity (8) and indomethacin induced gastric mucosal damage (9).

Lipid peroxidation could be prevented by reducing the formation of free radicals by (i) destroying the free radicals that are already formed, (ii) supplying a competitive substrate for unsaturated lipids in the membrane, and (iii) accelerating the repair mechanism of damaged cell membrane. Many natural (10) and synthetic antioxidants are in use to prevent the lipid peroxidation (11). In cases of reduced or impaired *in vivo* antioxidant defense and excess generation of free radicals that are not counterbalanced by endogenous defense mechanism, exogenously administered antioxidants may be helpful to overcome the oxidative damage caused by free radicals.

Gentamicin is an aminoglycoside antibiotic, which has wide utility in many bacterial infections. It has a broad spectrum of activity against some common pathogens, both Gram-positive and Gram-negative. It has strong activity against *P. aeruginosa* and other Gram-negative enteric bacilli (12). Though it is an important antibiotic yet it displays remarkable toxic potentials, which restrict its use in many cases. Most remarkable toxicities of gentamicin include ototoxicity and nephrotoxicity, are reported to be free radical associated and due to enhanced lipid peroxidation (13-18). Ascorbic acid, an antioxidant vitamin, has free radical scavenging property (19-22). It has also been reported to have

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protective role against cadmium-induced thyroid dysfunction due to its antioxidant action (23). Use of antioxidants as adjuvant of toxic drugs may become a promising approach for reducing iatrogenic disorders.

In our ongoing process of exploring drug-lipid interactions and drug-induced lipid peroxidation, we present here our current *in vivo* observations on gentamicin induced lipid peroxidation and its control with ascorbic acid.

EXPERIMENTAL

The study was conducted after obtaining institutional animal ethical committee clearance. The study was performed on New Zealand white rabbits (*Oryctolagus cuniculus*). Some common laboratory markers of lipid peroxidation including malonyldialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), reduced glutathione (GSH), and the nitric oxide (NO) content of blood were used in the present study. Rabbits were selected because the general physiology of rabbit is similar to human, and therefore, the rabbit has been used as a model for experimental studies with some frequency. The rabbit is large enough to provide adequate quantities of tissue for experimental work without pooling of samples but is small enough to be economical for most studies.

Collection of blood from animals

Animals were kept in 18 h fasting condition and divided into different experimental groups: control (C), drug treated (D), drug co-administered with antioxidant (DA), and only antioxidant treated (A). Gentamicin was administered intramuscularly at a dose of 40 mg/kg body weight (24) to animal groups marked as D and DA. Ascorbic acid was administered via the same route at a dose of 40 mg/kg body weight (25) to animal group marked as DA and A. After 3 h and 24 h of drug and/or antioxidant administration, blood was collected from marginal ear vein of animals in centrifuge tubes marked accordingly. Then equal volume of trichloroacetic acid (10% w/v) was added to each centrifuge tube and mixed well using glass rods. Then it was centrifuged at 4000 × g for 30 min and then filtered. The protein free supernatant was used for estimation of different lipid peroxidation parameters.

Estimation of malonyldialdehyde (MDA) level in blood (26)

Two mL of filtrate from each sample was transferred to stoppered glass tubes. Two mL of thiobarbituric acid (TBA) reagent was added to each tube and heated in a boiling water bath for 30 min. Then it was cooled to room temperature and the absorbance was measured at 530 nm against a blank prepared using 2 mL of distilled water and 2 mL of TBA reagent and heated similarly. The concentration of MDA present in the sample was estimated from the standard curve prepared using tetraethoxypropane (TEP) and TBA following the method of Tarladgis et al. (26).

Preparation of standard curve: Different aliquots from standard TEP solution were taken in 10 mL graduated stoppered glass tubes. Two mL of TBA reagent was added to each tube. Volume of each solution was made up to 4 mL with distilled water and the mixture was heated in steam bath for 30 min. The solutions were cooled to room temperature and the absorbance was noted at 530 nm against a blank containing 2 mL of reagent and 2 mL of distilled water. The best-fit equation was

$$\text{MDA content (nM/mL)} = (A_{530} - 4.92 \times 10^{-4}) / 5.84 \times 10^{-3}$$

where A_{530} = absorbance at 530 nm, r = 0.994, SEE = 0.005

Estimation of 4-hydroxy-2-nonenal (HNE) level in blood (27)

One mL of filtrate from each sample were taken in glass tubes marked accordingly. One mL of 2,4-dinitrophenylhydrazine (DNPH) solution (100 mg% in 0.5 M HCl) was added to each tube and the tubes were kept at room temperature for 1 h. Then the samples were extracted with hexane, which was evaporated at 40°C. After cooling, 2 mL of methanol were added to each sample and the absorbance was measured at 350 nm against methanol as a blank. The concentration was estimated from the standard curve.

Preparation of standard curve: HNE (1.0 mg or 6.4 μM) was taken in 6.4 mL of methanol to give 1 mM solution. The solution was standardized by diluting 100 μL to 5 mL with methanol and measuring the absorbance at 220 nm using methanol as a blank. The concentration of HNE in the primary standard was calculated considering molar extinction coefficient of HNE at 220 nm to be 13750. A series of dilutions of HNE in phosphate buffer solution were prepared from which 2 mL samples were pipetted, to which 1 mL of DNPH reagent was added and the mixture was maintained for 1 h. Then it was extracted with 2 mL aliquots of hexane, the extract was evaporated to dryness at 40°C and reconstituted in 1 mL of methanol. The absorbance

Table 1. Gentamicin-induced MDA content increase and its reduction with ascorbic acid

Animal Set	Time (h) after drug / drug-antioxidant / antioxidant administration					
	3			24		
	D	DA	A	D	DA	A
1	22.52 (t 14.40)	11.18 (t 9.27)	-12.92 (t 5.19)	9.07 (t 3.91)	4.68 (t 3.49)	-9.52 (t 5.74)
2	24.98 (t 20.99)	8.87 (t 11.15)	-7.41 (t 4.35)	12.11 (t 7.65)	6.46 (t 4.89)	-3.04 (t 3.81)
3	24.18 (t 18.54)	11.60 (t 8.47)	-6.31 (t 4.82)	17.11 (t 14.92)	5.91 (t 3.79)	-3.56 (t 3.67)
4	20.64 (t 9.43)	7.64 (t 9.27)	-8.94 (t 6.08)	11.02 (t 14.22)	3.53 (t 4.47)	-6.60 (t 18.50)
5	25.48 (t 40.89)	10.99 (t 6.56)	-8.17 (t 7.42)	13.66 (t 24.18)	5.35 (t 9.43)	-4.66 (t 3.20)
Average ± S.E.	23.56 ± 1.98	10.06 ± 1.71	-8.75 ± 2.52	12.59 ± 3.02	5.19 ± 1.13	-5.48 ± 2.64

D, DA and A denote animals treated with gentamicin, gentamicin-ascorbic acid and ascorbic acid, respectively. Percent changes in MDA content with respect to control, along with 't' values are shown. All percent change values are significant at p = 0.05 (n = 5). S.E. = standard error (n = 5).

Table 2. Gentamicin-induced HNE content increase and its reduction with ascorbic acid

Animal Set	Time (h) after drug / drug-antioxidant / antioxidant administration					
	3			24		
	D	DA	A	D	DA	A
1	21.99 (t 63.52)	16.64 (t 25.53)	-10.03 (t 14.30)	15.09 (t 24.62)	7.28 (t 24.33)	-4.29 (t 7.56)
2	16.06 (t 28.67)	9.01 (t 15.77)	-4.94 (t 24.43)	9.54 (t 16.14)	6.26 (t 7.59)	-2.77 (t 9.03)
3	15.81 (t 67.16)	8.42 (t 23.98)	-7.21 (t 14.83)	9.73 (t 17.96)	4.31 (t 14.68)	-2.86 (t 8.23)
4	16.42 (t 31.01)	8.68 (t 33.84)	-8.94 (t 13.19)	11.02 (t 15.36)	3.53 (t 5.53)	-6.60 (t 7.22)
5	21.47 (t 65.28)	12.52 (t 18.28)	-6.30 (t 7.76)	8.07 (t 19.04)	4.77 (t 10.62)	-2.69 (t 7.71)
Average ± S.E.	18.35 ± 3.09	11.05 ± 3.53	-7.36 ± 1.93	10.14 ± 2.87	5.27 ± 1.46	-3.24 ± 0.69

D, DA and A denote animals treated with gentamicin, gentamicin-ascorbic acid and ascorbic acid, respectively. Percent changes in MDA content with respect to control, along with 't' values are shown. All percent change values are significant at p = 0.05 (n = 5). S.E. = standard error (n = 5).

was measured at 350 nm using 0 μM standard as the blank (27). The best-fit equation was

$$\text{HNE content (nM/mL)} = (A_{350} - 5.60 \times 10^{-3}) / 3.26 \times 10^{-3}$$

where A_{350} = absorbance at 350 nm, r = 0.999, SEE = 0.007.

Estimation of reduced glutathione (GSH) level in blood (28)

Reduced glutathione was measured by its reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to give a compound that absorbs at 412 nm (Ellman's method) (28). One mL of the filtrate from each sample (obtained as above) was taken in corresponding glass tubes. Three mL of DTNB solution (0.01% in phosphate buffer 0.1 M, pH 8) was added to each tube and then was diluted with 4 mL of phosphate buffer 0.1 M, pH 8. The absorbance of each solution was determined at 412 nm against a blank containing 5 mL of phosphate buffer and 3 mL of DTNB solution. Concentration of GSH present in the samples was estimated from a standard curve.

Table 3. Gentamicin-induced GSH content decrease and its suppression with ascorbic acid

Animal Set	Time (h) after drug / drug-antioxidant / antioxidant administration					
	3			24		
	D	DA	A	D	DA	A
1	12.97 (t 31.47)	-7.25 (t 44.00)	8.72 (t 25.09)	-9.02 (t 26.00)	-4.09 (t 9.33)	3.92 (t 12.61)
2	-14.58 (t 74.22)	-7.72 (t 70.07)	4.30 (t 15.65)	-5.14 (t 9.70)	-2.55 (t 8.14)	2.25 (t 15.38)
3	-14.18 (t 53.11)	-7.48 (t 21.06)	3.63 (t 9.22)	-7.35 (t 33.00)	-4.86 (t 12.95)	1.58 (t 6.18)
4	-13.00 (t 54.42)	-6.83 (t 20.77)	5.28 (t 11.66)	-6.13 (t 17.55)	-3.24 (t 8.79)	1.63 (t 7.67)
5	-14.13 (t 64.92)	-6.73 (t 58.03)	4.83 (t 21.99)	-9.47 (t 27.78)	-3.87 (t 8.19)	2.02 (t 6.05)
Average ± S.E.	-13.77 ± 0.73	-7.20 ± 0.41	5.35 ± 1.98	-7.42 ± 1.84	-3.72 ± 0.87	2.28 ± 0.95

D, DA and A denote animals treated with gentamicin, gentamicin-ascorbic acid and ascorbic acid, respectively. Percent changes in MDA content with respect to control, along with 't' values are shown. All percent change values are significant at p = 0.05 (n = 5). S.E. = standard error (n = 5).

Table 4. Gentamicin-induced NO content decrease and its suppression with ascorbic acid

Animal Set	Time (h) after drug / drug-antioxidant / antioxidant administration					
	3			24		
	D	DA	A	D	DA	A
1	-34.707 (t 8.91)	-18.24 (t 2.98)	24.91 (t 7.02)	-21.80 (t 5.28)	-8.89 (t 4.21)	15.12 (t 7.90)
2	-34.15 (t 59.00)	-18.52 (t 9.43)	33.57 (t 6.23)	-20.84 (t 10.85)	-9.84 (t 6.66)	16.20 (t 3.72)
3	-40.94 (t 16.28)	-17.06 (t 6.90)	19.33 (t 10.00)	-20.85 (t 10.48)	-10.61 (t 14.00)	6.44 (t 2.72)
4	-32.27 (t 12.73)	-16.34 (t 14.60)	20.02 (t 5.00)	-18.79 (t 10.03)	-7.35 (t 7.06)	10.62 (t 4.06)
5	-29.69 (t 43.29)	-13.93 (7.05)	23.83 (t 15.53)	-14.66 (t 11.31)	-5.49 (t 9.48)	9.16 (t 3.29)
Average ± S.E.	-34.35 ± 4.17	-16.82 ± 1.84	24.33 ± 5.69	-19.39 ± 2.86	-8.44 ± 2.04	11.51 ± 4.09

D, DA and A denote animals treated with gentamicin, gentamicin-ascorbic acid and ascorbic acid, respectively. Percent changes in MDA content with respect to control, along with 't' values are shown. All percent change values are significant at p = 0.05 (n = 5). S.E. = standard error (n = 5).

$$\text{GSH content (nM/mL)} = (A_{412} - 8.55 \times 10^{-6}) / 6.82 \times 10^{-3}$$

where A_{412} = absorbance at 412 nm, r = 0.999, SEE = 0.003.

Estimation of nitric oxide (NO) level in blood (29)

The NO content was determined by reaction with Griess reagent [1:1 sulfanilamide (1% w/v in 3 M HCl) and 0.1% w/v N-(1-naphthyl)ethylenediamine dihydrochloride]. One mL of the filtrate from each sample was transferred to corresponding glass tubes. The pH of the mixture was adjusted to 6.7 with Na_2HPO_4. Subsequently, 3 mL of Griess reagent was added. After 10 min, the absorbance of the solutions was measured at 540 nm against a blank prepared using 1 mL of distilled water and 3 mL of Griess reagent. The concentrations were estimated from a standard curve.

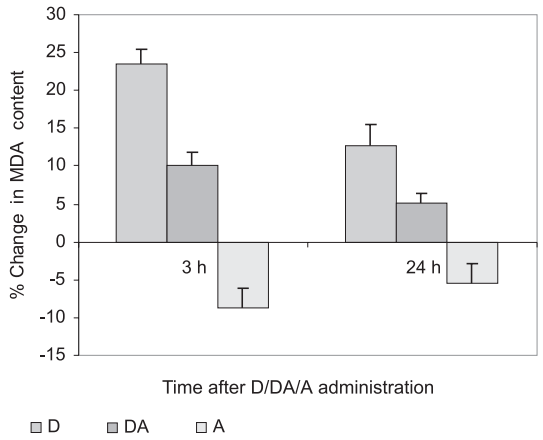

Figure 1. Gentamicin-induced MDA content increase and its reduction with ascorbic acid

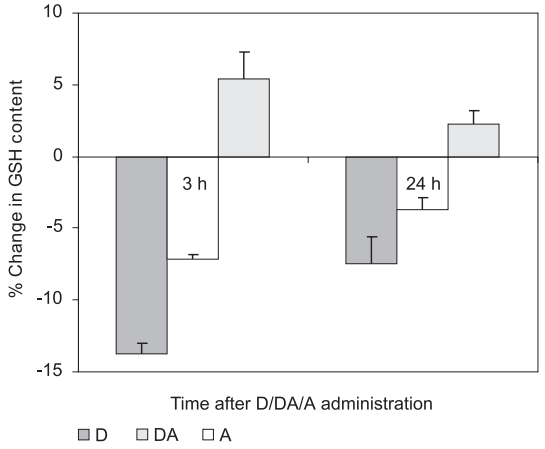

Figure 3. Gentamicin-induced GSH content decrease and its suppression with ascorbic acid

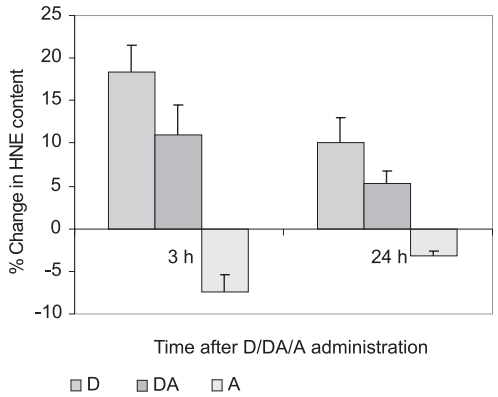

Figure 2. Gentamicin-induced HNE content increase and its reduction with ascorbic acid

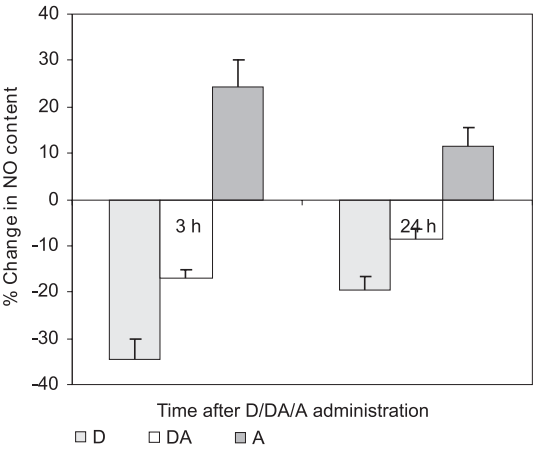

Figure 4. Gentamicin-induced NO content decrease and its suppression with ascorbic acid

Preparation of standard curve: Different aliquots from standard sodium nitrite solution were taken in graduated glass tubes. To each solution 3 mL of Griess reagent was added, and the volume was adjusted up to 4 mL with distilled water. The absorbance of each solution was determined at 540 nm against a blank containing 1 mL of distilled water and 3 mL of Griess reagent. The best-fit equation was

$$\text{NO content (nM/mL)} = (A_{540} - 4.52 \times 10^{-4}) / 5.30 \times 10^{-2}$$

where A_{540} = absorbance at 540 nm, r = 0.998, SEE = 0.002.

The percent changes in MDA, GSH, HNE, and NO level of different samples at different hours of interval were calculated with respect to the control, and the changes in MDA, GSH, HNE, and NO level were considered as an indicator of the extent of lipid peroxidation.

RESULTS AND DISCUSSION

The results of the study are shown in Tables 1 – 4, and further illustrated in Figures 1 – 4. The results were validated by the Student's *t*-test and found statistically significant. From Tables 1 and 2, it was suggested that gentamicin has increased the MDA and HNE contents of the blood of animals treated with the drug to a significant extent. In case of combinational therapy with both drug and antiox-

idant, the MDA and HNE contents were lower with respect to drug treated group. The treatment with only ascorbic acid resulted in the levels of MDA and HNE lower than those of control values. The results suggest that gentamicin could induce lipid peroxidation significantly, which might be a cause of its toxicity. The results also indicate that ascorbic acid could suppress the gentamicin induced lipid peroxidation to a significant extent. Tables 3 and 4 reveal that gentamicin decreases the GSH and NO level of the blood to a significant extent. A decrease in GSH and NO content was associated with an increase in lipid peroxidation. GSH plays a very important role in the defense mechanism of tissues against the reactive oxygen species (30). NO can protect cells against the detrimental effects of reactive oxygen species, and it has been demonstrated that NO can serve as a chain-breaking antioxidant in cell membranes (31). When the animals were treated with both drug and antioxidant, the GSH and NO levels were increased in comparison with the drug-treated group of the corresponding time period. Again, when the animals were treated with ascorbic acid alone, the GSH and NO contents were increased in comparison with the control samples. This increased GSH and NO levels indicate the antiperoxidative potential of ascorbic acid.

Both Tables and Figures show similar types of changes in both 3 and 24 h time period, but the changes were more prominent in the former. This difference might be due to significant elimination of the drug and antioxidant from the body within 24 h.

The results of the experiment revealed that the drug, gentamicin has lipid peroxidation induction potential. The clinical studies revealed that gentamicin has its own toxicities (13-16), including ototoxicity, nephrotoxicity and genotoxicity, which might be correlated with its lipid peroxidation inducing capacity. Further, while ascorbic acid was co-administered, the lipid peroxidation induction potential of the drug was reduced significantly. Thus, ascorbic acid co-administration might be beneficial to reduce gentamicin–induced toxicity, but a detailed study using more parameters may be required to draw any definite conclusion.

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