PROTECTIVE EFFECT OF ASCORBIC ACID ON NETILMICIN-INDUCED LIPID PROFILE AND PEROXIDATION PARAMETERS IN RABBIT BLOOD PLASMA

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Abstract: A drug may cause alteration in blood-lipid profile and induce lipid peroxidation phenomena on administration in the body. Antioxidant may play beneficial role to control the negative alteration in lipid profile and lipid peroxidation. In view of this context, the present in vivo study was carried out to evaluate the role of ascorbic acid as antioxidant on netilmicin-induced alteration of blood lipid profile and peroxidation parameters. Rabbits were used as experimental animals and blood was collected to estimate blood-lipid profiles, such as total cholesterol (TCh), high density lipoprotein cholesterol (HDL-Ch), low density lipoprotein cholestrol (LDL-Ch), very low density lipoprotein cholesterol (VLDL-Ch), triglycerides (Tg), phospholipids (PL), and total lipids (TL), as well as peroxidation parameters, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), reduced glutathione (GSH) and nitric oxide (NO). The results revealed that netilmicin caused significant enhancement of MDA, HNE, TCh, LDL-Ch, VLDL-Ch, Tg levels and reduction in GSH, NO, HDL-Ch, PL, TL levels. On co-administration, ascorbic acid was found to be effective in reducing netilmicin-induced negative alterations of the above parameters.

Keywords: lipid profile, lipid peroxidation, netilmicin, ascorbic acid

On administration, a drug passes through many barriers, most of them are lipoidal in nature. During its passage through biomembranes, the drug may interact with membrane lipids that lead to alteration in lipid pattern and composition, as well as lipid peroxidation which is a measure of the membrane damage. Lipid peroxidation is a highly destructive process that induces a wide variety of alterations in the structure and function of cellular membranes (1), and an important cause of certain diseases or disorders like atherosclerosis (2), diabetes mellitus, gastric ulcer, neurodegenerative diseases (3), aging (4) etc. Indomethacin induced gastric mucosal damage (5) and doxorubicin-induced cardiomyopathy (6) are the consequences of drug-induced lipid peroxidation and reactive oxygen species (ROS) generation.

Drug-induced alteration of lipid pattern is measured by estimating blood-lipid profile. A lipid profile is a measure of three components: cholesterol (Ch), triglycerides (Tg), and lipoproteins (LP) (high and low density). Total cholesterol (TCh) comprises all the cholesterol found in various lipoproteins such as high density LP (HDL), low density LP (LDL) and very low density LP (VLDL). High density lipoprotein cholesterol (HDL-Ch) is believed to play a key role in the process of reverse cholesterol transport that promotes the efflux of excess cholesterol from vessel wall to the liver for excretion (7, 8). On the contrary, low density lipoprotein cholesterol (LDL-Ch) contains the high percentage of cholesterol and is responsible for cholesterol deposit on the wall of the artery, resulting in heart disease – atherosclerosis. Triglycerides (Tg) are neutral fats found in the tissue and blood. Adipose tissue is the major reservoir of calories and consists of Tg. Very low density lipoprotein cholesterol (VLDL-Ch) is a large group of macromolecules synthesized and secreted mainly by liver and intestinal mucosal cells, and contain large quantities of Tg (9). Tg may also contribute to the disorders related to coronary heart disease (CHD) (10). Phospholipids (PL) are the major component of all biological membranes and blood. An evidence suggests that oxidized PL (a major lipid of LDL-Ch) is formed in atherogenesis and plays an
important role in the oxidative modification during LDL-Ch oxidation (11). An elevated lipid profile is associated with many diseases. Diabetic patients have increased level of TCh, Tg, LDL-Ch, VLDL-Ch and decreased level of HDL-Ch (12). CHD is also associated with an elevated level of serum TCh, Tg, LDL-Ch and decreased HDL-Ch (13). Serum TCH, HDL-Ch and LDL-Ch are found to be inversely associated with incidence of cancer like leukemia and Hodgkin’s disease, but Tg is found to be significantly elevated in patients (14). In AIDS patients, a disease progression is accompanied by a decrease in TCh, HDL-Ch and LDL-Ch, and an increase in Tg and VLDL-Ch levels (15). Patients with chronic kidney disease (CKD) are at an increased risk for cardiovascular disease and have a higher prevalence of hyperlipidemia (16).

Netilmicin, an aminoglycoside antibiotic, is a semisynthetic derivative of sisomicin and has a broader spectrum of activity than gentamicin (17). It is relatively resistant to aminoglycoside inactivating enzymes and thus effective against many gentamicin resistant strains. It is more active against Klebsiella, Enterobacter and Staphylococci, but less active against Ps. aeruginosa (17). In spite of its utility, it possesses certain adverse reactions including oto-toxicity and nephrotoxicity that limits its use. Some of these toxicities are reported to be free radical associated and due to drug-induced lipid peroxidation (18–20). In the present study, an attempt has been made to explore the effect of ascorbic acid on netilmicin-induced alteration in blood-lipid profile and peroxidation parameters. Ascorbic acid, a promising antioxidant, has free radical scavenging capacity (21–24). Use of antioxidants as adjuvants of drugs may become a promising approach in reducing drug-induced abnormalities. A change in blood-lipid profile which may occur on drug administration and due to drug-induced lipid peroxidation is also regulated by ascorbic acid (25–29).

EXPERIMENTAL

Estimation of drug-induced lipid peroxidation was carried out using common laboratory markers, like malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), reduced glutathione (GSH) and nitric oxide (NO) levels, and lipid profile like TCh, HDL-Ch, Tg, LDL-Ch, VLDL-Ch, PL and TL. New Zealand White rabbit (Oryctolagus cuniculus) was used as animal model. Institutional Animal Ethical Committee approved the design of study protocol.

Collection of blood

Animals, kept in 18 h fasting condition were divided into different experimental groups: control (C), drug treated (D), drug co-administered with

<table>
<thead>
<tr>
<th>Equation No.</th>
<th>Parameter</th>
<th>Equation</th>
<th>Statistics</th>
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<tbody>
<tr>
<td>1</td>
<td>MDA</td>
<td>MDA (nM/mL) = (A_{530} - 4.92 \times 10^{-4})/5.84 \times 10^{-3}</td>
<td>r = 0.994, se = 0.005, F = 1190.43 (df 1, 13), n = 15</td>
</tr>
<tr>
<td>2</td>
<td>HNE</td>
<td>HNE (nM/mL) = (A_{350} - 5.60 \times 10^{-3})/3.26 \times 10^{-3}</td>
<td>r = 0.999, se = 0.007, F = 1656.01 (df 1, 2), n = 4</td>
</tr>
<tr>
<td>3</td>
<td>GSH</td>
<td>GSH (nM/mL) = (A_{412} - 8.55 \times 10^{-6})/6.82 \times 10^{3}</td>
<td>r = 0.999, se = 0.003, F = 53157.77 (df 1, 24), n = 26</td>
</tr>
<tr>
<td>4</td>
<td>NO</td>
<td>NO (nM/mL) = (A_{540} - 4.52 \times 10^{4})/5.30 \times 10^{3}</td>
<td>r = 0.998, se = 0.002, F = 4403.86 (df 1, 13), n = 15</td>
</tr>
<tr>
<td>5</td>
<td>TCh</td>
<td>TCh (mg/100 mL) = (A_{505} - 6.36 \times 10^{-3})/1.532 \times 10^{3}</td>
<td>r = 0.999, se = 0.004, F = 3788.81 (df 1, 5), n = 7</td>
</tr>
<tr>
<td>6</td>
<td>HDL-Ch</td>
<td>HDL-Ch (mg/100 mL) = A_{520}/4.574 \times 10^{3}</td>
<td>r = 0.999, se = 0.002, F = 19215.95 (df 1, 8), n = 9</td>
</tr>
<tr>
<td>7</td>
<td>Tg</td>
<td>Tg (mg/100 mL) = A_{505}/1.068 \times 10^{4}</td>
<td>r = 0.999, se = 0.004, F = 6572.48 (df 1, 6), n = 7</td>
</tr>
<tr>
<td>8</td>
<td>PL</td>
<td>PL (mg/100 mL) = A_{505}/1.503 \times 10^{4}</td>
<td>r = 0.999, se = 0.004, F = 25165.12 (df 1, 6), n = 7</td>
</tr>
<tr>
<td>9</td>
<td>TL</td>
<td>TL (mg/100 mL) = (A_{505} - 4.311 \times 10^{-3})/3.87 \times 10^{4}</td>
<td>r = 0.998, se = 0.005, F = 3116.71 (df 1, 7), n = 9</td>
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A = absorbance at \lambda_{max} in subscript; r = correlation coefficient; se = standard error; F = variance ratio; df = degrees of freedom; n = no. of standard sample
antioxidant (DA) and only antioxidant treated (A). The drug, netilmicin, was administered intramuscularly at a dose of 6 mg/kg b. w. (30) to animal groups marked as D and DA. The antioxidant, ascorbic acid was administered at a dose of 40 mg/kg b. w. (31) to animal groups marked as DA and A. After 3 and 24 h of drug and/or antioxidant administration, blood was collected from marginal ear vein of animals. The blood samples were subjected to test for determination of drug-induced lipid peroxidation and its effect on lipid profiles.

**Determination of lipid peroxidation**

Drug-induced lipid peroxidation was measured by estimating level of MDA, HNE, GSH and NO. The blood-protein was precipitated by adding trichloroacetic acid (10% w/v), centrifuged at 4000 ◊ g for 30 min, and filtered. The filtrate sample was used for estimation of lipid peroxidation parameters as follows:

**Estimation of malondialdehyde (MDA) (32)**

The filtrate was added to equal volume of thio-barbituric acid (TBA) reagent and heated in a boiling water bath for 30 min. The absorbance of the colored sample was measured at 530 nm against a blank. The concentration of MDA present in the sample was estimated from the best fit equation (eq. 1 in Table 1) of standard curve prepared using tetraethoxypropane (TEP) and TBA.

**Estimation of 4-hydroxy-2-nonenal (HNE) (33)**

Equal volume of filtrate was mixed with 2,4-dinitrophenylhydrazine (DNPH) solution (100 mg % in 0.5 M HCl) and incubated at room temperature for 1 h. The mixture was extracted with hexane followed by addition of methanol. The absorbance of the methanol sample was measured at 350 nm. The concentration was estimated from the standard curve (eq. 2 in Table 1).

**Estimation of reduced glutathione (GSH) (34)**

GSH was measured by reacting with 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB) to give a color complex of absorbance at 412 nm (λmax) (Ellman’s method) (34). The protein free sample was mixed with DTNB (1:3) solution (0.01% in phosphate buffer 0.1 M, pH 8) and absorbance of the solution was measured at 412 nm against a blank. Concentration of GSH present in the blood samples was estimated from a standard curve (eq. 3 in Table 1).

**Estimation of nitric oxide (NO) (35)**

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]. The pH of the mixture was adjusted to 6.7 with Na2HPO4 and the absorbance of the solution was measured at 540 nm. The concentration of NO was estimated from the standard curve (eq. 4 in Table 1).

The percent changes in peroxidation parameters, MDA, GSH, HNE, and NO levels of different samples at different hours of interval were calculated with respect to the control.

**Determination of lipid profiles**

Drug-induced changes in lipid profile were measured by estimating the level of TCh, HDL-Ch, LDL-Ch, VLDL-Ch, Tg, PL and TL in the blood serum. The commercially available enzyme kits used for estimation of lipid profiles were obtained from Span Diagnostics Ltd., Surat, India and Labkit, Barcelona, Spain.

**Estimation of total cholesterol (TCh)**

Estimation of total cholesterol was performed by cholesterol oxidase (CHOD) – peroxidase aminoantipyrine phenol (PAP) method (36–39). Ten µL of serum was mixed with 1 mL of cholesterol reagent, containing Good’s buffer pH 6.7, cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine and stabilizers. The mixture was incubated at 37°C for 10 min. The absorbance was measured at 505 nm against cholesterol reagent as a blank. The concentration of TCh was calculated from a standard curve (eq. 5 in Table 1) prepared using cholesterol standard samples.

**Estimation of HDL cholesterol (HDL-Ch)**

HDL cholesterol was also estimated by CHOD – PAP method (36–39). 200 µL of clear serum was mixed with 200 µL of precipitating reagent containing PEG 6000 (200 mM/L), stabilizer and preservative. The mixture was kept at room temperature for 10 min and centrifuged for 15 min at 2000 rpm and the clear supernatant was separated. Hundred µL of supernatant was mixed with 1 mL of cholesterol reagent and incubated at 37°C for 10 min. The absorbance was measured at 505 nm. The concentration of HDL-Ch was calculated from a standard curve (eq. 6 in Table 1) prepared using HDL-Ch standard samples.

**Estimation of triglycerides (Tg) (36, 37, 40)**

Ten µL of serum was mixed with 1 mL of triglyceride mono reagent containing pipes buffer, 4-chlorophenol, magnesium, ATP, lipase, peroxidase, glycerolkinase, 4-aminoantipyrine, glycero-
phosphate oxidase, detergents, preservative and stabilizer. The mixture was incubated at 37°C for 10 min. The absorbance of the colored solution was measured at a wavelength of 505 nm. The concentration of Tg was calculated from a standard curve (eq. 7 in Table 1) prepared using triglyceride standard samples.

**Estimation of LDL-Ch and VLDL-Ch**

Concentrations of LDL-Ch and VLDL-Ch in the samples were calculated using Friedewald’s equations (41):

\[
\text{LDL-Ch content} = \text{TCh content} - \left(\frac{\text{Tg content}}{5}\right) - \text{HDL-Ch content}
\]

\[
\text{VLDL-Ch content} = \frac{\text{Tg content}}{5}
\]

**Estimation of phospholipids (PL) (42)**

Ten µL of clear serum was mixed with 1 mL of working reagent containing TRIS buffer pH 7.55, dichlorophenol, phospholipase D, choline oxidase, peroxidase and 4-aminophenazone. The mixture was incubated for 5 min at 37°C and the absorbance of the solution was measured at a wavelength of 505 nm. The concentration of PL was calculated from a standard curve (eq. 8 in Table 1) prepared using phospholipid primary standards.

**Estimation of total lipids (TL) (43, 44)**

Hundred µL of serum was mixed with 2.5 mL of sulfuric acid and then heated for 10 min in a boiling water bath (100°C) and thereafter cooled in iced water.
Fifty µL of the acid digested sample was mixed with 1 mL of phosphovanilline reagent and incubated for 15 min at 37°C. The absorbance was measured at a wavelength of 520 nm. The concentration of TL was calculated from a standard curve (eq. 9 in Table 1) prepared using total lipid primary standards.

The percent changes in lipid profile, TCh, HDL-Ch, LDL-Ch, VLDL-Ch, Tg, PL and TL levels of different samples at time interval were calculated with respect to the control.

RESULTS AND DISCUSSION

Results of the study are listed in Tables 2 and 3 and are illustrated in Figures 1, 2. Interpretation of the results is supported by Student’s t-test and the results are statistically significant. The observation indicated that netilmicin has significant lipid peroxidation induction potential which might cause elevation of MDA and HNE levels, as these are considered as the end products of lipid peroxidation (45). But the elevated levels of these parameters are controlled by ascorbic acid co-administration. Further, the contents of GSH and NO, which are related to the antioxidant defense mechanism (46, 47), are declined with respect to control due to lipid peroxidation induction capacity of netilmicin. The antioxidant effect of ascorbic acid also caused enhancement of GSH and NO levels in animal groups treated with drug and antioxidant.
Table 2. Percent changes in peroxide contents with respect to control on netilmicin-induced lipid peroxidation and on co-administration with ascorbic acid.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% Change and concentration (nM/mL ± SE) at time interval</th>
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<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>MDA</td>
<td></td>
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<tr>
<td></td>
<td>27.04 ± 2.05</td>
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<tr>
<td>HNE</td>
<td></td>
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<td></td>
<td>(155.08 ± 4.28)</td>
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<tr>
<td>GSH</td>
<td></td>
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<td></td>
<td>(62.78 ± 6.94)</td>
</tr>
<tr>
<td>NO</td>
<td></td>
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<td></td>
<td>(0.61 ± 0.07)</td>
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</tbody>
</table>

C, D, DA and A denote control, netilmicin treated, netilmicin-ascorbic acid treated and only ascorbic acid treated animals, respectively. The changes are significant at p < 0.05 (n = 25). SE = standard error.

Table 3. Percent changes in profile contents with respect to control on netilmicin-induced lipid peroxidation and on co-administration with ascorbic acid.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% Change and concentration (mg / 100 mL ± se) at time interval</th>
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<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Tch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(34.46 ± 5.44)</td>
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<tr>
<td>HDL-Ch</td>
<td></td>
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<tr>
<td></td>
<td>(12.74 ± 2.03)</td>
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<tr>
<td>Tg</td>
<td></td>
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<tr>
<td></td>
<td>(59.57 ± 10.38)</td>
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<tr>
<td>LDL-Ch</td>
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<td></td>
<td>(9.81 ± 1.64)</td>
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<tr>
<td>VLDL-Ch</td>
<td></td>
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<tr>
<td></td>
<td>(11.91 ± 2.07)</td>
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<tr>
<td>PL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(55.37 ± 5.68)</td>
</tr>
<tr>
<td>TL</td>
<td></td>
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<tr>
<td></td>
<td>(191.13 ± 23.85)</td>
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</tbody>
</table>

C, D, DA and A denote control, netilmicin treated, netilmicin-ascorbic acid treated and only ascorbic acid treated animals, respectively. The changes are significant at p < 0.05 (n = 25). se = standard error.
Changes in lipid profile due to drug effect and its control by ascorbic acid are illustrated in Table 3 and Figure 2. The results indicated that netilmicin caused significant elevation of TCh, LDL-Ch, VLDL-Ch and Tg levels which further decreased on co-administration of ascorbic acid. Table 3 and Figure 2 also show a decreased level of HDL-Ch in D animals with respect to C group. The DA group showed increased level of HDL-Ch when compared to D group, while A group showed elevated level of HDL-Ch with respect to C group. Enhancement of TCh, LDL-Ch, VLDL-Ch, Tg and reduction in HDL-Ch may be related to drug-induced lipid profile alteration (48–50), though changes in lipoprotein level may occur due to drug-induced protein metabolism (51). The results further corroborated with lipid peroxidation phenomena that caused enhancement of TCh, Tg, LDL-Ch and VLDL-Ch levels and reduction in HDL-Ch level (26, 28). Studies also showed that the liposuppressive effect of the antioxidant: ascorbic acid plays a beneficial role over abnormal alteration in lipid profile as a result of increased lipid peroxidation phenomena (25–29). The results also demonstrated the reduction in PL and TL levels in all animal groups. In case of D group, the reduction in PL and TL might be due to binding of the netilmicin with lipids (52). These observations could be corroborated with the work of Saha et. al. (53, 54) that efficient binding of the drugs, desogestrel and norgestrel (oral contraceptives) to the PL and TL might cause significant reduction of those parameters, vis a vis increased level of peroxidation parameters.

The drug, netilmicin, is widely used as an antibiotic to treat many bacterial infections (17), has also potential lipid peroxidation capacity. The drug-induced alteration in lipid profile may be correlated with its lipid peroxidation inducing capacity. This negative phenomenon may be controlled on co-administration of ascorbic acid, having free radical scavenging property (21–24). Antioxidant co-therapy may be useful in reducing drug-induced adverse reactions, and it can be a useful tool for the physicians to enhance patient compliance and to increase therapeutic index of the drug. Any conclusion can only be drawn after a detailed study using more specific parameters.

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REFERENCES


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