Liposomes as a model for studying
UV induced oxidative changes
Summary

In order to understand the mechanistic details of ultraviolet induced pro-oxidative changes on biomembranes, simulated studies were conducted with liposomes as a model. Sonicated vesicles were prepared from rat brain lipids with and without pro-oxidants and anti-oxidants and exposed to UV radiations as a simulator for different periods of time to measure the peroxidative decomposition of polyunsaturated fatty acids followed by the thiobarbituric acid (TBA) assay. UVC and to a less extent UVA caused lipid peroxidation specially in presence of Fe++. Ascorbic acid, especially at higher concentrations was found to have an antioxidant effect. Superoxide dismutase also showed biphasic effect with lower concentrations enhancing and higher concentrations inhibiting the TBARS formation. Liposomes prepared from pure lecithin also showed similar effects. The hydrophobic antioxidant tocopherol also caused better entrapping of Ca^{2+} in the liposomes. Thus the relation between Ca dynamics and peroxidative damage, is likely also in UV induced effects.
Introduction

Increasing concern is being expressed regarding the depletion of stratospheric ozone layer and the ensuing climatic changes and enhanced dermal risks due to higher UV incidence (Koehler and Hajost, 1990; Yoytek, 1990). Human health hazard due to photochemical activation of dermally exposed occupational and environmental xenobiotics is a problem in tropical countries, necessitating further studies in this direction. Formation of active oxygen species, especially singlet oxygen and superoxide anion, have been implicated as a factor in photochemotherapy and phototoxicology (Pathak and Joshi, 1983; Joshi and Pathak, 1984). In the photosensitizing effect of psoralens also, oxygen dependent type II photodynamic reactions involving \( \text{O}_2^- \) and \( \text{O}^- \) were found (Pathak and Joshi, 1984) to cause membrane damaging reactions including oedema or inflammation. Data from the toxic effects of chemicals indicate that the membrane damage by active oxygen radical formation could lead \textit{inter alia} to peroxidative degradation of polyunsaturated fatty acids (Fraga et al., 1987; Kakkar and Viswa-nathan, 1990).

For studying the processes involved in lipid peroxidation, liposomes are convenient models (Chatterjee and Agarwal, 1988). P-phenylene diamine, a potential dermal irritant component of hair dyes, was found to elicit lipid peroxidation to lecithin vesicles (Gupta et al., 1989). A possible interrelation between oxygen radical mediated membrane damage and altered \( \text{Ca}^{2+} \) fluxes was indicated by \textit{in vitro} studies with hepatocytes (Bonventre, 1990) and liver mitochondria (Mehrotra et al., 1991). In view of the above considerations, the effect of ultraviolet radiations on liposomized lecithin was studied using thiobarbituric acid reactive substance (TBARS) formation, calcium ion fluxes and the influence of free radical scavengers and antioxidants.

Materials and Methods

Chemicals

Superoxide dismutase from bovine erythrocytes, lecithin and \( \alpha \)-tocopherol were procured from Sigma chemical Co., USA. All the other chemicals used were either BDH Analar or E. Merck Extrapure.
Animals

Male albino Wistar rats weighing 100-150 g from Industrial Toxicology Research Centre Animal Colony were used for the study and were kept on standard pellet diet (M/S Lipton, Bombay) and Water ad libitum.

Extraction of phospholipids

Rats were killed by decapitation and liver and brain were immediately taken out, rinsed with normal saline, dried, freed of connective tissue and weighed. A 10 per cent homogenate w/v was prepared in chloroform-methanol (2:1) in a Potter Elvehjem glass homogenizer at 0-4 °C. 0.88 per cent KCl was added to this homogenate in 5:1 ratio (Folch et al., 1957). The homogenate was then left to cool for the separation of the two layers. The upper methanol layer was discarded and the lower chloroform layer containing phospholipids was evaporated in a dessicator.

Preparation of liposomes

The extracted phospholipids were lyophilized. Liposomes were prepared by sonication the phospholipids in presence of Tris KCl buffer (0.01 M, pH 8.5 in 0.1 M saline) at 25 KCs/sec \(^1\) (Kinsky et al., 1969). Liposomes were centrifuged at high speed to remove impurities. To prepare lecithin liposomes, pure lecithin (Sigma) was used instead of tissue phospholipids.

Preparation of calcium entrapped liposomes

To prepare calcium entrapped liposomes, 1 mg Ca ml\(^{-1}\) of 10 mM Tris-KCl buffer pH 8.5 was sonicated along with the phospholipids for 9 minutes at 25 KCs sec\(^{-1}\). Excess calcium was removed by dialyzing the liposomes against 10 mM Tris-KCl buffer pH 8.5 in 0.1 M saline for 24 h at 0.4 °C.
Incorporation of α-tocopherol in liposomes

α-Tocopherol was added to the extracted phospholipids so that the final concentration was 10 mM and the phospholipids were kept for chloroform evaporation. The lyophilized sample was sonicated in presence of the above Tris-KCl buffer at 25 KCs/sec for 9 minutes to obtain tocopherol incorporated liposomes.

Exposure to UV and estimation of calcium

Ca\(^{2+}\) entrapped liposomes were directly exposed under UV lamp (Indian Equipment Corporation) for varying periods of time at 254 nm (UVC) and 365 nm (UVA) in a closed chamber. The irradiated liposomes were dialyzed against 10 mM Tris-KCl buffer pH 8.5 for 24 h to remove the calcium leached out of membrane.

After dialysis, liposomes were digested with HNO\(_3\) to remove interference of phospholipids in Ca\(^{2+}\) estimation. The digested samples were made upto a uniform volume and estimated for calcium on Perkin Elmer 5000 Atomic absorption spectrophotometer.

Lipid peroxidation

Malonaldehyde formed as a product of the lipid peroxidation was estimated by the method of Ohkawa et al. (1979). The reaction system containing 10 per cent w/v homogenate was incubated at 37 °C with constant shaking and after two hours aliquots were withdrawn for the assay of thiobarbituric acid reactive material formed. The O.D. was measured at 535 nm. Molecular extinction coefficient of 1.56 x 10\(^5\) x M\(^{-1}\) was used to calculate n moles malonaldehyde formed mg protein\(^{-1}\) h\(^{-1}\).

Estimation of Pi and phospholipid content

Phospholipid phosphorus was estimated by the method of Bartlett (1959). The liposome sample was digested with perchloric acid and colour developed by boiling
Fig. 1: Malonaldehyde Formation in Liposomes Prepared from Rat Brain Phospholipids on Exposure to UVC & UVA
Fig. 2: MDA Formation in Liposomes Prepared from Rat Brain Phospholipids + Iron on Exposure to UV
with ANSA reagent and 5 per cent ammonium molybdate solution for 8 minutes. Readings were taken at 660 nm. Pi was estimated and the value multiplied by 25 to give phospholipid value.

Statistical analysis

In each group, estimations were done in four separate animals and the statistical evaluations of effect of UVC and UVA exposure on membrane lipids was done by student's 't' test taking p value < 0.05 as significant (Fisher, 1950).

Results
Lipid Peroxidation in Liposomes on UVA and UVC Exposure

Malonaldehyde formed on exposing liposomes formed from rat brain lipids to UVA and UVC have been recorded in Figure 1. Exposure with UVC recorded a higher rate of TBARS formation compared to exposure with UVA. This was specially marked after 20 minutes exposure when TBARS formation with UVC exposure was found to be 2.12 times more than the TBARS formation with UVA exposure (p < 0.001; p < 0.001, respectively). This gap was reduced at 25 min exposure and after 30 min exposure the TBARS formation in both cases was almost equal. There was however, no linear relation between MDA formation and time of exposure. This shows that UVC exposure causes more damage to lipids initially than UVA, but after long and sustained exposure it is found that the damage in both cases is almost equal.

Lipid peroxidation in liposomes on UVA and UVC exposure in presence of Fe

Exposure of rat brain lipid liposomes to UVA and UVC in presence of 0.5 mM Fe revealed the prooxidant qualities of iron (Figure 2). TBARS formation was enhanced in liposomes containing Fe. Though, the pattern obtained after UVA and UVC exposure at different time intervals was the same as the earlier experiment conducted in the
Fig. 3: MDA Formation in Liposomes Prepared from Rat Brain Phospholipids + 10 mM Ascorbic Acid on Exposure to UV
Fig. 4: MDA Formation in Liposomes Prepared from Rat Brain Phospholipids + Fe+10 mM Ascorbic acid on Exposure to UV
absence of Fe, it was observed that the maximum difference in TBARS formation on UVA and UVC exposure was seen at 25 minute, *i.e.* TBARS formation in UVC exposed liposomes was found to be 1.49 times the TBARS formed in UVA exposed liposomes (*p* < 0.001; *p* < 0.02, respectively).

**Lipid peroxidation in liposomes on exposure to UVA and UVC in presence of high concentrations of Ascorbic acid**

Exposure of brain lipid liposomes to the antioxidant effect of high concentrations of ascorbic acid (10 mM) in presence of UVA and UVC radiation (Figure 3) revealed that Ascorbic acid acted as an antioxidant on 5 minutes exposure to both UVA and UVC. There was an increase in TBARS formation in both cases at 10 min exposure. At 15 minutes exposure, however, there was a marked increase in TBARS active substances in the UVC exposed liposomes (1.13 times, *p* < 0.01) while there was a drastic reduction in UVA exposed liposomes (51.6%, *p* < 0.001). This trend was revised on further exposure and at 30 minute exposure, the antioxidant effect of ascorbic acid was more marked in liposomes exposed to short UV than those exposed to long UV.

**Lipid peroxidation in liposomes on exposure to UVA and UVC in presence of iron and high concentration of Ascorbic acid**

The antioxidant effect of a high concentration of ascorbic acid was observed when malonaldehyde formation was recorded in brain lipid liposomes exposed to UVA and UVC for different time intervals in presence of Fe and 10 mM. Ascorbic acid (Figure 4). UVC exposed liposomes showed an inconsistent rise in TBA reactive substances with increasing time duration of exposure. Maximum TBARS formation was observed at 30 min exposure. With UVA exposure, however, there was an initial increase in malonaldehyde formation at five minutes (15.4%, *p* < 0.001) followed by a decline at 10 mins (20%, *p* < 0.001) and 15 mins exposure (28.3%, *p* < 0.001). An insignificant increase in TBARS formation at 20 and 25 mins exposure was again followed by a decline at 30 minutes exposure (33.4%, *p* < 0.001). Thus, it is seen that high concentrations of ascorbic acid have an antioxidant effect on lipid peroxidation.
Fig. 5: Malonaldehyde Formation in Liposomes Prepared from Lecithin and Rat Brain Phospholipids on UVC Exposure
Fig. 6: Malonaldehyde Formation in Liposomes Prepared from Lecithin and Rat Brain Phospholipids on UVA Exposure
Table 1

Effect of 10 mins of UVA and UVC Exposure on Rat Brain Liposomes in presence of Varying Amounts of α-Tocopherol

<table>
<thead>
<tr>
<th>System</th>
<th>UVC Exposure for 10 mins. (nM MDA/mg Phospholipid/hr)</th>
<th>UVA Exposure for 10 mins. (nM MDA/mg Phospholipid/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>13.35±0.41</td>
<td>18.85±0.41</td>
</tr>
<tr>
<td>Liposomes + 1 mM Tocopherol</td>
<td>25.06±0.76</td>
<td>18.85±0.28</td>
</tr>
<tr>
<td>Liposomes + 5 mM Tocopherol</td>
<td>18.28±0.41</td>
<td>16.51±0.49</td>
</tr>
<tr>
<td>Liposomes + 10 mM Tocopherol</td>
<td>19.55±0.28</td>
<td>15.21±0.34</td>
</tr>
</tbody>
</table>

Values are arithmetic mean± S.E. of four determinations in each case.
Lipid peroxidation in liposomes on UVC exposure

The formation of malondialdehyde on exposing liposomes prepared from pure lecithin and rat brain lipids to UVC are recorded in Fig 5. Rate of TBARS formation was significantly higher with brain lipids than lecithin. The increase in peroxidation with UV exposure was higher with brain preparations. However, there was no linear relation between MDA formation and time of exposure. Maximum enhancement of about 70% was obtained at 20 min (p < 0.001). On the other hand UVC exposure caused no statistically significant effect on peroxidation of lecithin vesicles. Thus, apparently, some component of brain lipids other than phosphatidyl choline is the likely target of UV induced in vitro peroxidation.

Lipid peroxidation in liposomes exposed to ultraviolet A radiations

The formation of malonaldehyde on exposing liposomes prepared from pure lecithin and rat brain lipids to long UV (UVA) are recorded in Figure 6. Rate of TBARS formation was found to be higher in brain lipid liposomes formed from pure lecithin. The increase in peroxidation with UVA exposure was also higher with brain preparation as compared to lecithin preparations, but this increase became more and more insignificant at 5, 10 and 15 min. exposure with UVA. At 20 mins. exposure, brain lipid liposomes showed an increase in TBARS formation as compared to lecithin liposomes which registered a decline. This gap increased significantly at 25 mins (p < 0.001; p < 0.001) respectively. At 30 mins again, this gap was narrowed. This establishes the long term damages of UVA radiation on both brain lipid and lecithin liposomes.

Lipid peroxidation in liposomes exposed to UVA and UVC for 10 mins in presence of varying amounts of \( \alpha \)-tocopherol

The formation of malonaldehyde in liposomes exposed to UVA and UVC for 10 min in presence of varying amounts of \( \alpha \)-tocopherol has been recorded (Table 1). Liposomes exposed to short UV showed increased TBARS formation in presence of 1
Fig. 7: Protective Effect of SOD on UV Induced LPO in Rat Brain Lipid Liposomes
Fig. 8: Influence of Time on the Effect of UVC and UVA on Calcium Retention by Lecithin Liposomes
Fig. 9: UVC & UVA Induced Ca Leaching from Liposomes Prepared from Rat Brain & Liver Phospholipids
mM α-tocopherol (1.8 times). Clearly, 1 mM α-tocopherol does not have a protective effect in liposomes exposed to UVC, 5 mM and 10 mM. Tocopherol, however, had a protective effect on UVC exposed liposomes but the protection accorded was not complete. However, in case of UVA exposed liposomes, 1 mM, 5 mM and 10 mM α-tocopherol accorded increasing protection to liposomes.

**Effect of superoxide dismutase on lipid peroxidation in liposomised brain lipids**

The formation of TBA reactive substances on 10 minutes exposure to UV caused significant increase as compared to controls. However, UVA was about 50% more active (p < 0.05). When SOD was added (Figure 7), in case of UVC there was a consistent and statistically significant increase in peroxidation products, even though there was no strict close effect relation. Maximum increase of about 70% was with 30 units SOD/mg protein (p < 0.01). In the case of UVA, lower amount of SOD caused marginal increase in peroxidation, while higher amounts were inhibitory. However, 40 units SOD/mg protein caused about 35% inhibition (p < 0.001). This shows that superoxide radicals are produced during UV irradiation, specially UVA irradiation, which are quenched by SOD.

**Effect of irradiation on liposome entrapped Ca$^{2+}$**

When liposomised liver lipids were exposed to UVC, for 15 min and 30 min, about 20 and 40% of the entrapped Ca$^{2+}$ was found to be lost (Figure 9). With UVA, there was 20% decrease which was unaltered on further irradiation. With liposomes from brain lipids also, UVC caused more loss of calcium as compared to UVA exposure, but the magnitude of change was less. However, with brain lipids, the Ca$^{2+}$ loss by either UV exposure for varying time periods was not statistically significant. When pure lecithin was used to prepare liposomes, the pattern was similar (Figure 8). UVA did not cause any effect but UVC caused 25 and 45% loss of entrapped Ca$^{2+}$ (p < 0.05; p < 0.001 respectively). Thus, showing enhanced damage to liposomes on UVC irradiation.
Fig. 10: Effect of UVC on Lecithin Liposomes for Varying Time Periods and Protective Effect of 10 mM tocopherol
Incorporation of tocopherol in the lecithin vesicles, caused almost two fold higher calcium retention as compared to controls (Figure 10). Thus \( \alpha \)-tocopherol lecithin system can trap calcium ions more effectively than lecithin alone. UVC exposure for 15 and 30 mins caused 29 and 52\% (\( p < 0.01 \)) loss of \( \text{Ca}^{2+} \). Thus, \( \alpha \)-tocopherol was not effective in preventing \( \text{Ca}^{2+} \) loss, but could cause better entrapment.

**Discussion**

The present data indicate that liposomized preparations could be used as convenient models for studying UV induced changes on membranes through active oxygen species and \( \text{Ca}^{2+} \) fluxes. In this respect, the liposome model has many similarities with the response of biomembranes towards xenobiotic induced oxidative stress. A direct correlation with the lipid peroxidative processes and \( \text{Ca}^{2+} \) fluxes was indicated from the studies of Bonventre (1990) and Rasmussen *et al.* (1990). *In vitro* studies with fresh liver mitochondria from this laboratory also showed an interrelation among active oxygen species, membrane functions and calcium dynamics (Mehrotra *et al.*, 1991). The liposomal model could be convenient for further studies in this direction.

Short range UV caused more membrane damage than long range UV. However, long and sustained exposure to UVA caused increased damage to membrane. \( \text{Fe}^{2+} \) was seen to have a prooxidant effect while high concentrations of Ascorbic acid had an antioxidant effect. Presence of \( \alpha \)-tocopherol is seen to have a pronounced protective effect in UVA exposed liposomes but not in UVC exposed liposomes. Presence of \( \alpha \)-tocopherol enhances \( \text{Ca}^{2+} \) entrapment but has very little influence on calcium ion leaching on UV exposure. Thus the protective effect of \( \alpha \)-tocopherol on membranes is confirmed.

The enhancement of malonaldehyde formation in the presence of SOD specially in UVA is interesting. It appears that \( \cdot \text{OH} \) formed by dismutation could be responsible for initiating the reactions as reported in many cases (Gardner, 1989).

Influence of trace amounts of iron could be involved with SOD which at higher concentration reduced the LPO by UVA but not UVC radiations. It seems probable that
the target molecules for the UV attack differ in the two cases. It could be likely that the UVC reactions involve \( ^1O_2 \) and the UVA reactions, \( O_2^- \). Recently, it has been reported that short UV radiations induce a strong peroxidation of the lipid content of low density lipoprotein (Dousset et al., 1990). In the present in vitro system the interconverting mechanisms like Haber-Weiss may not be operating due to lack of transition metal cations and electron acceptors. The inclusion and retention of larger amounts of Ca\(^{++}\) in the presence of \( \alpha \)-tocopherol could be indicative of a more stabilized state of the vesicles.

Apart from UVC and UVA induced oxidative membrane damage and the beneficial effect of antioxidants, the present data also suggest the potential of liposomes in the study of photochemical membrane damage.