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PHOTOBIOSTIMULATION AS A FUNCTION OF DIFFERENT WAVELENGTHS

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In the current study we compare the effect of different light sources in the visible and near infra-red (IR) range on cell stimulation. It is obvious that in order to interact with the living cell, light has to be absorbed by intracellular chromophores. In a search for chromophores responsible for photobiostimulation, endogenous porphyrins, mitochondrial and membranal cytochromes were found to be suitable candidates, as they possess absorption bands in the visible and near I.R. ranges. The above-mentioned chromophores are photosensitizers that generate reactive oxygen species (ROS) following irradiation. In our opinion the first step in photobiostimulation might be ROS formation. To confirm ROS formation by various light sources, we used the electron paramagnetic resonance (EPR) associated with spin trapping techniques. All wavelengths used (360, 630, 660, 830nm), including a broad band in the visible range (400-800nm), stimulated hydroxyl radical formation in sperm cells. Measuring the amount of OH radicals as a function of the irradiating wavelength shows that shorter wavelengths might be more effective on the cell than longer ones.

Key words: Photobiostimulation, ROS, Laser Therapy, Wavelength

Introduction

A large number of reports document significant positive effects of low energy laser (LEL) radiation on biological systems at the biochemical level. And many patients who responded favorably to LEL therapy are among those who did not respond to standard medical treatments. Various wavelengths (wl) in the visible and near IR are being used in the laboratory and in the clinic for activating the cell. The most common light source for photobiostimulation is the He-Ne (632nm) laser, but many other lasers such as Ar+(488nm), GaAlAs-diode-laser (805nm) and light emitting diodes (660, 780, 830 and 904nm) are successfully used as well.

In the current study we wanted to assess whether the cell responds differently to various wl in the visible range. Because we believe that the first step in photobiostimulation is ROS formation, we estimated the amount of ROS in irradiated cells as a function of the irradiating wl.

Unfortunately, the detection of ROS in biological systems is not simple because of their very short lifetime. An indirect way of measuring ROS is by using a probe molecule, which reacts with the ROS to give a long-life product that may be detected spectroscopically. For example, 9,10 dimethylanthracene (DMA) serves as a molecular trap for $^1O_2$ [1]. Another example of this approach is the EPR spin trapping technique. The addition of an appropriate diamagnetic compound called a spin trap to a certain ROS results in a long-life secondary free radical, called a spin adduct, which may be detected by the EPR technique [2]. In this study we have followed hydroxyl radical formation in irradiated sperm cells as a function of the wl, using the EPR spin trapping technique.

Materials and Methods

EPR measurements:

We used 5,5 dimethyl-1-pyrroline-N-oxide (DMPO) to trap the hydroxyl radicals. DMPO (0.02M) was added to 106-107 bovine sperm cells/ml in a 96 multiwell dish and the cells were irradiated with various light sources. Then, 100 ml of the cell culture was drawn with a syringe into a gas-permeable teflon capillary (Zeus Industries, Raritan, NJ), of 0.032" inner diameter, 0.015" wall thickness, and 15 cm length. Each capillary was folded twice, inserted into a narrow quartz tube which was open at both ends, and placed into the EPR cavity. The EPR spectra were recorded on a Bruker ER 100d X-band spectrometer. The microwave of the EPR was set at 9.67GHz and the power at 20 mW. Modulation frequency and modulation amplitude were 100 KHz and 1G respectively, and the swept width was 65G. Time constant (TC) was 655 ms and measurements time, 168 s.

Irradiation

For the illumination we used: a) A VL-206BL light source emmitting UVA light at 320-400nm with a maximum at 360nm, the irradiance being 1.5mW/cm^2 and time of irradiation 1min. b) An He-Ne laser (632nm), 2mW/cm^2 for 5 min c) A multiwave band (400-800nm) centered at a

600nm light device, 40mW/cm² for 4 min d) A 660nm light emmitting diode 10mW/cm² for 7 min e) A 830nm light emmitting diode (Lasotronic), 26mW/cm² for 9min.

Results

Irradiation of sperm cells with visible or UVA light in the presence of DMPO produces a four-line EPR spectrum with hyperfine splitting ($_aN=_aH=14.9G$) characteristic of a DMPO-OH spin adduct. In Fig. 1, an example of an EPR spectra of DMPO-OH before and after illumination of sperm cells with a 830nm diode laser 26mW/cm², for 9 min is depicted.

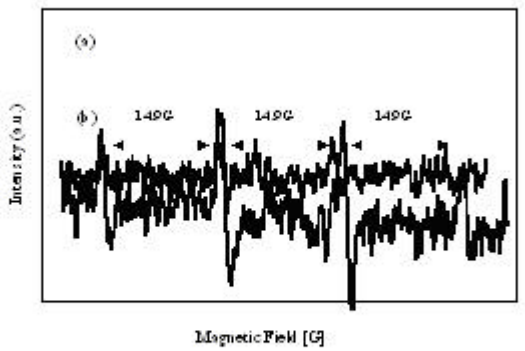

Fig 1: EPR spectra of sperm cells, (a) Before irradiation. (b) After 9 min of illunination with 830 nm diode laser. The spectra parameters were set to: receiver gain $2*10^5$; sweep width 57 G; resolution 2048; conversion time 82 ms; time constant 655 ms; sweep time 168 s; power 20 mW: number of scan 3; frequency 9.7 GHz.

The amount of OH radicals is proportional to the integrated area under the EPR signal. Assuming a linear dependence of the generated hydroxyl radicals on cell number and fluence of the light source, we normalized the first peak area (of the DMPO-OH quartet) to one cell/cm² illuminated with an energy dose of 1J/cm². The normalized area for each wl is shown in Figure 2. It can be seen that the 660nm diode and the He-Ne laser produce similar amounts of OH radicals. The broad band visible light device produces half of this amount, and the 830nm light emitting diode produces a very small amount of hydroxyl radicals. As expected, the UVA light generates a huge amount of OH radicals in comparison with light in the visible range.

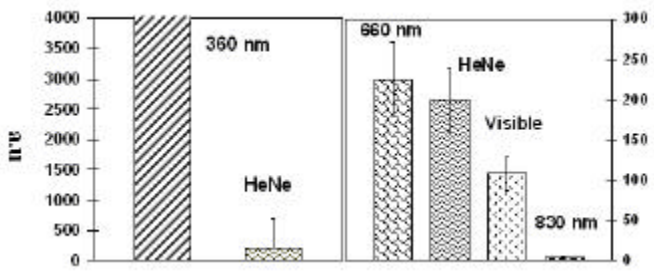

Fig 2: Integrated area of DMPO spin adduct signal normalized to the cell number (cell/ml) and the fluence (J/cm²), for the various illuminating light sources.

Discussion

Although the mechanism of light-induced cell activation is still under debate, it is strongly believed that ROS are involved in light-enhanced biostimulation. [3,4,5]

Recent evidence demonstrates that relatively low and controlled concentrations of ROS play an important role in the activation of many cell processes. For example, ROS stimulates fibroblast proliferation [6] and DNA synthesis in the haemopoietic cell line U937. [3] In the case of spermatozoa, ROS such as a superoxide anion, H_2O_2, and nitric oxide were found to induce sperm hypercapacitation and acrosome reaction. [7] Suzuki et al. [8] have shown that ROS stimulate signal transduction processes for transcription factor activation, gene expression, muscle contraction and cell growth. Thus, ROS should not be viewed merely as agents that damage cells but may also be mediators of physiological functions and serve as a second messenger.

We strongly believe that small amounts of ROS are being produced in irradiated cells as a result of light absorption by endogenous porphyrins, mitochondrial cytochromes and flavoproteins, [9,10,4] as they possess absorption bands in the visible and near I.R. ranges (Figure 3).

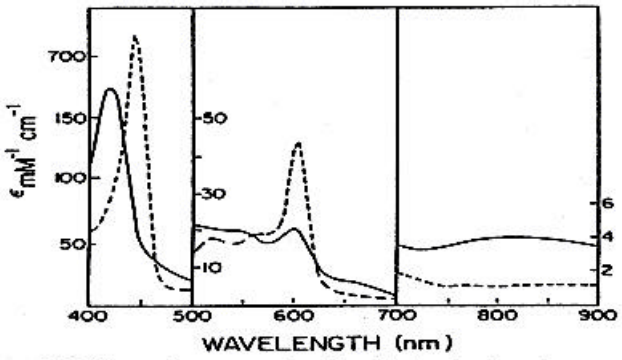

Fig 3: Absorption spectra of oxidized (-) and reduced (---) cytochrome oxidase.

Since these chromophores are photosensitizers, they create ROS upon irradiation. Karu [11] discusses at length the possibility of Cytochrome C oxidase as the main photoacceptor for visible and near infrared light. We suggest that NADPH OXIDASE, which has been found to exist in non-blood cells, [12] and possesses a flavoprotein, can also be a target of light. As porphyrin-like molecules have an intense absorption band in the 360nm region, and additional bands with decreasing intensity at 502nm, 540nm, 560nm, 630nm, and 830nm, (Figure. 3), we expected all these wl to stimulate ROS formation, the shorter wl being more effective. To follow ROS formation in irradiated sperm cells we use the EPR spin trapping

technique. In the past we have succeeded in detecting singlet oxygen, superoxide anion, and hydroxyl radicals in various irradiated cell cultures. [13,14,15]. In the present study we have estimated the amount of OH radicals generated in sperm cells as a function of the irradiating wl, and we found that all wl (used by us) in the UVA and the visible range stimulate OH radical formation (Figure 2), UVA light being more effective, as expected, from the absorption spectrum of porphyrin-like molecules (Figure 3). The action spectrum in Figure 2 also shows that white light (400-800nm) is capable of ROS stimulation, and hence, might be used for photobiostimulation. Supporting our results are those of Sroka et al., [16] who found that the action spectrum of the cellular response determined as the mitotic rate induced by light application of an irradiation of $4J/cm^2$ at an irradiance of $10mW/cm^2$, fits the absorption bands of the respiratory chain components. In addition, the action spectrum of cell adhesion (which plays a regulative role in the wound repair process) correlates with the absorption spectrum of cytochrome a/a3 [17].

UVA light (which generates high amounts of ROS, Figure 2) is known for its damaging effect on cells, but if it is used at a very low fluence, it can stimulate the cell. For example, it has been found that to enhance fibroblast proliferation, $0.6J/cm^2$ of UVA light is needed in comparison to 4-$10J/cm^2$ at 600nm. [19]

According to our results (Figure 2), the stimulatory effects of near IR light sources (for example, 830nm) would be very small as they generate low amounts of ROS. Surprisingly, there are many positive reports in the literature where the stimulating light is in the near IR region. [20,21] For example, to obtain fibroblast proliferation with a 780nm laser only $0.5J/cm^2$ is needed. [5] It is therefore possible that wl in the far-red region interact with the cell through a different mechanism. Karu [22] and Friedmann [4] have suggested that near IR lasers may activate enzymes, and probably C^a channels, in the membranes.

When irradiating tissues, the penetration of various wl (going down from near IR to UVA) must obviously be considered. Al Watban et al. [23] studied acceleration of wound healing as a function of the wl, and found that it was not attributed to laser skin transmission. They achieved accelerated wound healing at 442nm where the skin transmission is very low, but (according to Figure 3) it is compensated by the high extinction coefficient of this wl.

We conclude that UVA and visible light can either stimulate or harm the cell, depending on the amount of ROS they generate. For each wl the irradiance and the time of irradiation has to be assessed in order to get the desired optimal bioeffect.

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