Effects of photodynamic treatment on biological antioxidant systems in rats

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Effects of photodynamic treatments on inherent antioxidant metabolites and cellular defence enzymes have been investigated in rats. Wistar rats were grouped into untreated controls, light controls, hematoporphyrin derivative (Hpd) (treated with 5 and 10 mg Hpd / kg body weight and kept in dark) and sets treated with both Hpd and red light (dose 172 and 344 J/m²). After 2, 24, 48 and 72 hr of Hpd injection the rats sacrificed, livers quickly excised to analyze Hpd uptake, activities of enzymes like catalase, GSH-Px and antioxidants like GSH, vitamin A, vitamin E and vitamin C. The results showed that the loss of Hpd from liver as a function of post-injection time was non-linear. An increased generation of lipid radicals was observed in the groups treated with 5 mg Hpd and higher dose of light and in groups treated with 10 mg Hpd at both the doses of light. Combination of light and Hpd reduced hepatic GSH content with a concomitant reduction in GSH-Px. At higher doses of Hpd and light, there was a significant reduction in hepatic vitamin A levels. Combination of Hpd and light in all doses reduced vitamin E content in liver. The decreased biological antioxidant contents and GSH-Px may be attributed to their utilization for the scavenging of free radicals generated by Hpd and light in tissues. However, no change in catalase activity and vitamin C content in liver was noted in experimental rats. The results suggest that exposure to higher doses of Hpd with light alters oxidant stress system and TBARS content in rat.

Photodynamic therapy is one of the recent modalities of cancer treatment which is based on the ability of photosensitizing dye to differentially localize in tumour tissue and to generate cytotoxic radicals upon photoirradiation of the tumour tissue. Hematoporphyrin derivative (Hpd) is being widely used as a photosensitizing agent for therapy and early detection of solid tumours and several thousand patients have been treated all over the world by this modality. During treatment Hpd is however taken up to a large extent by liver, spleen and kidney along with tumours. The photodynamic action of Hpd has been shown to be mediated through the generation of the reactive oxygen species like singlet oxygen (to a large extent) O₂*, H₂O₂, •OH. These oxygen radicals can react both directly and indirectly with many biological molecules like lipids, proteins and nucleic acids, leading to damage which could be lethal or sub lethal based on the extent of involvement of these macromolecules in the crucial biological functions. The extent of cellular damage is determined by the status of the inherent cellular defence systems. Cell protection is dependent upon the structural integrity and the antioxidant status of the cell. It is known that the living organisms have inherent defence mechanisms to counter the onslaught by the oxidative radicals. These mechanisms include a number of radical scavenging antioxidants like glutathione (GSH) ascorbic acid, tocopherols, uric acid etc. In addition there are a number of defence enzymes such as catalase, glutathione peroxidase (GSH-Px) and glutathione reductase (GSSGR). Rougeee et al. have shown that molecules containing –SH groups could be targets of singlet oxygen. Atlante et al. have also demonstrated the effects of photodynamic treatment (PDT) on carrier thiols which are involved in Pi transport in mitochondria. Prevention of such oxidative damages by quenching of singlet oxygen by α-tocopherol upon prior incubation of cells with this antioxidant in vitro has been reported. However, it is not known as to what will be the status of inherent antioxidant metabolites or the cellular defence enzymes in either the tumour tissue or the normal tissue in vivo after the photodynamic treatment, whether these are sufficient to protect the cells from the oxidative onslaught, whether their induction is increased or do they get inactivated. This study, has therefore been undertaken to investigate the effects of photodynamic treatment on protective enzymes and on the antioxidant metabolites in normal rat liver using two doses of Hpd and two doses red light. Hpd was synthesized as described earlier. The Hpd thus synthesized had
been shown to be highly effective in vitro\(^{19,20}\) when compared to the commercially available pure compound Photofrin II. However, it was not known whether it will be effective in vivo as well, this study, therefore is also aimed at investigating the efficacy of Hpd in vivo. For this study non tumour bearing normal and healthy rats were selected so as to establish a data base on the effects in normal tissues compared to the commercially available pure compound.

Carried out in tumour bearing rats as well. Liver tissue therefore is also aimed at controls, (b) light controls, (c) Hpd- treated (5 and 10 mg/kg) and 172 \(\mu\)g/\(\text{m}^2\) as well as in groups treated with 5 mg Hpd and higher dose of light (dose II) as well, this study, as a result of oxygen.

Materials and Methods
Rats—Male Wistar rats weighing between 100-120 g were randomly grouped into (a) untreated controls, (b) light controls, (c) Hpd- treated (5 and 10 mg/kg) and kept in dark, (d) Hpd treated (5 and 10 mg/kg) and irradiated with red light (dose I, see below) and (e) Hpd treated (5 and 10 mg/kg) and irradiated with red light (dose II). Food and water were provided ad libitum.

Hpd preparation and injection—Hpd was prepared from hematoporphyrin dihydrochloride as described earlier\(^{18-20}\) essentially according to Lipson et al\(^{21}\). Rats were injected with Hpd dissolved in phosphate buffered saline (PBS) i.p at a dose of 5 and 10 mg / kg body weight. Control rats were injected PBS.

Analysis of Hpd uptake by liver—A portion of liver tissue just before irradiation was homogenised with the tissue solubilizer soluene, centrifuged and the optical density of supernatant was measured at \(\lambda_{\text{max}}\) 330nm and concentration was calculated from the absorbance plot of known amounts of Hpd in soluene.

Photodynamic treatment—At the time of irradiation (after 24 hr of Hpd injection) rats were sacrificed, livers were quickly excised, washed in ice cold PBS to remove adhering blood, cut into 2 cm\(^2\) slices of 1mm thickness and exposed to red light (630 ± 10) from a Philips lamp (M/S Phillips India). To a total dose of 172 \(\mu\)g/\(\text{m}^2\) (dose I) and 344 \(\mu\)g/\(\text{m}^2\) (dose II).

Before irradiation the liver was kept in ice cold (4\(^{\circ}\)C) PBS containing 5 mM glucose, till the tissue attained that temperature. Intracellular Hpd was better retained at 4\(^{\circ}\)C as compared to 22, 37 or 40\(^{\circ}\)C.\(^{19}\) Rise in temperature during irradiation with red light was 6\(^{\circ}\)C in 5 min (dose I) and 15\(^{\circ}\)C in 10 min (dose II).

Hepatic thiobarbituric acid reactive substances (TBARS)\(^{22}\), vitamin A\(^{23}\), total tocopherols\(^{24}\), ascorbic acid\(^{25}\), glutathione\(^{26}\) catalase\(^{27}\) and GSH-Px activities\(^{28}\) were estimated essentially according to the prescribed procedures. Student’s \(t\) test was employed to evaluate level of significance between treatments.

Results and Discussion
Uptake and retention of Hpd by liver—Hpd content of liver was estimated 2, 24, 48 and 72 hr after injection. After 2 hr, 76.6 and 78.13% of the injected Hpd could be extracted from livers of the rats treated with 5 and 10 mg Hpd/kg body weight respectively (Table 1). At 72 hr the Hpd content was 9.6 and 20.2% respectively. The loss of Hpd from liver as a function of post- injection time was non-linear. The Hpd retained by the liver at every time point was higher in the rats injected with 10 mg / kg body weight as compared to those injected with 5 mg / kg body weight.

Lipid peroxidation—Fig. 1 shows the effects of Hpd and light on the peroxidation of liver lipids. The values in the untreated controls were \(8.1 \times 10^{-9}\) moles/g tissue. The values for the two light controls and the two Hpd controls were not significantly different from those of untreated controls. However, a significant increase was observed in the groups treated with 5 mg Hpd and higher dose of light (dose II) as well as in groups treated with 10 mg Hpd at both the doses of light. Hpd in combination with light is known to cause TBA reactive species. \(^{22-29}\) Lipid peroxidation, a chain reaction, is a result of oxygen

<table>
<thead>
<tr>
<th>Hpd conc. (mg/kg body wt.)</th>
<th>0</th>
<th>2</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
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<td>5</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Values taken as blank</td>
<td>45±4.3</td>
<td>200±7.8</td>
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</tbody>
</table>

Table 1 — Hpd uptake and retention (in \(\mu\)g) by whole liver as a function of post- injection time.
[Values are mean ± SD of 6 rats]
radicals generated due to photodynamic treatment of Hpd loaded cells. This could also be a cause of further destruction of other macromolecules due to the generation of lipid radicals which would ultimately lead to exhaustive tissue injury.

Effect of Hpd on antioxidant metabolites — Effects of Hpd treatment in dark on the various antioxidants were monitored up to 72 hr after injection and one time point i.e 24hr was selected for irradiation since the Hpd content was significantly high when compared to 48 or 72 hr. Hepatic glutathione provides vital antioxidant reserve against reactive oxygen species and xenobiotics. The GSH content in control rats was 1.56 ± 0.11, 1.61±0.18, 1.81±0.17 and 1.79±0.18 m moles / g liver after 2, 24, 48 and 72 hrs, it was found to increase upon Hpd treatment though not significant. The irradiation however reduced the GSH levels (Table 2).

The vitamin A content of liver was not affected by treatment with either Hpd alone (5 or 10 mg / kg body weight up to 72 hr or with irradiation alone (light dose I and light dose II). Even the combination of Hpd and light at lower dose i.e 5 mg / kg body weight and light dose I did not cause any effect on the vitamin A content whereas the higher doses (5 mg Hpd + light dose II, 10 mg Hpd + light dose I and 10 mg Hpd + light dose II) reduced the vitamin content significantly (Table 2).

Total tocopherols content in untreated controls was 18.32 mg/g liver tissue. No significant change could be observed in the two light controls or the two Hpd controls (up to 72 hr). Combination of Hpd and light in all doses studied however, reduced the content of tocopherols significantly, although the difference in the effects in between the dose was not significant. Ascorbic acid content of liver was enhanced by the combination of light and high dose of Hpd (Table 2). The decreased biological antioxidant contents in liver viz. vitamin A and vitamin E may be explained as utilization of these antioxidants towards the scavenging of free radicals generated by Hpd and light in the tissues.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vitamin A (IU/g)</th>
<th>Vitamin E (mg/g)</th>
<th>Vitamin C (mg/g)</th>
<th>GSH (m moles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.91±4.27</td>
<td>18.19±1.19</td>
<td>2.30±0.32</td>
<td>1.61±0.18</td>
</tr>
<tr>
<td>Light control (dose I)</td>
<td>50.83±6.18</td>
<td>20.20±2.31</td>
<td>2.41±0.39</td>
<td>1.61±0.17</td>
</tr>
<tr>
<td>Hpd (5mg/kg)</td>
<td>50.91±4.29</td>
<td>17.79±1.68</td>
<td>2.68±0.28</td>
<td>1.78±0.11</td>
</tr>
<tr>
<td>Hpd (10mg/kg)</td>
<td>53.23±4.18</td>
<td>18.09±1.81</td>
<td>2.62±0.30</td>
<td>1.69±0.14</td>
</tr>
<tr>
<td>Hpd (5mg) light (dose I)</td>
<td>50.99±7.68</td>
<td>15.21±2.01</td>
<td>2.72±0.40</td>
<td>1.59±0.13</td>
</tr>
<tr>
<td>Hpd (5mg) light (dose II)</td>
<td>47.61±5.60</td>
<td>12.9±1.72</td>
<td>2.52±0.31</td>
<td>1.32±0.15</td>
</tr>
<tr>
<td>Hpd (10mg) light (dose I)</td>
<td>45.69±6.19</td>
<td>11.28±1.31</td>
<td>2.81±0.40</td>
<td>1.31±0.11</td>
</tr>
<tr>
<td>Hpd (10mg) light (dose II)</td>
<td>46.88±5.52</td>
<td>11.59±1.40</td>
<td>2.89±0.41</td>
<td>1.11±0.18</td>
</tr>
</tbody>
</table>

Values not sharing the same superscripts are significantly different (P<0.05).
Effects of Hpd and light on the antioxidant enzymes—Effects of the photodynamic treatment on two of the antioxidant enzymes viz. glutathione peroxidase and catalase have been investigated (Table 3). Neither Hpd alone nor light alone had any effect on the activity of glutathione peroxidase. Combination of Hpd and light at both the doses reduced the activity of this enzyme significantly, which shows the role played by GSH-Px in scavenging hydroperoxides. Activity of catalase, however, was not affected either with Hpd alone, light alone or with the combination treatment. GSH-Px is more important than catalase in removing H2O2 and lipid peroxides in mammalian cells.

Reactive oxygen species and their subsequent modification of macromolecules (such as DNA, lipid and protein) play an important role in photodynamic cytotoxicity. Cell protection is dependent on various inherent features of cellular systems. Protection of vital cellular constituents appears to be dependent on structural integrity of cell, the compartmentalization of both functions and constituents and presence of antioxidant/defence enzymes. The cellular defence mechanism can be classified into; preventive antioxidants and chain-breaking antioxidants. The specific enzymes which have a major responsibility towards cellular protection against oxygen mediated toxicity include the GSH-Px, GSSGR and catalase. These antioxidant enzymes are preventive antioxidants because they are capable of scavenging oxygen and organic free radicals such as O2•-, H2O2 and hydroperoxides. Antioxidants of small molecular size such as glutathione, vitamin E and vitamin C can annihilate oxidizing radicals directly and therefore are chain-breaking antioxidants. GSH accounts for about 90% of the intracellular non-protein thiol content. GSH is resistant to typical proteases by virtue of the γ-glutamyl linkage. Thus GSH is an ideal cellular constituent for the cell protection system. Formation of GSH conjugates with many carcinogens has been shown and also the role of GSH as the main intracellular interceptor of reactive, electrophilic compound is becoming well established. Vitamin E and C are well known tissue antioxidants and efficient inhibitor of lipid peroxidation in vivo. Autoxidation, in simple terms, is a chain reaction and these vitamins break and shorten this oxidation chain. The decreased levels of tocopherols and vitamin A would lead to the loss of reductive environment in tissues at higher doses of Hpd and light as observed in this study. The effects on lipid peroxidation (Fig. 1) also show that Hpd in combination with light increases oxidative stress. The increased hepatic ascorbic acid level in rats injected with high dose of Hpd may help in mitigating the oxidative stress. The present study shows that photodynamic treatment does not affect the activity of catalase. The result is very interesting since it is known that photosensitization with Hpd and light is induced by singlet oxygen to a large extent and not through H2O2 radicals which are scavenged by catalase.

Analyses were carried out in liver because liver not only takes up the maximum amount of the photosensitizer but also is the major organ for conjugation and detoxification of exogenous materials in the body. It will be therefore relevant to investigate the effects of the photosensitizer in dark as well as in combination with light in this tissue. Slicing and ex vivo irradiation had to be resorted to due to the problems in access of the tissue for irradiation and due to the problems of light penetration. Irradiation
was carried out at 4°C because earlier work\textsuperscript{19,34} has shown that Hpd is retained in the cells better at this temperature with the rise in temperature seen after irradiation with red light is not sufficient release Hpd from the intracellular sites. In clinical applications photodynamic therapy therefore, is more applied to tumours in hollow organs (buccal cavity, uterus, bladder etc.) and tumours in areas, easily accessible for irradiation, for eg. Breast, prostate etc. Therefore, it is necessary that a database be established on the effects of PDT on cellular defence systems in normal and malignant tissues of every organ. This type of knowledge could be very useful in planning the differential elimination of malignant tissue.

Acknowledgement

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References

2 Gomer, CJ & Dougherty TJ, Determination of \textsuperscript{3}H and \textsuperscript{14}C hematoporphyrin derivative distribution in malignant and normal tissues, \textit{Cancer Res.} 39 (1979) 146.
25 Roe JH & Kauther CA, The determination of ascorbic acid in whole blood and urine through 2,4 DNP derivative of dehydroascorbic acid. \textit{J.Biochem}. 147, (1943) 399.
32 Hill R, Smail DB, Murant RS, Leaky PB & Gibson SL, Hematoporphyrin derivative induced photosensitivity of mitochondria succinate dehydrogenase and selected cytosolic
