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Studies on Protective Effects of Glutathione and α-Tocopherol on Norethindrone Induced Lipid Peroxidation

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Studies on possible antiperoxidative property of glutathione (GLU) and α-tocopherol (TOC) on lipid peroxidation (LP) induced by norethindrone (NE), an antifertility drug, were carried out using goat liver homogenate as the lipid source. The study was aimed at exploring possible potential of the antioxidants to reduce drug-induced toxicity that may be mediated through free radical mechanism. The degree of peroxidation was quantitated by thiobarbituric acid reactive substance (TBARS) content. The present study revealed that both GLU and TOC could significantly suppress NE-induced lipid peroxidation. Considering lipid peroxidation as a toxicity mediating process, the findings are interesting and provide a scope for further extensive studies on the antioxidants with an aim to increase therapeutic index of the drug.

Spontaneous oxidation of lipid molecules in membranes by oxygen at room temperature can be termed as lipid peroxidation[1]. Lipid peroxidation is a measure of membrane damage. It is a highly destructive process that induces a plethora of alterations in the structure and function of cellular membranes[2]. It is involved in a number of diseases and poisoning by several toxins[3]. The disrupted tissues are known to undergo lipid peroxidation faster than the healthy ones. Free radicals are capable of inducing lipid peroxidation in biological membranes[4]. The extent of lipid peroxidation is more in the presence of some metal ions. Lipid peroxidation-induced damage is involved in aging[5], atherosclerosis[6], neuronal ceroid lipofuscinosis[7], intermittent claudication[8], oxygen toxicity[9] and liver injury caused by orotic acid and ethanol[10-12]. The effects of free radicals on human beings have recently been considered to bear close relation to toxicity, diseases and aging[13,14].

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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[15] and synthetic antioxidants are in use to prevent the lipid peroxidation[16].

Lipid peroxidation, a molecular mechanism of cell injury, has been implicated as the underlying cause of toxicity of some drugs[17,18]. Recently, doxorubicin-induced cardiotoxicity has been reported to be mediated through free radical reaction sequence[19]. In view of toxicity of lipid peroxidation end products, drug induced lipid peroxidation may contribute significantly to the toxicity. In our ongoing exploratory studies on drug-induced lipid peroxidation[20-26], we have now included norethindrone, a newer 19-nortestosterone antifertility agent, as representative drug in view of its toxicity and general recommendation of wide spread use of oral contraceptives for combating population explosion.

Drug-induced lipid peroxidation has been focussed in an attempt to correlate toxic potential of the drug to its lipid peroxidation induction capacity. In cases of reduced or impaired *in vivo* antioxidant defense mechanism and excess generation of free radicals that are not counterbalanced by endogenous defense mechanism, exogenously administered antioxidant may be helpful to overcome the oxidative damage. Antioxidants therapy has been helpful in certain disease conditions, e.g., probucol in atherosclorosis, and ebselen in inflammation[17]. Antioxidants may also be helpful to contravene toxicity due to drugs or chemicals. Probucol provides protection against adriamycin-induced cardiomyopathy without interfering antitumor properties of the antibiotic[27]. Ascorbic acid has been reported to have protective role against cadmium-induced thyroid dysfunction due to its antioxidant action[28]. Use of antioxidants as adjuvants of toxic drugs may become a promising approach for reducing iatrogenic disorders. Thus, studies of the possible role of antioxidants in suppressing drug-induced lipid peroxidation might create a new scope for possible use of these agents in reducing drug-induced toxicity. In the ongoing search for antioxidants that may reduce drug-induced lipid peroxidation, presented here are studies on evaluation of glutathione and α-tocopherol as suppressors of norethindrone-induced lipid peroxidation. This has been done to explore possible potential of the antioxidants in reducing drug-induced toxicity on co-administration *in vivo.*

MATERIALS AND METHODS

Antifertility drug, norethindrone (NE) was provided by Bristol-Mayers Squibb Pharmaceutical Research Institute, USA. Thiobarbituric acid (TBA), glutathione (GLU), and α-tocopherol were purchased from Loba Chemie, Mumbai. Trichloroacetic acid (TCA) was procured from Merck, Mumbai and 1,1,3,3-tetraethoxy propane (TEP) was obtained from Sigma Chemical Co., USA.

Preparation of tissue homogenate:

Goat liver was collected in a sterile vessel containing phosphate buffer saline (pH 7.4) in cold condition. After collection of liver it was immediately excised and a homogenate (1 g/ml) was prepared using phosphate buffer saline (pH 7.4) in cold condition. It was centrifuged (2000 g) for 10 min, the supernatant was collected and finally suspended in phosphate buffer saline to contain approximately 0.8-1.5 mg portion in 0.1 ml of suspension to perform *in vitro* experiments.

Treatment and incubation of tissue homogenate with drug and/or antioxidant:

For each antioxidant, the tissue homogenate was divided into four parts of 50 ml each. The first portion was kept as the control (CL) while the second portion was treated with the drug (NE) at a concentration of 1.2 μg/g liver homogenate. The third portion was treated with drug and antioxidant. In case of GLU, the effective concentration was 0.04 mg/g and in case of TOC the same was 0.1 mg/g liver homogenate. The fourth portion was treated with only antioxidant (GLU/TOC). After treatment with drug and/or antioxidant, liver homogenates were stirred for 1 h below 20^0 and then incubated at 15^0 for 28 h along with the control sample. For each antioxidant, replicate determinations in five animal sets were done.

Estimation of lipid peroxidation breakdown products measured as TBARS:

The extent of lipid peroxidation was estimated in terms of malonaldehyde (MDA) content using thiobarbituric acid (TBA). The estimation was done at 3, 6, 24, and 28 h of incubation and additionally at 0 h (during addition of drug and/or antioxidant) for the control sample. In each case four samples of 2.5 ml of incubation mixture were transferred to tubes containing 2.5 ml of 10% trichloroacetic acid. Then the tubes were centrifuged at 3000 rpm for 30 min to precipitate the protein part. The TCA soluble fraction was fully separated and then a colour reaction was developed as described by Yagi *et al.*[29] with slight modifications.

In brief, to the tube containing TCA soluble fraction, 5 ml 0.002 M TBA solution was added and volume was made up to 10 ml with distilled water. The mixture was heated in boiling water bath for 30 min, and then tubes were cooled to room temperature and the absorbance was determined at 530 nm against a TBA blank (prepared from 5 ml TBA solution and 5 ml distilled water). The malonaldehyde content values were determined from the standard curve obtained by using 1,1,3,3-tetraethoxypropane (TEP).

RESULTS AND DISCUSSION

The MDA content of different treatment samples were compared to that of control of corresponding incubation period. Drug treated liver homogenate (NE) showed significant increase in the malonaldehyde content with

TABLE 1: EFFECTS OF GLUTATHIONE (GLU) AND α-TOCOPHEROL (TOC) ON NORETHINDRONE-INDUCED LIPID PEROXIDATON

Anti-oxidants	Hours of incubation	Percentage changesψ in MDA content Samples			Multiple Comparison Pooled variance* (Critical difference#)	Ranked means**
	D	DA	A			
	3	47.01 (±13.54)	76.07 (±18.83)	16.70 (±15.92)	509.28 31.11	(DA, D) (D,A)
	6	69.84 (±14.66)	29.82 (±15.48)	8.69 (±16.38)	259.26 22.19	(D) (DA, A)
GLU	24	73.63 (±17.31)	36.06 (±10.50)	50.29 (±56.66)	4172.14 89.04	(D) (DA) (A)
	28	59.01 (±14.89)	36.62 (±12.73)	11.98 (±16.37)	21.93 6.45	(D) (DA) (A)
	3	50.83 (±6.36)	18.01 (±7.38)	-1.8 (±7.78)	144.63 16.57	(D) (DA) (A)
	6	49.08 (±5.64)	22.37 (±6.88)	-0.89 (±3.66)	39.78 8.69	(D) (DA) (A)
TOC	24	41.58 (±7.80)	14.65 (±9.00)	0.38 (±4.38)	86.44 12.81	(D) (DA) (A)
	28	33.91 (±9.37)	16.44 (±10.49)	-4.99 (±2.09)	146.23 16.67	(D) (DA, A)

Ψ-Average per cent changes (with respect to controls of the corresponding hours) ± standard error (No. of animal sets = 5) values (degree(s) of freedom-df = 4) are shown. D, DA, and A indicate drug-treated, drug and antioxidant-treated, and antioxidant-treated samples, respectively. * Represents error mean square (df = 12). # Indicates critical difference (p=0.05) according to least significant difference procedure[30]. ** Denotes two means not included within same parentheses are statistically significantly different at $p < 0.05$.

respect to control Table 1. The increase in malonaldehyde (which is a lipid peroxidation breakdown product) content due to presence of drug (NE) is possibly due to enhanced lipid peroxidation. Lipid peroxidation, being a cell injury mechanism generating reactive oxygen species (ROS), which play important role in different disease conditions may be related to the toxic effects of the drug.

Effects of the antioxidants, GLU and TOC, on NE induced lipid peroxidation have been presented in Table 1. In case of liver homogenate treated with both drug and antioxidant and only antioxidant, different effects were observed depending on the antioxidant used. In case of liver homogenate treated with the drug NE and antioxidant GLU, the increase in MDA content was significantly reduced than that of drug treated liver homogenate except in 3 h Table 1. In case of antioxidant TOC, the increase in MDA content due to NE-induced lipid peroxidation was more significantly suppressed than that with GLU Table 1.

It was further observed that when TOC was used without drug in liver homogenate, it produced slight decrease in MDA content with respect to control of different hours but GLU did not show any such change in MDA content. Interpretation of the results is supported by student's 't' test and also from statistical multiple comparison analysis by least significant different procedure[30] Table 1.

In conclusion, glutathione and α-tocopherol appear to possess the potential to inhibit generation of peroxidation breakdown products responsible for expression of various toxic effects of drugs and this may be exploited during future formulation design to enhance the therapeutic indices of the drug. However, further extensive study is required to confirm the potential of the free radical scavengers for reducing iatragenicity of drugs.

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