Hydrocortisone-Induced Lipid Peroxidation and its Inhibition with Various Antioxidants

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Liver, the metabolic machinery of the body, is under constant threat of oxidants. Lipid peroxidation being a toxicity mediating process, drug induced lipid peroxidation may be a probable mechanism of drug induced toxicity. In the present study, drug induced lipid peroxidation has been studied taking hydrocortisone as representative drug and liver homogenate as the experimental model. The antioxidant effects of ascorbic acid, probucol and α-tocopherol on hydrocortisone induced lipid peroxidation were also studied in an attempt to explore the possible potential of these agents in reducing drug-induced toxicity. It was observed that hydrocortisone could cause time-dependent induction in lipid peroxidation which may be related to the toxic potential of the drug. Further, ascorbic acid, probucol and α-tocopherol could suppress hydrocortisone-induced lipid peroxidation to significant extent. The present findings are interesting and provide a scope for further extensive studies on the antioxidants with an aim to reduce toxic potential of the drug.

Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated lipids. It is a highly destructive process involving free radicals and alters the structure and function of cellular membrane[1]. It leads to the generation of peroxides and lipid hydroperoxides that can decompose to yield a wide range of cytotoxic products most of which are aldehydes, as exemplified by malondialdehyde (MDA) and 4-hydroxynonenal[2]. Involvement of lipid peroxidation in several diseases like porphyria[3], eye disorder[4], iron and copper overload[5], Parkinson's disease[6], Alzheimer's disease[7], diabetes[8], emphysema[9], haemolytic diseases[10], malaria[11], aging[12] and atherosclerosis[13] is well known, and hence, free radicals have recently received much attention in medical sciences. Protection of the cell membrane

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from lipid peroxidation becomes a necessity to prevent, cure or delay the aforesaid pathologies.

Many drugs and medicinal substances like adriamycin, menadione, paraquat and alloxan have capacity to produce peroxides[14]. Free radicals or reactive oxygen species (ROS) are produced in vivo from various biochemical reactions and also from the respiratory chain as a result of occasional leakage[15]. These free radicals may be atoms or molecules with one or more unpaired electrons like superoxide anion radical, hydroxyl radical, peroxyl radical and alkoxyl radical.

Lipid peroxidation and its pathophysiology being considered to result from an oxidative imbalance between stress and antioxidant defence, it may be presumed that it is possible to limit oxidative tissue damage and, hence, to prevent or ameliorate disease progression, by supplementing antioxidant exogenously[16].

Drug-induced lipid peroxidation may be related to drug-related toxicities. Use of suitable antioxidants as adjuvants with toxic drugs appears to become a promising approach for reducing iatrogenic disorders[17]. As a part of our ongoing effort to explore drug-induced lipid peroxidation, we present here our recent findings on lipid peroxidation induction potential of antiinflammatory steroidal drug hydrocortisone, a glucocorticoid compound commonly used in inflammatory and allergic conditions. Hydrocortisone, being a steroidal (and hence, lipophilic) compound, studies on its effects on lipid constituents (e.g., lipid peroxidation) seem to be interesting and relevant. Considering lipid peroxidation a possible mediator of toxicity of the drug, we have attempted to explore the capacity of free radical scavenging actions of some conventional antioxidant compounds, ascorbic acid, α-tocopherol and probucol on hydrocortisone induced lipid peroxidation.

Liver being the major site for detoxification, is the primary target for environmental or occupational toxic exposure[18]. It has been suggested that the metabolism of toxic compounds including free radicals mainly, though not exclusively, occurs in liver. The metabolites from liver may diffuse into various extra hepatic tissues, cause lipid peroxidation and cellular injury[19]. The whole study was done using goat liver homogenate as the lipid source and replicate determinations were made at different periods of incubation using MDA content as the indicators of lipid peroxidation.

MATERIALS AND METHODS

Antiinflammatory steroidal drug hydrocortisone sodium succinate (HC) was procured from Glaxo, Bangalore, India; thiobarbituric acid (TBA), ascorbic acid (AA) and α-tocopherol (TOC) were purchased from Loba Chemie, Mumbai; trichloroacetic acid (TCA) was purchased from Merck, Mumbai; probucol (PR) and 1,1,3,3-tetraethoxy propane (TEP) were purchased from Sigma, USA.

Preparation of tissue homogenate:

Goat liver was collected in a sterile vessel containing phosphate buffered 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 buffered saline (pH 7.4) in cold condition. It was centrifuged (2000 g) for 10 min, the supernatant was collected and finally suspended in phosphate buffered saline to contain approximately 0.8-1.5 mg protein in 0.1 ml of suspension to perform in vitro experiments.

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

For each set of experiments with a particular antioxidant, the tissue homogenate was divided into different parts of 50 ml each. The first portion was kept as the control (CL) while the second portion was treated with the drug (HC) at a concentration of 0.04 mg/g liver homogenate. The third portion was treated with drug and antioxidant. The fourth portion was treated with only antioxidant. TOC and PR were used at effective concentrations of 0.2 mg/g and 0.04 mg/g respectively. In case of AA, two dose levels of antioxidants 0.05 mg/g (AA1) and 0.1 mg/g (AA2) were used. Thus, for the sets of experiment with AA as antioxidant, six parts of tissue homogenate were prepared. After treatment with drug and/or antioxidant, liver homogenates were stirred for 1 h below 20° and then incubated at 15° for 28 h along with the control sample.

Estimation of lipid peroxidation breakdown products measured as malondialdehyde:

The extent of lipid peroxidation was estimated in terms of malondialdehyde (MDA) content using thiobarbituric acid (TBA). The estimation was done at 4, 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 three samples of 2.5 ml of incubation mixture was transferred to a tube 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 was developed as described by Yagi et al.[20] 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 upto 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 values were determined from the standard curve that is obtained by using 1,1,3,3-tetraethoxy propane (TEP) according to the method of Tarladgis et al.[21,22].

RESULTS AND DISCUSSION

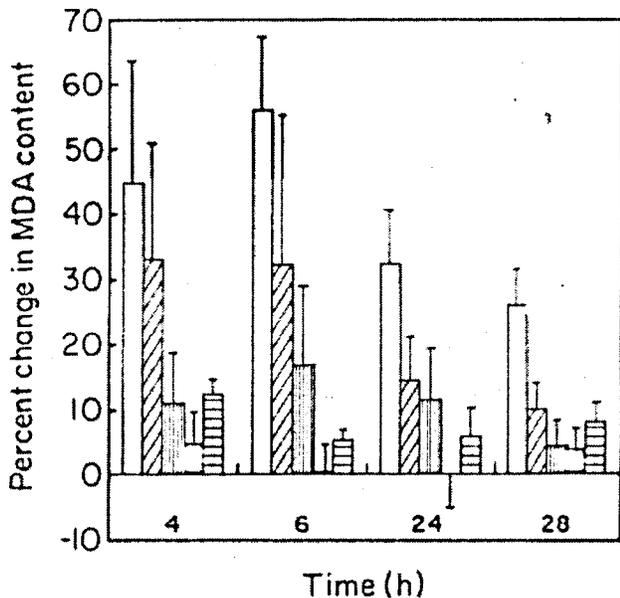The percent changes in MDA content with respect

Fig. 1: Effects of AA on HC induced lipid peroxidation

☐ Drug-treated', ▓ Drug and antioxidant-treated 0.1 mg/g, ⊟ Antioxidant-treated 0.1 mg/g, ▨ Drug and antoxidant-treated 0.05 mg/g, ☐ Antioxidant-treated 0.05 mg/g

Means of five animal sets with standard errors are shown

to the control of different hours of incubation for different samples of different animal sets and their averages are shown along with statistical analysis in figs. 1, 2 and 3. The figures project the effects of AA, PR and TOC as free radical scavengers. Interpretation of the results is supported by statistical multiple comparison analysis using least significant different procedure[23,24] (Tables 1, 2, 3 and 4).

Incubation of liver homogenate with HC caused increase in malondialdehyde (which is a lipid peroxidation breakdown product) content with respect to control (figs. 1, 2 and 3). This is possibly due to enhanced lipid peroxidation induced by HC. Lipid peroxidation, being a cell injury mechanism generating reactive oxygen species (ROS), which plays important role in different disease conditions, may be related to the toxic effects of the drug[14,25]. It will not be out of the context to mention here that recently doxorubicin induced cardiotoxicity has been reported to be caused by a free radical mediated process[26]. Further, insulin deficiency diabetes induced by alloxan[14] is reportedly mediated through free radical mechanisms. Thus, drug induced lipid peroxidation may be a probable mechanism of drug induced toxicity.
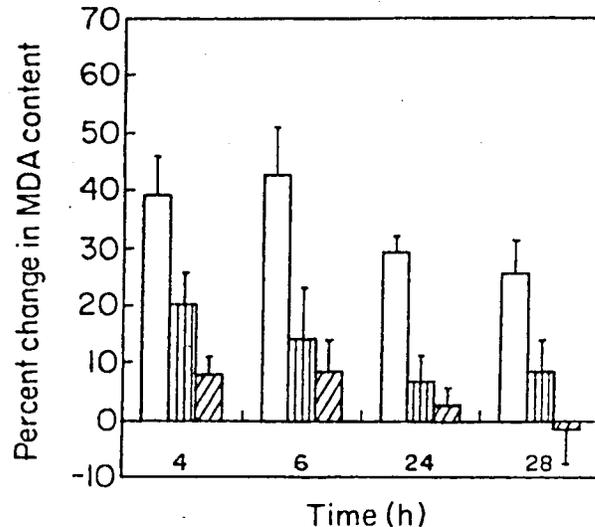

Fig. 2: Effects of PR on HC induced lipid peroxidation

☐ Drug-treated, ⊞ Drug and antioxidant-treated, ▨ Antioxidant-treated

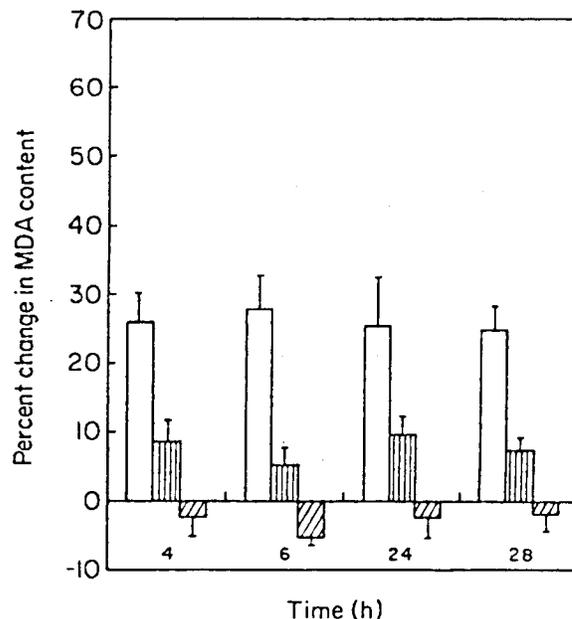Means of five animal sets with standard errors are shown

Fig. 3: Effects of TOC on HC induced lipid peroxidation

☐ Drug-treated, ⊞ Drug and antioxidant-treated, ▨ Antioxidant-treated

Means of five animal sets with standard errors are shown

When liver homogenate was treated with both drug (HC) and antioxidant (AA / PR / TOC), the increase in MDA content was significantly reduced in each case in

comparison to the drug (HC) treated samples (figs. 1, 2 and 3). This implies that the antioxidants ascorbic acid (AA), probucol (PR), and α-tocopherol (TOC) could significantly reduce the extent of HC induced lipid peroxidation. This is attributed to their property of scavenging various free radicals that may have been generated within the system due to the presence of the drug. Thus, it may be presumed that these free radical scavengers may have role in reducing the toxicities of HC, which may be due to enhanced lipid peroxidation. Inhibitory actions of different antioxidants, e.g., ascorbic acid, probucol and glutathione on lipid peroxidation induced by some other drugs also were discussed in previous communications[17,27-33]

Free radicals and oxidative stress have been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defences[34]. Potential antioxidant therapy should, therefore, include either natural free radical scavenging antioxidant enzymes or agents which are capable of augmenting the activity of enzymes superoxide dismutase, catalase and glutathione peroxidase[16].

Ascorbic acid is regarded as the first line natural

antioxidant defence in plasma and a powerful inhibitor of lipid peroxidation. It also regenerates the major antioxidant α-tocopherol in lipoproteins and cell membranes[35]. Ascorbic acid is thought to be an important antioxidant with protective effects against respiratory diseases, atherosclerosis and carcinogens[23,36,37]. α-Tocopherol is a potent chain breaking antioxidant which inhibits lipid peroxidation in membranes by scavenging peroxyl and alkoxyl radicals[38,39]. The free radical scavenging action of probucol, a highly lipophilic compound[40-45], is well reported[14]. Probucol is also known to modulate lipid peroxidation in human low-density lipoproteins induced by environment in space and have a protective effect on peroxidative stress[46]. It was found that antiatherogenic effect of probucol is far greater than expected from its cholesterol-lowering ability[9]. The antiatherosclerotic action of probucol may be due to its capacity to prevent LDL oxidation[47].

It was further observed from the present study that AA produced slight but significant increase in MDA content with respect to control (0 h) when used without drug (HC) but PR and TOC did not produce any such change in MDA content. The increase in MDA levels in the liver homogenates treated with only AA (AA1, AA2) may be due to that AA (in absence of HC) reduces Fe^{3+}

TABLE 1: EFFECTS OF ASCORBIC ACID ON HYDROCORTISONE-INDUCED LIPID PEROXIDATION

| Incubation time (h) | F ratio | | Pooled variance+ | Critical difference# at 5% level with ranked sample means## | |
	Between sample*	Between animals**		LSD+	Ranked means
4	3.66ˢ	06.22ˢ	391.78	26.53	(D,DAA1) (DAA1,AA2, DAA2) (DAA2, AA1, AA2)
6	6.18ˢ	06.80ˢ	412.04	27.21	(D,DAA1)(DAA1,AA2,DAA2) (DAA2, AA1, AA2)
24	13.06ˢ	13.89ˢ	57.40	10.15	(D) (DAA1, DAA2) (DAA2, AA2) (AA2, AA1)
28	13.26ˢ	12.65ˢ	30.66	7.42	(D) (DAA1, AA2, DAA2, AA1)

S denotes that F values are significant at $p < 0.05$. D, DAA1, DAA2, AA1 and AA2 indicate drug-treated, drug and antioxidant-treated 0.05 mg/g, and 0.1 mg/g, and only antioxidant-treated 0.05 mg/g, and 0.1 mg/g samples respectively. * indicates degrees of freedom (d.f.) = (4,16); ** means degrees of freedom (d.f.) = (4,16); + denotes least significant difference procedure; ♣ denotes error mean square; # indicates critical difference according to least significant difference procedure (Refs. 23 and 24). ## indicates that two means not included within same parenthesis are statistically significantly different at $p < 0.05$

TABLE 2: EFFECTS OF PROBUCOL ON HYDROCORTISONE- INDUCED LIPID PEROXIDATION

Incubation time (h)	F ratio		Pooled variance+	Critical difference# at 5% level with ranked sample means##	
	Between sample*	Between animals**		LSD+	Ranked means
4	3.80	1.47	255.04	23.29	(D,DPR) (DPR, PR)
6	13.54[S]	12.40[S]	84.30	13.39	(D),(DPR, PR)
24	8.34[S]	2.39	71.88	12.36	(D) (DPR, PR) (DAA2, AA2) (AA2, AA1)
28	12.73[S]	11.14[S]	49.59	10.27	(D) (DPR, PR)

S denotes that F values are significant at $p < 0.05$. D, DPR, and PR indicate drug-treated, drug and antioxidant-treated and antioxidant-treated samples respectively. * means degrees of freedom (d.f.) = (2, 8); ** means degrees of freedom (d.f.) = (4, 8); + denotes least significant difference procedure; + denotes error mean square; # indicates critical difference according to least significant difference procedure (Refs. 23 and 24). ## indicates that two means not included within same parenthesis are statistically significantly different at $p < 0.05$.

to Fe^{2+} which promotes generation of hydroxyl radicals and other highly reactive species accelerating lipid peroxidation[48,49]. The double role (antioxidant and pro-oxidant in presence of metal ions, particularly, iron and copper) of AA is already reported[50,51].

While comparing free radical scavenging capacity of three antioxidants AA (AA1 and AA2), PR and TOC (Table 4), it has been seen that free radical scavenging capacity of AA, PR and TOC on HC induced lipid peroxidation are not significantly different (Table 4: In

ANOVA, F values is not significant at $p = 0.05$ level). However AA at 0.1 mg/g dose has higher antiperoxidative capacity than at 0.05 mg/g dose.

The present study certainly indicates that lipid peroxidation induced TBA-reactive substance (MDA) formation in liver homogenate is suppressed by addition of different antioxidants AA, PR, TOC. However further extensive study is required to confirm the potential of the free radical scavengers for reducing toxicity of the drug.

TABLE 3: EFFECTS OF α-TOCOPHEROL ON HYDROCORTISONE- INDUCED LIPID PEROXIDATION

Incubation time (h)	F ratio		Pooled varience+	Critical difference# at 5% level with ranked sample means##	
	Between sample*	Between animals**		LSD+	Ranked means
4	48.40[S]	9.36[S]	21.23	6.72	(D) (DTOC) (TOC)
6	22.95[S]	1.34	62.33	11.51	(D) (DTOC, TOC) (TOC)
24	25.44[S]	7.80[S]	37.36	8.91	(D) (DTOC) (TOC)
28	76.05[S]	6.77[S]	11.99	5.05	(D) (DTOC) (TOC)

S denotes that F values are significant at $p < 0.05$. D, DTOC, and TOC indicate drug-treated, drug and antioxidant-treated and antioxidant-treated samples respectively. * means degrees of freedom (d.f.) = (2, 8); ** means degrees of freedom (d.f.) = (4, 8); + denotes least significant difference procedure; + denotes error mean square; # indicates critical difference according to least significant difference procedure (Refs. 23 and 24). ## indicates that two means not included within same parenthesis are statistically significantly different at $p < 0.05$.

TABLE 4: COMPARISON OF INHIBITION POTENTIAL OF ANTIOXIDANTS ASCORBIC ACID, PROBUCOL AND α-TOCOPHEROL ON HYDROCORTISONE-INDUCED LIPID PEROXIDATION

Incubation time (h)	Percentage of inhibition changes[ψ] Different antioxidants				Analysis of variance and Multiple comparison
	AA1	AA2	PR	TOC	
4	45.65	113.74	25.80	68.2	$F_1 = 2.27^S$ [df = (3,9)] $F_2 = 0.63^S$ [df = (3,9)]
6	58.75	82.69	76.84	76.89	Pooled variance $(s^2)^*$ =369.79 Critical difference (p = 0.05)[#]
24	63.86	67.55	76.34	62.18	 LSD = 30.75
28	70.77	106.2	84.38	61.76	Ranked means** (AA2, TOC, PR) (TOC, PR, AA1)
Av. (± SE)	59.75 (±5.30)	92.54 (±10.63)	65.84 (±13.47)	67.25 (±03.53)	

ψ denotes that per cent changes with respect to controls of corresponding hours are shown (average values of five animal sets were used for computation). S denotes that F values are not significant at $p < 0.05$. F_1 and F_2 correspond to variance ratios between samples and between animals respectively. AA1, AA2, PR, TOC indicates Ascorbic acid 0.05 mg/g, Ascorbic acid 0.1 mg/g, Probucol and α-Tocopherol respectively. Av. indicates average data of each column; S.E. indicate standard error (d.f. = 3); d.f. means degree(s) of freedom. * denotes error mean square, # denotes critical difference according to least significant difference procedure (Refs. 23 and 24). ** denotes that two means not included within same parenthesis are statistically significantly different at $p < 0.05$

To conclude, our findings show that the antioxidants ascorbic acid, probucol and α-tocopherol have significant protective role against HC-induced lipid peroxidation. However, these in vitro results give only predictive idea about the nature of actions of the antioxidants on lipid peroxidation and are not to be treated as conclusive. Variations may be found between in vitro and in vivo results and this necessitates further in vivo studies to confirm the predictions.

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