PHOTODYNAMIC ACTIVITY ON HUMAN ERYTHROCYTES

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ABSTRACT

Photohemolysis of erythrocytes using five lasers of different wavelengths and two photosensitizers viz. acridine orange and fluorescein was studied. The per cent hemolysis in the presence of laser and sensitizer was higher when compared to laser alone. Of the two sensitizers, acridine orange had a higher effect in photomodification of cells than fluorescein. Hemolysis was found to increase towards shorter wavelengths. Per cent hemolysis depends on post incubation time, indicating the photochemical nature of the damage due to the delayed photo-oxidation via the cross-linking between biomolecules.

INTRODUCTION

PHOTODYNAMIC action (PDA), also termed as photoradiation therapy (PRT), is a relatively new method of treating malignant tumours by a combination of light and a photosensitizing agent in the presence of molecular oxygen. The use of lasers in PDA has assumed great importance because of its specificity and its capability to regress tumours even in internal organs. The reaction mechanism accepted for inactivation of biological systems is an energy transfer process from the excited triplet state of sensitizer to oxygen, producing singlet oxygen, which causes irreversible oxidation of essential cellular components[1]. The schematic of this PDA may be represented as

$$S + h\nu \longrightarrow {}^1S^*,$$

$${}^1S^* \longrightarrow S + h\nu',$$

$${}^1S^* \longrightarrow {}^3S^*,$$

$${}^3S^* + {}^3O_2 \longrightarrow S + {}^1O_2^*,$$

$${}^1O_2^* + X \longrightarrow X(0).$$

Where S represents the sensitizer, O the molecular oxygen and X the cellular substrate. The superscript 1 represents the singlet state, 3 the triplet state, and * the excited state.

The characterization of this PDA is still incomplete. For example, the localization of sensitizer, the site of damage at the cellular level has not been precisely specified[2–4]. This may be due to the inhomogeneity of the cells, which contain many microenvironments with different physicochemical properties. Because of these complexities, very little is known about the details of photosensitized reactions in cells or in specific lesions which results in cell injury and death. A preliminary approach to the study of such complexity is to work with the so-called model system that stimulates certain properties of the living cells.

In this connection, the photohemolysis of human erythrocytes (a simple model system) has been studied using different lasers in combination with acridine orange (AO) and fluorescein (FL) as photosensitizers. Since photoinduced cellular damage is always on the membrane it is hoped this study would improve our understanding of the mechanism of photodynamic regression of malignancy.

MATERIALS AND METHODS

Human blood (obtained from the Cancer Institute, Madras) and the erythrocytes were washed thrice with tris buffer of pH 7.4. The buffy coat was removed after the final spin. The washed erythrocytes were diluted in buffered saline in the ratio 1:29.

The photosensitizers AO and FL were used in the micromolar concentrations. The solutions were prepared using tris buffer. Lasers were used as irradiation source at five different wavelengths and its characteristics are summarized in table 1.

Nitrogen laser

Nitrogen laser used was constructed in our laboratory for dye laser gain studies. This provides an intense UV laser pulses of 100 kW peak power, with a pulse duration of 10 ns at 337.1 nm at a repetition rate of 1 Hz. This laser is used to irradiate samples and generate dye lasers at different wavelengths by transverse pumping of dye solution as shown in figure 1.

Table 1 *Details of lasers*

Laser and wavelength (nm)	Mode	Power (kW)	Pulse duration (ns)	Pulse energy (mJ)	Divergence (mr)	Spectral brightness ($MW.m^{-2}Sr^{-1}$)
Nitrogen (337.1)	Pulsed	100	10	1 0	10	3
Nitrogen pumped dye lasers						
POPOP in Toulene (415)	Pulsed	20	5	0 1	15	3000
C 7 in methoxy ethonal (505)	Pulsed	20	5	0.1	15	3000
Rhod B in ethonal (630)	Pulsed	20	5	0 1	15	3000
All these lasers work in Superradiant mode						
He-Ne	CW	15	CW	15	1	300

Dye lasers

One significant advantage of dye laser is the possibility through proper dye selection for obtaining intense and coherent light at almost any wavelength from 350 nm to 1000 nm. Also the lasing wavelength of a single dye laser is continuously tunable over a few hundred angstroms, either by varying the solution or cavity parameters. The dye lasers obtained by pumping the dye transversely using nitrogen laser, and their corresponding solvents are given in table 1. He-Ne laser (Spectra Physics, USA) works at 632.8 and delivers 15 mW continuous power.

Estimation of photohemolysis

The incubated erythrocytes were irradiated by expanding the laser beam to cover the entire surface area of the sample. The irradiated samples were centrifuged and the amount of haemoglobin released from the cell suspension was measured in terms of the optical density of the supernatant of the centrifuged solutions. The per cent hemolysis (PH) was measured as

$$\frac{\text{O.D of haemoglobin in experimental samples}}{\text{O.D of haemoglobin due to distilled water}} \times 100.$$

RESULTS AND DISCUSSION

Figure 2 shows PH as a function of nitrogen laser (337.1 nm) irradiation time with and without the sensitizer AO of 10 μM concentration. The PH increases by more than 40% when the sensitizer labelled cells were irradiated (figure 2). The 5% PH due to the nitrogen laser alone, may be due to the slight absorption of UV radiation by water and its content of amino acids.

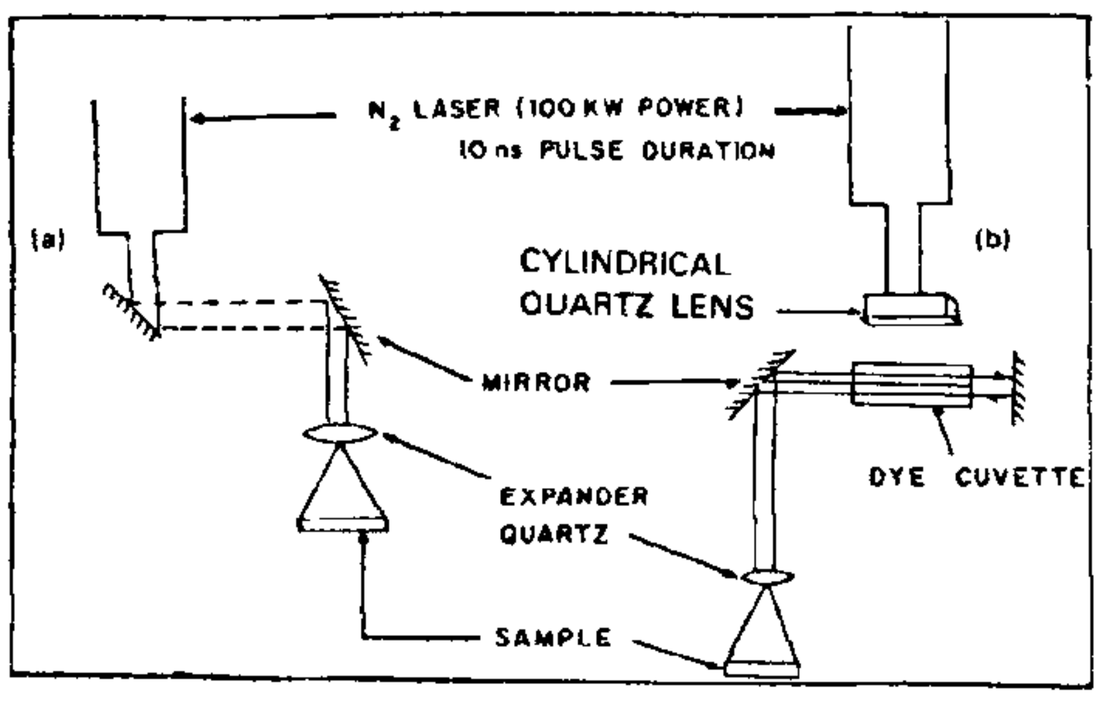

Figure 1a, b. (a) nitrogen laser set up. (b) Dye laser set up.

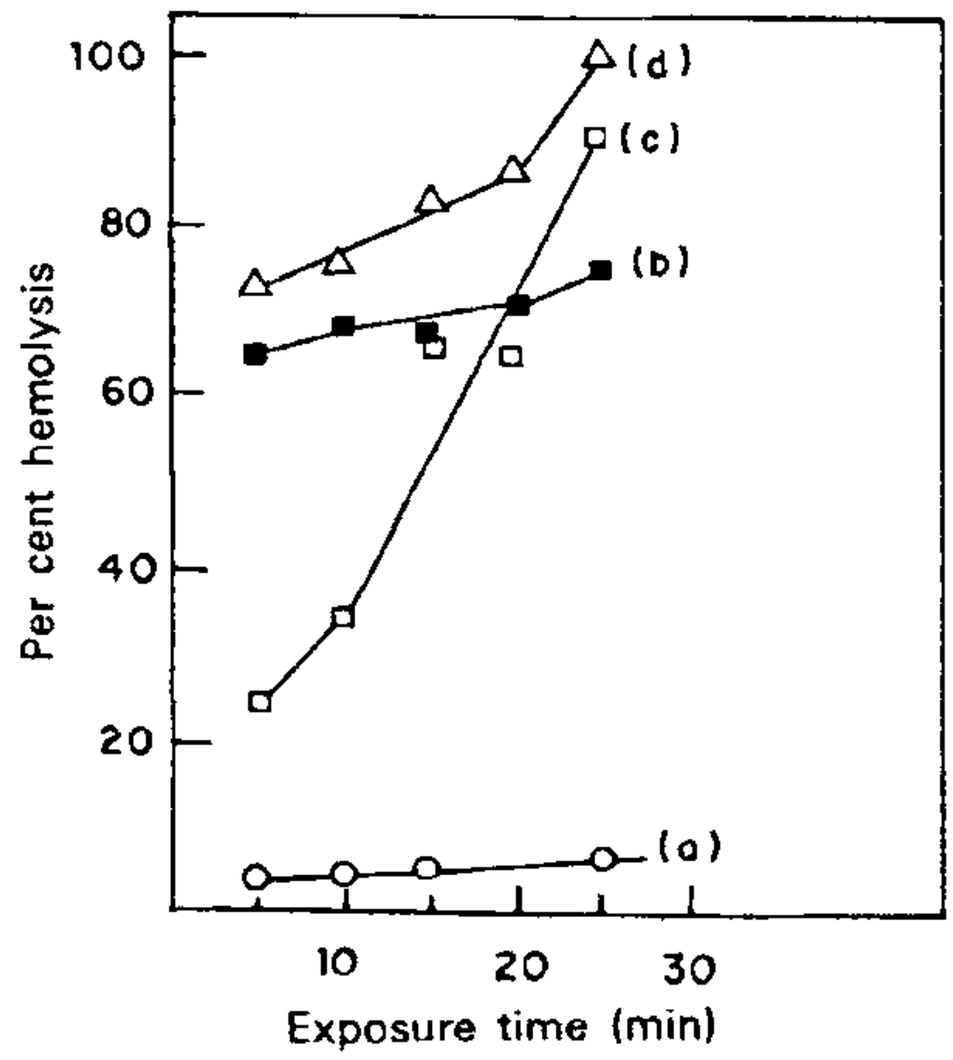

Figure 2. Erythrocytes irradiated with: (a) N_2 laser alone; (b) N_2 laser alone (after 24 h post incubation); (c) N_2 laser with 10 μM AO; (d) N_2 laser with 10 μM AO (after 24 h PI).

An eight-fold increase in PH was noticed due to the presence of the sensitizer (figure 2). It is found that AO penetrates the cell membrane and accumulates either on the nucleus (wherever the latter is present) or around the cytoplasm. Under this situation, the enhanced PH due to the photosensitizers may be viewed as follows: The laser photons penetrate through the cell membrane (note that the cell membrane has little absorption for the laser wavelengths used) and reach the AO, and raise AO to the higher excited state. Some of the AO exist in the excited singlet state but many of them relax to the triplet state. The photo-oxidation of the inner wall of the cell membrane originates from these excited triplet AO in large proportion and singlet AO in small proportion.

The above hypothesis gets reinforced from the results shown in figure 3 where the PH vs irradiation time with varying sensitizer concentrations. It clearly shows that the PH is higher at higher concentrations of the sensitizer.

The PH with fluorescein observed as a sensitizer under similar conditions is shown in figure 4 for comparison. It is seen that the PH is better with AO than with FL. This may be attributed to the lower triplet yield of FL in comparison with AO[5,6]. This indicates that PH increases with increasing triplet yield of the sensitizer.

Erythrocytes were irradiated with He-Ne laser in the presence of 10 μM concentration of AO. Figure

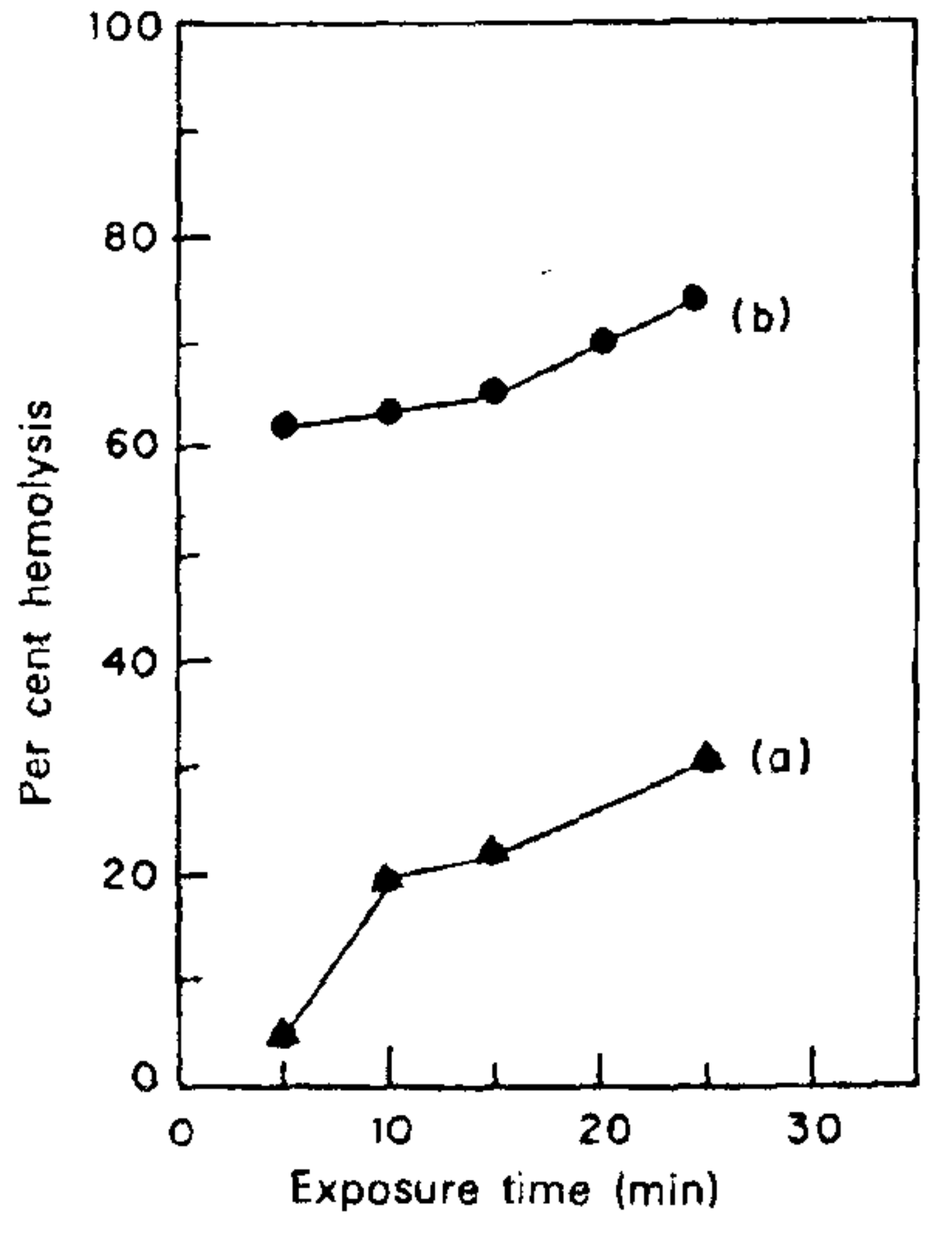

Figure 3. Erythrocytes irradiated with N_2 laser (a) with 5 μM AO, (b) with 10 μM AO.

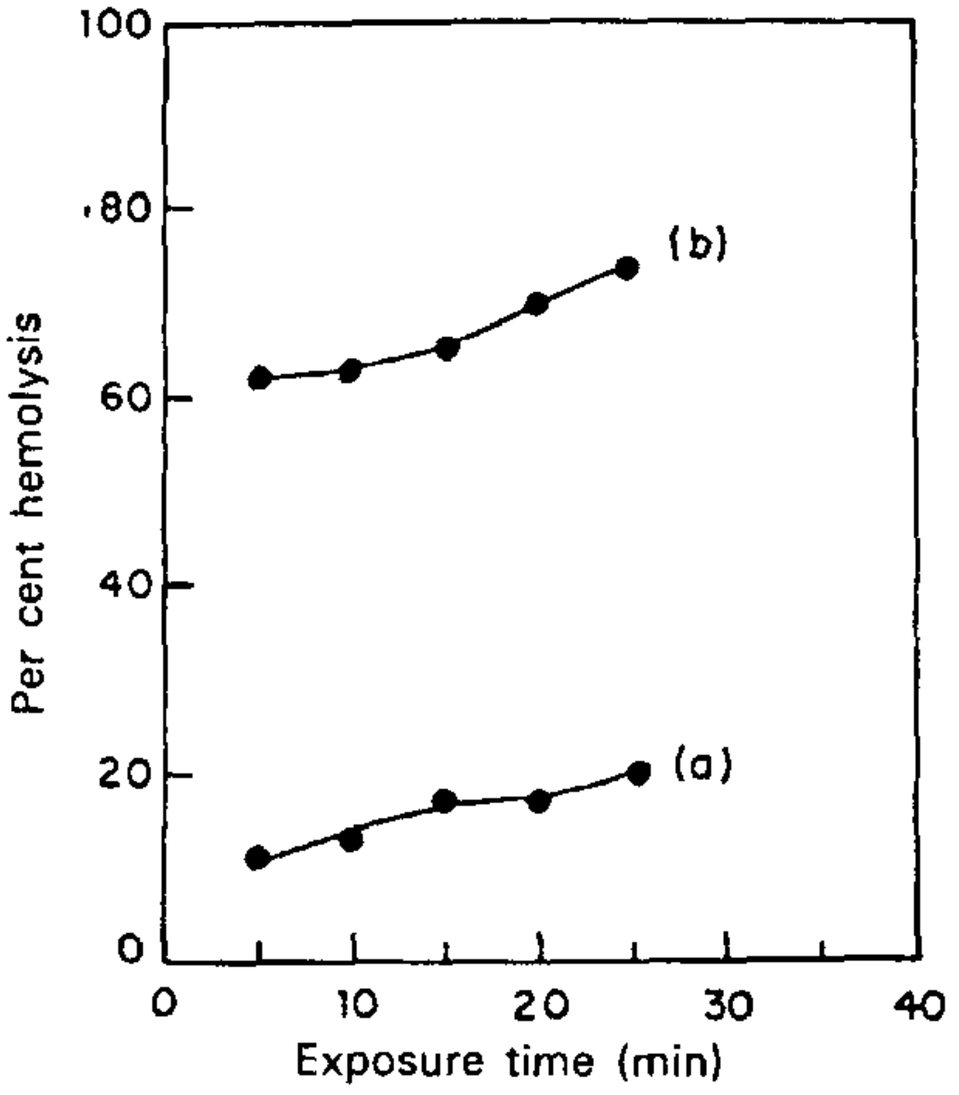

Figure 4. Erythrocytes irradiated with N_2 laser in conjunction with (a) 10 μM FL; (b) 10 μM AO.

4 shows the PH due to He-Ne laser. It may be noted that the total energy delivered by the He-Ne laser for 10 min exposure is 9 Joules; and by the nitrogen laser, it is 10 times less than that due to the He-Ne laser. The cell membrane and the sensitizer AO are transparent at 632.8 nm. Hence the probable way of hemolysis is due to vibrational excitation of cellular molecules at 632.8 nm and may also be due to the heating of water molecules at 632.8 nm leading to cell membrane damage. On the other hand, the dye has considerable absorption at the nitrogen laser wavelength, 337.1 nm and would lead to electronic excitation of the molecules.

To assess the dependence of PH on the wavelength used, experiments were carried out to measure the PH as a function of wavelength (using pulsed dye lasers of the same energy and keeping all other parameters unaltered). Figure 5 shows that the PH is only about 50% around 630 nm but increases rapidly as the wavelength of the laser used shifts towards the shorter region. Around 630 nm neither the biomolecules of the membranes nor the dye has good absorption. This 50% is attributed to the absorption of photon by haemoglobin and is also due to local heat generation on the cells. The PH due to pulsed laser at 630 nm was 50% whereas the PH due to CW laser (633 nm) was only 10% for 10 min exposure time (figures 4 and 5). This is because the number of photons from a pulsed laser per unit time is very large as compared to CW red laser. Thus it shows a pulsed red laser of small energy content in each pulse has better PH effect

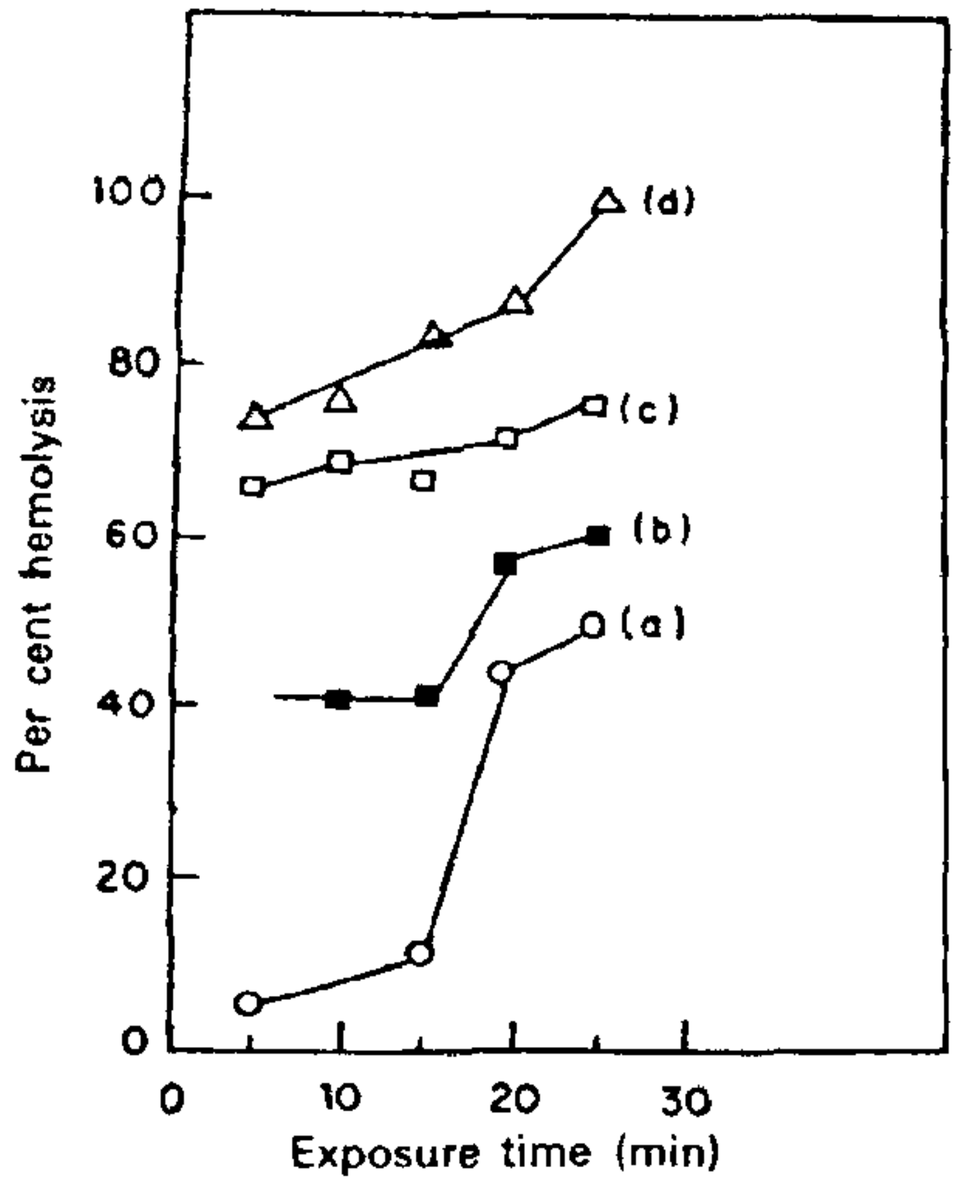

Figure 5. Erythrocytes irradiated with: (a) He-Ne laser in conjunction with 10 μM AO; (b) He-Ne laser in conjunction with 10 μM AO (after 24 h PI); (c) N_2 laser in conjunction with 10 μM AO; (d) N_2 laser in conjunction with 10 μM AO (after 24 h PI).

than a CW red laser of the same wavelength.

An attempt was made to study the effect of hemolysis by estimating the photohemolysis after irradiation as well as after a lapse of a few hours of incubation in dark. Figure 6 shows the result of PH as a function of incubation period, with energy of

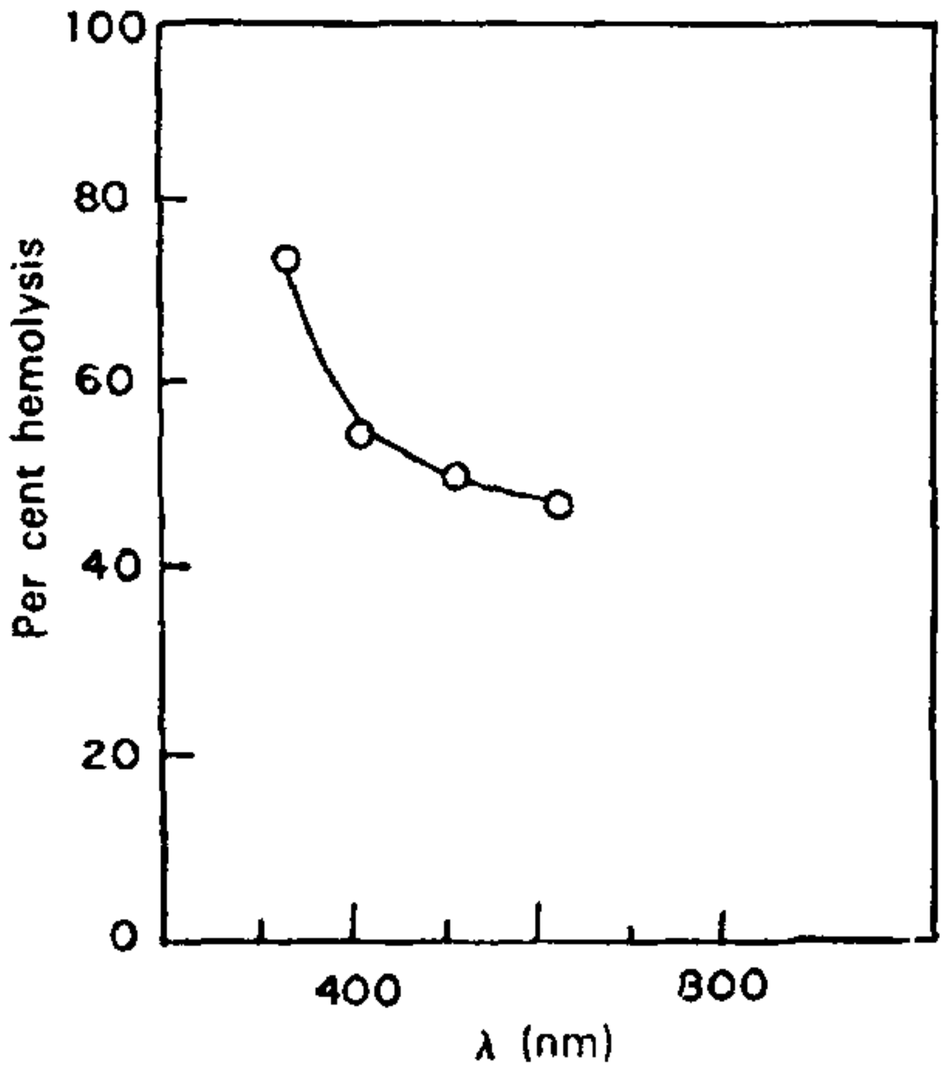

Figure 6. Erythrocytes irradiated at different wavelengths (pulsed lasers) in the presence of 10 μM AO.

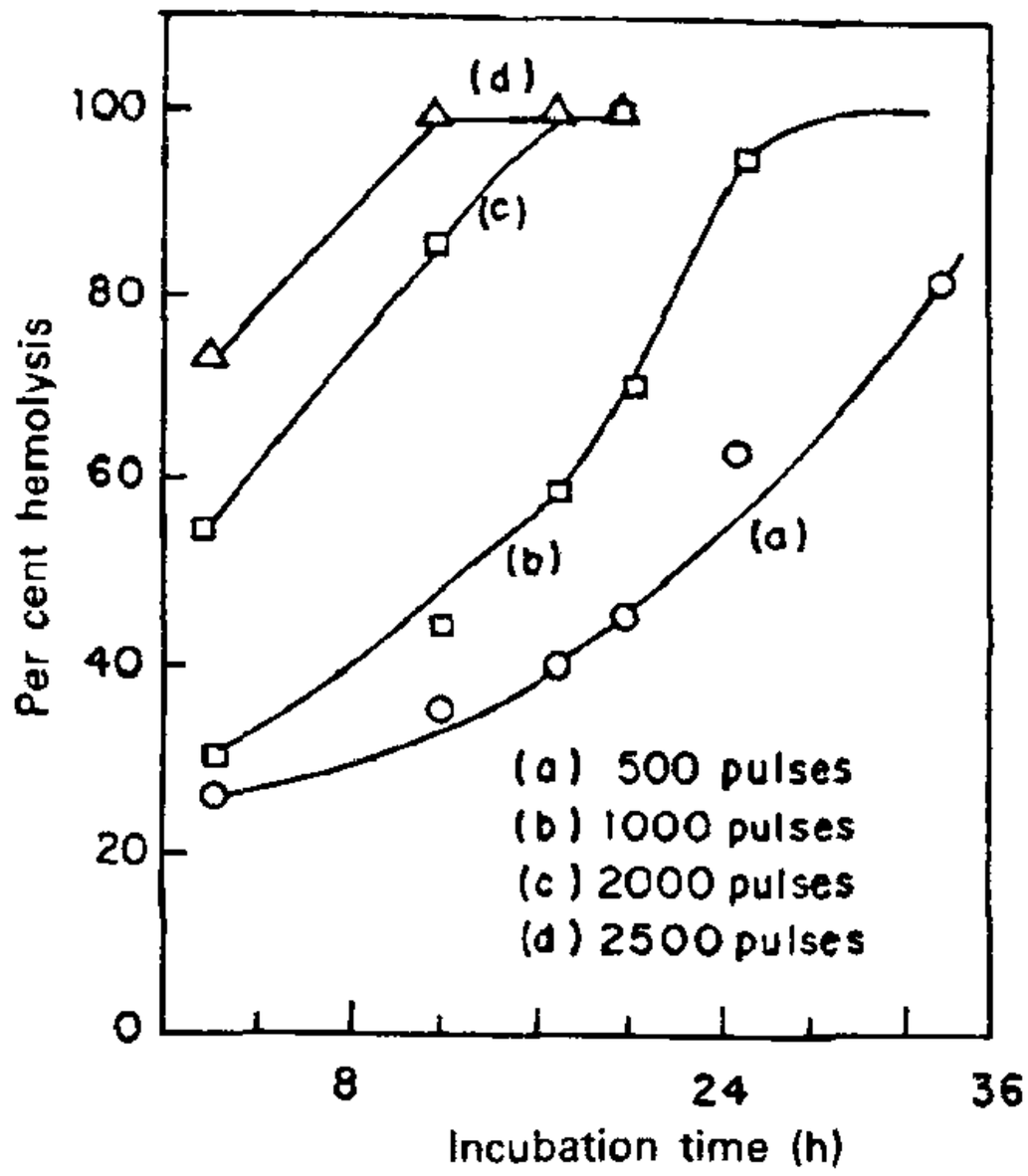

Figure 7. Erythrocytes are irradiated with N laser in conjunction with 10 μM AO and the PH is estimated at different post incubation time.

irradiation as varying parameter and indicates that PH increases rapidly with incubation period reaching a saturation for higher energy dosage/longer incubation period and indicating complete hemolysis. This might be due to the slow cross-linking between biomolecules leading to denaturation and degradation. The present results agree with those of Doubbleman *et al*[7] who observed that photo-oxidation of protein molecules when irradiated in conjunction with photosensitizers was continued even after irradiation (when kept in dark).

CONCLUSION

The present study indicates that the PH is mainly dependant on: (i) Wavelength and dosage of laser used; (ii) Type of sensitizer and its concentration (here AO has higher effect than FL), and (iii) Post incubation time.

ACKNOWLEDGEMENTS

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ANNOUNCEMENTS

INTERNATIONAL CONFERENCE ON BIODETERIORATION OF CULTURAL PROPERTY

The above conference will be held at the National Research Laboratory for Conservation of Cultural Property, Lucknow, India from 20 to 25 February 1989 sponsored by ICCROM and INTACH.

The conference will cover the following areas: a. Effect of micro-organisms and plant growths; b. Insects and rodents: On different art materials like wood, leather, parchment, fur, paper, photographic material, textiles, metals, wall paintings, oil paintings, building materials and monuments; c. Biodeterioration of various restoration materials, like adhesives and resins, canvas, paper, etc; d. Use of chemicals, radiation and other techniques for control of micro-organisms/insects, etc. in museums, and e. Ecology of biodeterioration of museum materials.

For further details please write to the Convener, Organizing Committee, International Conference on Biodeterioration of Cultural Property, National Research Laboratory for Conservation of Cultural Property, Sector E/3, Aliganj Scheme, Lucknow 226 020, India.

MODERN TRENDS IN OPTICAL WORKSHOP PRACTICES/TEST AND EVALUATION

Engineering Staff College of India, Visvesvaraya Bhavan, Khairatabad, Hyderabad, has organised a course on "Modern Trends in Optical Workshop Practices/Test and Evaluation from July 7 to 13 1988. The venue of the course is The Institution of Engineers (India), Karnataka State Centre, No. 3, Dr Ambedkar Veedhi (Vidhana Veedhi), Bangalore 560 001.

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