CHAPTER 5

PHOTODYNAMIC ACTIVITY ON HUMAN ERYTHROCYTES

In the preceding chapters, a brief survey of application of lasers in the medical field has been given. It has been indicated that specific tumour cell killing is possible by employing laser in conjunction with suitable sensitizer [49]. Though many researchers have carried out work in this line, the actual site of cell damage is not yet definitive [69]. In this context a study of photodynamic action on human erythrocytes is very important because the cell damage has necessarily to be confined to cell membrane or extra nucleus [70,71,72]. This is because mature erythrocytes lack nucleus. Further, erythrocytes in saline do not proliferate. Investigation on the photomodification of erythrocytes provide a semi-model system for the study of cancer cell regression using lasers, and help in developing proper protocol such as the right choice of laser, fluence, sensitizer and the sensitizer concentration. To the best of our knowledge this is the first report of such studies. The closest that comes to this work is by Monfrecola et al., [73] who has studied the effect of photohemolysis (PH) due to HPD and bonellin employing He-Ne laser.

5.1 EXPERIMENTAL PROTOCOL

In this study, erythrocytes are isolated from human blood, incubated with suitable sensitizer, irradiated with different lasers, and the damage was assessed. The photosensitizers used were Fluorescein (FL), Eosin-Y (EY), Rose Bengal (RB) (all belonging to xanthene family), HPD and
DHE (of porphyrin family), and Aluminum chloro sulphonated phthalocyanine (A1PCS) (representative of phthalocyanine family). The laser used were He-Ne laser of 15 mW (wavelength = 632.8 nm), Argon ion laser of 1 W (wavelength = 488 and 515.5 nm) and Nitrogen laser of 100 kW peak power (wavelength = 337.1 nm). The photohemolysis was studied as a function of concentration of sensitizer and for different fluence. Such analysis was carried out for all these sensitizers and for all these lasers. Investigations were carried out for the variation of photohemolysis as a function post incubation to look for delayed photochemical degradation of cells.

The erythrocytes were separated from plasma. This was obtained by taking 1 ml of blood and 1 ml of Isotonic Salt Solution (ISS), 149 mM of NaCl, 5 mM of Tris hydroxymethylaminomethane titrated to pH 7.4 with HCl and by mixing gently to avoid mechanical damage. The centrifugation was done at 1000 rpm for 10 minutes. The supernatant was sucked off and then discarded. The sediment was made up to 2.0 ml by adding ISS and this was once again washed. This process was repeated three to four times. Finally the sediment was made up to 40 ml by adding saline gently. 1 ml of this diluted erythrocyte suspension was taken and to this 1 ml of appropriate sensitizer was added and incubated in the dark for one hour. The diluted erythrocyte suspension at different experimental conditions were irradiated by expanding the laser beam to cover the entire surface area of the sample taken in the petri dish of diameter 2.2 cm.

Hemolysis was estimated from the amount of hemoglobin released from the erythrocytes. This was done by centrifuging the irradiated samples and measuring the O.D of
supernatant solution at 418 nm, the peak wavelength of absorption for hemoglobin by absorption spectrophotometer.

To obtain the rate of hemolysis in terms of percentage, 1 ml of diluted erythrocytes suspension was taken in a test tube containing 3 ml of distilled water. Due to the change in the permeability and pH, all the hemoglobin was released from the cell as a result of membrane rapture and the O.D measured at 418 nm under this condition corresponds to 100% hemolysis [72].

The erythrocytes were irradiated after sensitizing with different sensitizers to study the dependency of hemolytic rate as a function of irradiation time, concentration of the sensitizer, pre and post incubation and also the influence of oxygen.

5.2 PHOTOHEMOLYSIS BY LASER AND PORPHYRINS

The percentage of hemolysis of erythrocytes, labeled with HPD and DHE at different fluence (337.1 nm) is shown in Figure 5.1. The figure shows that the percentage of hemolysis increases with the fluence and the curve exhibits a sigmoidal shape with minimal effect at lower fluences and a saturation for high fluences. The rate of hemolysis was quantitatively determined by taking the slope at the linear portion of the graph (at 20 minutes) for DHE and HPD. Figure 5.1 shows the rate of hemolysis per unit time at this linear portion of the graph i.e., (dH/dt) at 20 µg/ml is 15% for HPD and 17% for DHE.

To study the dependency of sensitizer concentration at a fixed fluence, the samples were labeled with the photosensitizer over a range from 5 to 30 µg/ml. These
Figure 5.1 The percentage of hemolysis as a function of irradiation time at 337.1 nm for (a) HPD and (b) DHE
samples were then irradiated for 30 minutes. The percentage of hemolysis increases with increase of concentration and reaches the saturation beyond 10 to 15 μg/ml (Figure 5.2). The hemolytic efficiency at 30 minutes exposure for a concentration of 10 μg/ml, for DHE is 15% higher than that for HPD. It indicates that as the concentration increases, the number of photons absorbed by the sensitizer increases, resulting in higher photochemical reaction. At higher concentration the dye molecules shield the sample and affects the hemolytic rate. This may be the reason for the saturation at these concentrations [74].

In order to assess the dependency of pre and post incubation, the HPD and DHE are pre incubated for one hour and then they are irradiated as usual at different fluence. Figures 5.3 and 5.4 show the percentage of hemolysis as the function of irradiation time for samples with and without pre-incubation, for one hour. It is seen that preincubation increases the PH by 15% and in this respect the behavior of DHE and HPD are almost same.

In order to know whether there is any considerable delayed effect or not, the irradiated samples were post incubated for different duration (in hours). The hemolytic rate were measured at different post incubation time and are shown in Figures 5.5 and 5.6 for HPD and DHE respectively. From the figures it is observed that at 16 hour post incubation for 20 minutes of N2 laser irradiation, 100% hemolysis was seen for DHE whereas HPD has only 85%. This indicates that the DHE is having high hemolytic rate than HPD as in the case of PDA on different cells [75].

Since the tumours are usually growing under hypoxic condition, it is worth to check the photosensitizing
Figure 5.2 The percentage of hemolysis as a function of concentration (μg/ml) at 337.1 nm for (a) HPD and (b) DHE

Graph: A graph showing the percentage of hemolysis on the y-axis and concentration on the x-axis. The graph has two curves labeled HPD and DHE, with HPD being the lower curve and DHE the higher curve. The graph is labeled with "Exposure = 30 min."
Figure 5.3 The percentage of hemolysis as a function of irradiation time at 337.1 nm for HPD
(a) without preincubation
(b) with 1 hour incubation
Figure 5.4 The percentage of hemolysis as a function of irradiation time at 337.1 nm for DHE
(a) without preincubation
(b) with 1 hour incubation
Figure 5.5 The percentage of hemolysis as a function of irradiation time for different post incubation period at 337.1 nm for HPD
Figure 5.6 The percentage of hemolysis as a function of irradiation time for different post incubation period at 337.1 nm for DHE
properties of the sensitizers under the reduced oxygen condition. To do this, the samples were bubbled for 10 minutes by using $N_2$ gas as described by Moan et al., [76] and the irradiation was carried out as usual. Figures 5.7 and 5.8 shows the percentage of hemolysis as function of irradiation time by keeping aerobic and reduced oxygen concentration due to bubbling of nitrogen gas as the two different parameters for the dyes HPD and DHE respectively. From the Figures 5.7 and 5.8, it is seen that DHE is having higher reduction in photohemolysis than HPD, especially at higher irradiation time. In other words DHE mediated photohemolysis is more sensitive to oxygen concentration in comparison with HPD based photohemolysis.

The same experiment was repeated for all the parameters with He-Ne laser of wavelength 632.8 nm, energy density of 4 mJ/cm$^2$ and Ar ion laser of wavelength 514.5 nm of 10 mJ/cm$^2$, in order to compare the relative effect of these two lasers.

A convenient way of such intercomparison is hemolytic rate, $dH/dt$ (percentage of hemolysis per minute) for a given concentration of the sensitizer. Since among the laser used, $N_2$ is pulsed with average power of 1 mW, though peak power is 100 kW, and others are CW lasers with power of 15 mW and 40 mw, we have taken time of exposure, a practical unit for the measure of laser effect. This was calculated by taking the slope in the linear portion of the graph, at 20 minutes exposure time. Similarly ($dH/dt$), the percentage of hemolysis at 10 $\mu$g/ml concentration of the sensitizer, PH at 16 hours of post incubation after 20 minutes of exposure and variation in PH for pre incubation and $N_2$ gas bubbling are summarized for these two dyes, DHE and HPD in Table 5.1 for all these three lasers.
Figure 5.7 The percentage of hemolysis as a function of irradiation time at 337.1 nm for HPD
(a) due to nitrogen gas bubbling
(b) aerobic condition
Figure 5.8 The percentage of hemolysis as a function of irradiation time at 337.1 nm for DHE (a) due to nitrogen gas bubbling (b) aerobic condition
Table 5.1 The values of percentage of hemolysis for HPD, DHE, FL, EY and RB at 337.1 nm for different experimental conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPD</th>
<th>DHE</th>
<th>FL</th>
<th>EY</th>
<th>RB</th>
<th>A1PCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH/dT</td>
<td>15</td>
<td>17</td>
<td>2</td>
<td>5</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>PH(C)</td>
<td>60</td>
<td>73</td>
<td>8</td>
<td>14</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>PH(Pre)</td>
<td>14</td>
<td>15</td>
<td>8</td>
<td>11</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>PH(O$_2$)</td>
<td>11</td>
<td>15</td>
<td>3</td>
<td>11</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>PH(Post)</td>
<td>85</td>
<td>100</td>
<td>18</td>
<td>27</td>
<td>26</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 5.2 The values of percentage of hemolysis for HPD, DHE and AlPCS at 632.8 nm for different experimental parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPD</th>
<th>DHE</th>
<th>AlPCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH/dT</td>
<td>7</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>PH(C)</td>
<td>42</td>
<td>57</td>
<td>24</td>
</tr>
<tr>
<td>PH(Pre)</td>
<td>10</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>PH(O₂)</td>
<td>7</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>PH(Post)</td>
<td>67</td>
<td>74</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 5.3 The values of percentage of hemolysis for HPD, DHE, FL, EY and RB at 514.5 nm for different experimental parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPD</th>
<th>DHE</th>
<th>FL</th>
<th>EY</th>
<th>RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH/dT</td>
<td>10</td>
<td>12</td>
<td>8</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>PH(C)</td>
<td>51</td>
<td>58</td>
<td>19</td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td>PH(Pre)</td>
<td>16</td>
<td>15</td>
<td>6</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>PH(O₂)</td>
<td>6</td>
<td>9</td>
<td>6</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>PH(Post)</td>
<td>62</td>
<td>83</td>
<td>54</td>
<td>57</td>
<td>71</td>
</tr>
</tbody>
</table>
From Table 5.1 to 5.3, it can be seen that, in the case of HPD and DHE, DHE is 1.1, 1.9 and 1.2 times more effective than HPD at 337.1 nm, 632.8 nm and 514.5 nm respectively. Note that the fluence for 20 minutes exposure for $N_2$ laser is 1.2 J/cm$^2$, for He-Ne it is 4.8 J/cm$^2$ and for Ar ion laser it is 12 J/cm$^2$. Among the three lasers, $N_2$ laser is having higher hemolytic efficiency both for HPD and DHE than that at other wavelengths. This implies that even at lower fluences comparing to Ar ion laser and He-Ne laser, $N_2$ laser has higher effectiveness both for DHE and HPD. This is apparently due to the high absorption cross-section of the dyes at these wavelengths and clearly indicates the photochemical nature of reaction.

In terms of concentration, i.e., at a fixed concentration of 10 ng/ml for 30 minutes exposure time, DHE is having higher percentage of hemolysis for all these wavelengths and this is high at 337.1 nm.

Though pre incubation doesn't show up any considerable difference between DHE and HPD, in the case of post incubation, it is seen that at 16 hours post incubation, DHE has 100% hemolysis whereas HPD has only 85% and similar trend holds good for all other wavelengths.

In the case of reduced oxygen concentration, it is seen that, there is no great variation for HPD for 20 minutes exposure, but there is considerable reduction in PH for DHE. This indicates that DHE efficiency decreases with decrease in the concentration of the sensitizer and this trend holds good independent of nature of laser for excitation.
Since porphyrins are having poor absorption at red region, many sensitizers have been under investigation for maximum absorption at red region in order to increase the penetration length of the optical radiation in the tissues. In this point of view, pthalocyanines were introduced for PDA, especially its derivative A1PCS, which has good absorption at 675 nm and considerable amount of absorption both at 337 and 630 nm. Hence, A1PCS was also studied for all the experimental conditions described for DHE and HPD, and intercomparison was made.

5.3 PHOTOHEMOLYSIS DUE TO A1PCS

When the photohemolysis due to A1PCS is compared with that of HPD or DHE, percentage hemolysis of A1PCS, both at the wave-lengths 632.8 nm and 337.1 nm was very less effective in all respect, i.e., dependency of fluence at 10 μg/ml, concentration for 30 minutes, pre and post incubation condition. On the other hand certain other photohemolysis properties of A1PCS are quite interesting. The PH with or without pre-incubation is the same. This clearly indicates that the dye molecules which are responsible for hemolysis of erythrocytes are exogenous i.e., acting externally without binding. This agrees with the results of Ben-Hur et al., [77] from their study of PDA on Chinese hamster cell line. This differs from DHE and HPD (Figures 5.3 to 5.9) which bind on the membrane.

If the oxygen dependency was seen by N₂ gas bubbling, it is seen that there is no measurable difference in percentage hemolysis between aerobic and reduced oxygen condition (both for the He-Ne and N₂ laser). This indicates that though A1PCS is having low hemolytic rate, it is effective even in the hypoxic condition (Figure 5.10).
Figure 5.9 The percentage of hemolysis as a function of irradiation time at 337.1 nm for A1PCS, EY (a) 1 hour preincubation (b) no preincubation
Figure 5.10 The percentage of hemolysis as a function of irradiation time at 337.1 nm for AlPCS, EY (a) aerobic condition (b) due to nitrogen gas bubbling
5.4 PHOTOHEMOLYSIS DUE TO XANTHENES

It is seen from the sections 5.2 and 5.3 that DHE is more effective than HPD, and AlPCS has least effectiveness both at 337.1 and 632 nm, when compared to porphyrins (Tables 5.1 to 5.3). Since HPD has high retaining time and requires more time for specific accumulation at tumour, one has to wait for longer time to undergo treatment (24 to 72 hours) and avoid exposure from the sun which may produce photoallergies. Further HPD has neuro toxicity and poor absorption at red region. This led many to try on AlPCS. But for therapy i.e., for clinical purposes, so far it was not tried for want of pharmacological data. This led to go for other dyes [78,79,80]. In this context, xanthenes (FL, EY and RB) were studied for all the experimental conditions as in the case of porphyrins.

Tables 5.1 to 5.3 give the values of \( \frac{dH}{dt} \), the percentage of hemolysis for 30 minutes exposure at 10 \( \mu g/ml \), variation in pre incubation, oxygen influence etc.

From the Tables 5.1 and 5.3, it is seen that though the effectiveness of xanthenes are not as good as porphyrin, EY and RB are comparable with HPD. Among the three xanthenes, RB is having higher effectiveness than EY and FL.

Since PDA depends upon the triplet quantum yield and the later depends on the presence of heavy atoms in the sensitizers, Shea et al., [81] compared the effectiveness of rhodamine 123 with tetrabromo-R123 (TBR). In this respect, a study was carried out for xanthenes to know whether heavy atoms play a role in photomodification of cellular damage. For these the samples were irradiated at 337.1 nm for a
fixed optical density (O.D = 1) for all the three sensitizers. This ensures that the number of dye molecules excited to be the same. From Figure 5.11, it is seen that RB is having higher percentage of hemolysis, the next comes EY and the least one is FL.

5.5 DISCUSSION

Before introducing a new photosensitizer for clinical trial, it is necessary to check for their toxicity, effectiveness at different wavelengths and its delayed effect. For this purpose photohemolysis is used as a semi-model system. Since the release of hemoglobin is exclusively due to membrane damage, one can utilize this technique to know the effectiveness of the photosensitizer in the membrane damage. It is in this context, the photohemolysis effectiveness was studied for three different families of sensitizers before going for an in-vitro and in-vivo study on tumours.

Since porphyrins are used clinically, this was compared with AlPCS and xanthenes, under a few laser excitation conditions. The findings are as follows

Among all the sensitizers, DHE is more effective than HPD, FL, EY, RB and AlPCS. For example, for N₂ laser and for a concentration of 10 μg/ml, there is 73% for DHE hemolysis, 60% for HPD, 8% for FL, 14% for EY and 20% for RB and 24% for AlPCS. This indicates that at 337.1 nm both xanthenes and AlPCS have comparable percentage of hemolysis at 10 μg/ml and they are less than porphyrins. These differences are mainly due to difference in the absorption of laser wavelength.
Figure 5.11 The percentage of hemolysis as a function of irradiation time at 337.1 nm for FL, EY and RB at O.D = 1.
In order to verify the above mentioned dependency of PH on wavelength of absorption, the samples were irradiated using dye lasers of different wavelengths but of same energy. The sensitizer used was EY and results are shown in Figure 5.12. It can be seen that photohemolysis is the highest around 510 nm, which corresponds to the peak absorption of EY, least at 630 nm where absorption is also least and intermediate at 415 nm.

In the case of pre-incubation, porphyrin (both HPD and DHE) produces 10% photohemolysis in general, whereas, the AlPCS doesn't show any effect, which clearly indicates that AlPCS act exogeneously when compared to porphyrin and xanthene(Figures 5.3 and 5.9)

Both porphyrins and xanthenes are depending upon oxygen content (not very high) on the other hand AlPCS doesn't show any difference, this indicates, AlPCS may be used even in hypoxic condition. Among all the dyes DHE is most sensitive to the oxygen.

In order to assess the possibility of delayed secondary hemolysis due to the post incubation period, the irradiated samples were kept in dark. Figures 5.5, 5.6, 5.13-5.16 and Tables 5.1-5.3 show the photohemolysis dependency on post incubation period and percentage of hemolysis as a function of post incubation period for all the sensitizers respectively. It is seen that, in this aspect also DHE is having high hemolysis rate than other sensitizers i.e., percentage hemolysis for DHE>HPD>RB=EY>AlPCS>FL. From the above Figures, the saturation in the hemolytic rate indicates the complete hemolysis. The post incubation hemolysis is due to the osmotic change and cross-linking of biomolecules [82]. This
Figure 5.12 The percentage of hemolysis as a function of irradiation time at different wavelengths for EY, FL and RB.
Figure 5.13 The percentage of hemolysis as a function of irradiation time for different post incubation period at 632.8 nm for HPD
Figure 5.14 The percentage of hemolysis as a function of irradiation time for different post incubation period at 632.8 nm for DHE.
Figure 5.15 The percentage of hemolysis as a function of irradiation time for different post incubation period at 514.5 nm for HPD
Figure 5.16 The percentage of hemolysis as a function of irradiation time for different post incubation period at 514.5 nm for DHE
linking may be initiated by the molecule suffering primary excitation which leads to a slow chain reaction and denaturation. This agrees with those of van Steveninck et al., [83] who observed the delayed effect on proteins and erythrocytes. The observations made above hold good for other cases.

The variation in the percentage of hemolysis among xanthenes (Figure 5.11) clearly indicates that, due to presence of heavy atoms, Iodine in RB and Bromine in EY enhances the hemolytic rate and the less in FL may be due to the absence of heavy atoms. This agrees with the result of Oshea et al [81]. Hence, the dependency of photohemolysis on concentration, pre and post incubation and the presence of heavy atoms clearly indicate that the damage may be due to photochemical effect only and not a thermal one.

The results of protocol and optimization of operational parameters for PDA on erythrocyte are discussed in this chapter. With the experience and insight gained therein, further study was done on tumour cells which are presented in subsequent chapters.