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GENERATION OF REACTIVE OXYGEN SPECIES AND FREE ELECTRONS IN VISIBLE LIGHT ILLUMINATED CELLS AND PLASMA MEMBRANES

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Visible light has been used successfully over the last three decades for biostimulation of various tissues. Applications include stimulation of wound healing improving fertilizing and more. Previously, we suggested that low levels of reactive oxygen species (ROS) serve as messengers in light mediated stimulation. Using the EPR spin trapping coupled with the probe, we succeeded to detect ROS generation in isolated cells and sperm membranes illuminated with broad band visible light. We found that low energy visible light (LEVL) not only generates $^1O_2$, •OH and $O_2^{\bullet -}$ but might also enhances other radicals such as, phenyl and free electron. Our results also suggest that the membrane components NADPH oxidase system participate in ROS production by LEVL.

INTRODUCTION

Low energy in the visible and near IR light (LEVL) is emerging in recent years for the treatment of various pathologic conditions such as diabetic wounds (Forney & Mauro, 1999), injured bones (Guzzardella, Fini, Torricelli, Giavaresi & Giardino, 2002), severely injured peripheral nerves (Rochkind & Ouaknine, 1992), and more. The stimulatory effects of LEVL irradiation in-vitro includes induction of respiratory burst in neutrophils (Cohen, Lubart, Rubinstein & Breitbart, 1998), stimulation of the transforming growth factor-β (TGF-β) release from cultured fibroblasts (Yu, Naim & Lanzafame, 1994), production of vascular endothelial growth factors (Kipshidze, Nikolaychik, Keelan, Shankar, Khanna, Kornowski, Leon & Moses, 2001) and more. Studies with fibroblasts and keratinocytes indicate that at a specified relatively low energy doses of He-Ne laser or 780 nm diode laser, accelerated mitosis occurs (Yu *et al*., 1994; Grossman, Schneid, Reuveni, Halevy & Lubart, 1998). Ben-Dov *et al*. (Ben-Dov, Shefer, Irinitchev, Wernig, Oron & Halevy, 1999) found that He–Ne laser irradiation affects satellite cell differentiation, and in the area of fertilization, we have found that He-Ne laser irradiation of mouse sperm enhances the fertilization rate of metaphase-arrested eggs by 37% relative to control. The most important studies on the specificity of laser responses of various eukaryotic and prokaryotic cells were done by Karu and are summarized in her book (Karu, Ryabykh, Fedoseyeva & Puchkova, 1989).

The effect of light on cellular and medical processes led scientists to study LEVL-cell interaction. The mechanism of light-tissue interaction is very important as it might enable manipulating light induced cellular activities. Though no single mechanism has been unequivocally established, most researchers suggest that the first step following visible light irradiation is light absorption and reactive oxygen species (ROS) formation by endogenous cellular photosensitizers (Friedmann, Lubart, Laulicht & Rochkind, 1991; Karu, 1999; Lubart, Wollman, Friedmann, Rochkind & Laulicht, 1992). The absorption depends on the action spectrum of the chromophore, the wavelength of the light source, its intensity and energy dose. Suggested candidates for endogenous chromophores include porphyrins, flavins, mitochondrial cytochromes, and the plasma membrane NADPHoxidase components, flavoproteins and cytochrome b (Fraikin, Strakhovskaya & Rubin, 1996; Edwards & Silva, 2001; Klebanov, Strashkevich, Chichuk, Modestova & Vladimirov, 1998; Kim & Jung, 1992; Grzelak, Rychlik & Bartosz, 2001). These chromophores excited by light to their singlet state undergo an intersystem crossing to the triplet state which has a long relaxation time, thus enhancing many chemical reactions. If the excited photons are

transferred to near-by oxygen molecules, production of ROS occurs. It is well known that high amounts of ROS are lethal to the cell, a phenomenon exploited in photodynamic therapy (PDT), but if present at low concentrations, below that required for cytotoxicity, ROS have a wide range of positive stimulatory effects on the cell. Recent evidence has demonstrated that low ROS fluxes play role in transcription factors release, gene expression, muscle contraction, and cell growth (Das, 2001; Rhee, 1999; Suzuki & Ford, 1999; Sundaresan, Yu, Ferrans, Sulciner, Gutkind, Irani & Finkel, 1996). Thus ROS should not be merely perceived as agents that damage cells, but also as mediators of physiological functions and as secondary messengers.

Changes in ROS are always accompanied by $[Ca^{2+}]_i$ changes. When changes in intracellular calcium ($[Ca^{2+}]_i$) were studied while cardiomyocytes were illuminated with LEVL, together with production of ROS a transient increase in $[Ca^{2+}]_i$ is observed. The later reflects adaptation of the cells to oxidative stress and cell stimulation (Lavi, Shainberg, Friedmann, Shneyvays, Rickover, Eichler, Kaplan & Lubart, 2003). As the energy of LEVL does not damage the cell, studying the LEVL-cell interaction can be a model for learning about control of cellular processes.

Since LEVL-cell interaction is involved with radical production, the EPR can serve as a useful tool. In particular, the EPR spin trapping technique which enables to follow cellular ROS changes in situ, and distinguish among the oxy-radicals types using different probe traps. In this article, we summarize our results from the EPR coupled with a trapping probe concerning production of radicals such as superoxide anion, $O_2^{\bullet -}$ hydroxyl radicals-•OH- and singlet oxygen, in illuminated cells and sperm plasma membranes. The results are discussed in regard to visible light irradiation.

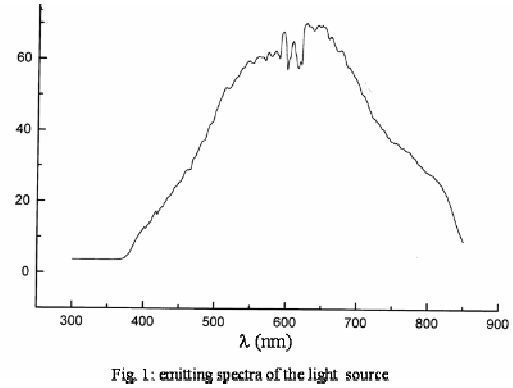

Fig. 1: emitting spectra of the light source

Fig. 1: emitting spectra of the light source.

MATERIALS AND METHODS

The light source

A conventional light source of visible light (400-800 nm, Fig. 1), producing a power density of 80 mW/cm^2, was used to illuminate the cells in the EPR cavity. The EPR cavity grid transmits less than 50% of the light energy to the cells; thus during a typical 3 min exposure, the cell culture was illuminated with 7.2 J/cm^2. The incident light produced no detectable heating.

Spin trapping EPR

To detect various radicals, we used the EPR-spin trapping coupled with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), purchased from Sigma. The DMPO was purified in a phosphate buffered saline (PBS), pH 7.4, with activated charcoal in the dark. After about 30 min, the solution was filtered and its concentration was determined spectrophotometrically using $\varepsilon_{227nm} = 8.0$ $mM^{-1} \times cm^{-1}$ (Kalyanaraman, 1982). The solution was stored at -20^{o}C for no longer than 2 weeks.

Samples of cells with the paramagnetic probe were drawn by a syringe into gas-permeable teflon capillary (Zeus, Raritan, NJ) and inserted into a narrow quartz tube which was open at both ends (Krishna & Samuni, 1993). Then the tube was placed into the EPR cavity and the spectra were recorded on a Bruker EPR 100d X-band spectrometer while illuminating the samples in the EPR cavity.

Simulation of the recorded spectra was performed using an algorithm provided by the WINSIM program, which is available from NIEHS, National Institutes of Health web site (http://epr.niehs.nih.gov/pest_mans/winsim.html).

RESULTS AND DISCUSSION

Detection of singlet oxygen in illuminated various cell culture

Detection of $^1O_2$ in illuminated cells is difficult especially because of the high reducing capability of the cells, especially under illumination, which tends to quench the spin adduct radicals that are being produced. Nevertheless, by using the probe 2,2,6,6-tetramethyl-4-piperidone (TEMP), we detected an increase in the triplet signal (a_N=15.6 G), of the 4-oxo-2,2,6,6-tetramethyl-piperidine-N-oxyl (4-O-TEMPO), which is attributed to $^1O_2$ production.

Since the cellular steady state concentration of $^1O_2$ during measurements is small, the signal to noise is very low (Lavi, Sinyakov, Samuni, Shatz,

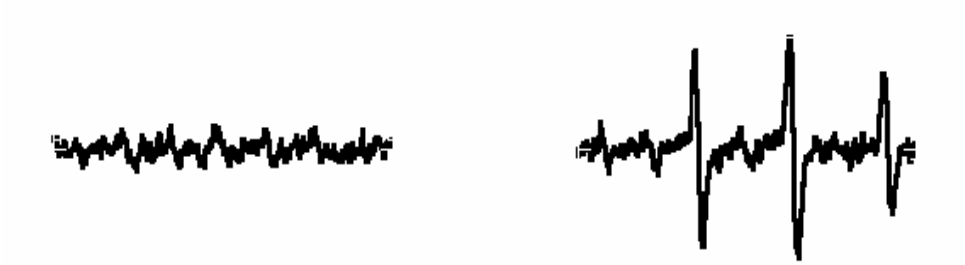

Fig. 2 : EPR spectrum of 1.5×10^7 NIH/ml suspension in PBS +18 mM DMPO (a) NO illumination (b) with 168s illumination while measurements. Measurement parameters: time constant 655 ms; resolution: 2048 points; modulation amplitude 1G; modulation frequency 100 KHz; conversion time 82 ms; gain 2×10^5; power 20 mW.

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Friedman, Shainberg, Breitbart & Lubart, 2004). To overcome this, we performed a wavelet transformation of the signal in a Matlab environment. This procedure eliminates noise, which does not have high similarity to a differential peak. Using this technique we detected a statistically significant increase in the 4-O-TEMPO triplet spectra of illuminated NIH and sperm cells.

Keeping a constant flow of oxygen in the fibroblasts suspension during illumination (up to 10 minutes) an increase in the 3-line signal intensity was shown progressively during illumination. The presence of oxygen during illumination of the cells increased the production rate of singlet oxygen, and consequently, that of the 4-O-TEMPO signal in these cells even though the reduction of 4-O-TEMPO during illumination prevents its accumulation. Moreover, oxidation of the corresponding hydroxylamine back to 4-O-TEMPO occurs constantly by O_2, thus an increase in the 3-lines signal was observed (Lavi *et al*., 2004).

We conclude that LEVL causes $^1O_2$ production which can be increased with O_2 concentration. The dependence of $^1O_2$ generation on oxygen pressure favors our assumption that LEVL causes ROS by photosensitization of cell chromophores.

Light generates a DMPO-OH signal in illuminated cells

In order to detect •OH and $O_2^{\bullet-}$ in cells, we used the spin trap DMPO. While illumination of PBS (control) did not result in any EPR signal (Fig. 2a), illumination of human sperm, ram sperm, rat skeletal muscle cultures, rat cardiomyocytes and NIH cells resulted in a quartet of $a_N = a_H = 15$ G type, which was attributed to the DMPO-OH signal. A representative spectrum of illuminated NIH is shown in Fig. 2b. The DMPO-OH signal can arise

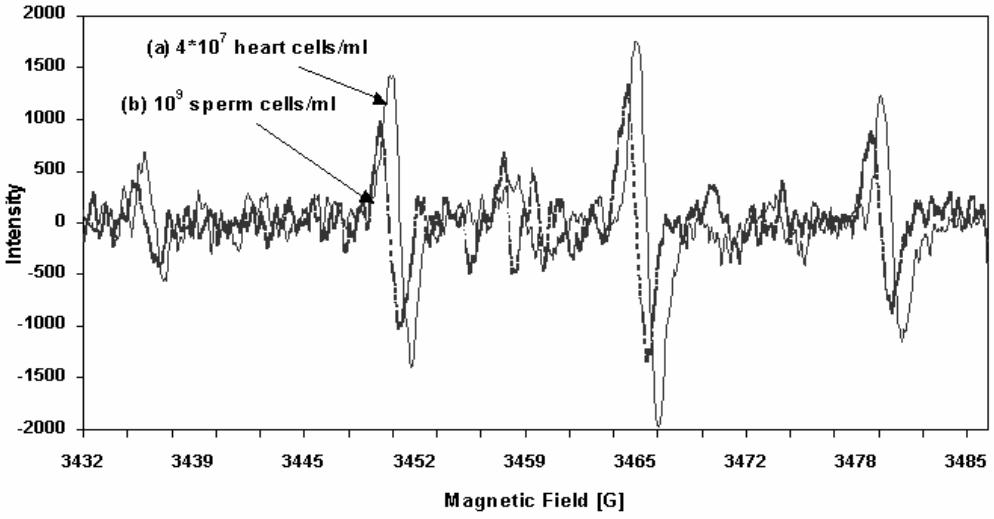

Fig. 3: Influence of cell type on the intensity of DMPO-OH production in 168s illuminated cells. The EPR spectra of 4×10^7 heart cells/ml (continue line___) and 10^9 sperm cells/ml (dash line - - -) illuminated suspension in PBS + 13 mM DMPO are presented. Measurement parameters: time constant 655 ms; resolution: 2048 points; modulation amplitude 1G; modulation frequency 100 KHz; conversion time 82 ms; gain 2×10^5; power 20 mW.

either by the addition of a genuine •OH radical to the double bond of DMPO, or by addition of $O_2^{\bullet-}$ to form DMPO-OOH, which then decomposes to DMPO-OH (Finkelstein, Rosen & Rauckman, 1982). We showed in the past, by using the spin trap 5-(diethoxyphosphoryl)-5-methyl–1-pyrroline-N-oxide (DEPMPO) (Lavi *et al*., 2003) that LEVL illuminated cardiomyocytes generate $O_2^{\bullet-}$ immediately during illumination. Taking into consideration these results, we can assume that the observed DMPO-OH signal arises from both •OH and $O_2^{\bullet-}$ in LEVL illuminated cell systems.

The influence of the cell type on the intensity of DMPO-OH production in illuminated cells

We compared the DMPO-OH signal from illuminated rat cardiomyocytes and ram sperms

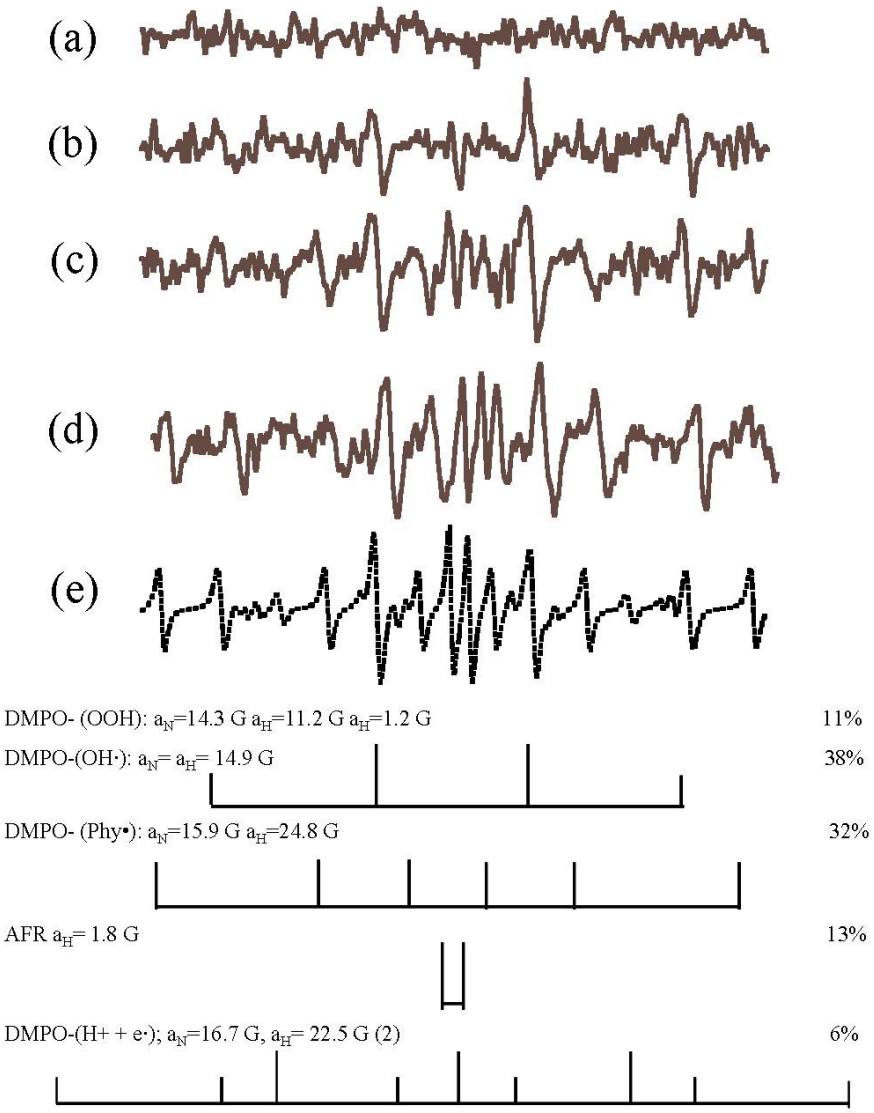

Fig. 4: EPR spectra of 18 mM DMPO with increasing concentration of sperm cells illuminated while measurement illumination. (a) $177*10^7$ cells/ml without illumination; (b) $20*10^7$ cells/ml during illumination; (c) $98*10^7$ cells/ml during illumination; (d) $177*10^7$ cells/ml during illumination; (e) simulated spectra of DMPO spin adducts species. Measurements parameters: time constant 655 ms; resolution: 2048 points; modulation amplitude 1G; modulation frequency 100 KHz; conversion time 82 ms; gain 4×10^5 ; power 20 mW.

(Fig. 3). The illuminated cells were shown to have a quartet type signal where in illuminated sperm cells spectra (Fig. 3b) the small doublet signal (around 3459 G), is attributed to the natural ascorbyl free radical (AFR). By comparing the intensities of the DMP0-OH quartet, it can be seen that the efficiency of the cells to produce DMPO-OH upon illumination is not similar, and were found to be at the order: cardiomyocytes > sperm. The high intensity of the DMP0-OH second low field peak in $4x10^7$ cells/ml illuminated cardiomyocytes (Fig. 3a) were found to be about 1.5 times of $1x10^9$ ram sperm cells/ml (Fig. 3b). This difference can be explained by the difference in the amount of endogenous photosensitizers in each cell. While cardiomyocytes are known to have a large number of mitochondria, where each mitochondrium is naturally enriched with photosensitizer molecules, the sperm cells have one mitochondrium. Moreover, it was suggested that mitochondria can act as a waveguides, therefore improve visible light absorption ability of the cell (Thar & Kuhl, 2004). These reasons make the cardiomyocytes a "better" sensitize cells than the sperm cells.

Observing a multi spin adducts spectra as the illuminated sperm cells concentration is increased

In order to study the correlation between the DMPO spin adduct signal intensity and illuminated cells number we measured the EPR spin trapping spectra in increasing concentration of illuminated sperm cells. As the concentration of the sperm cells is increased from 20×10^7 (Fig. 4b) to 177×10^7 (Fig. 4d) an increase in the intensity of the DMPO-OH quartet is observed (Fig. 4), indicating that indeed the cells are the source for •OH generation. Moreover, together with the increase of DMPO-OH signal an additional DMPO spin adduct signals are observed (Fig. 4d). To identify these signals, a simulation of the experimentally recorded spectra was performed. The simulation spectra (Fig. 4e), was constructed from: 38% DMPO-OH $a_N = a_H =$ = 14.9G, 11% DMPO-OOH $a_N = 14.3$ $a_H = 11.2$G $a_H = 1.2$G, 32% phenyl radial DMPO spin adduct (DMPO-Ph) $a_N = 15.9$ $a_H = 24.8$G, 6% DMPO hydrated electron adduct (DMPO-H) $a_N = 16.7$ $a_H = 22.5$G (× 2) and 13% AFR $a_H = 14.9$G. The existence of the AFR radical is a consequence of the natural sperm ascorbic acid oxidation by light, where the signal of DMPO-OOH arise from $O_2^{\bullet-}$ production upon illumination as discussed in the previously sections. The phenyl radical can be a consequence of secondary radical production from cytochrome c reaction with H_2O_2 (which is derived from •OH) (Yue Qian, Chen, Deterding, Fann, Chignell, Tomer & Mason, 2002). Nevertheless, since the hyperfine splitting of the DMPO-Ph has a similarity with other conjugated ring spin adducts

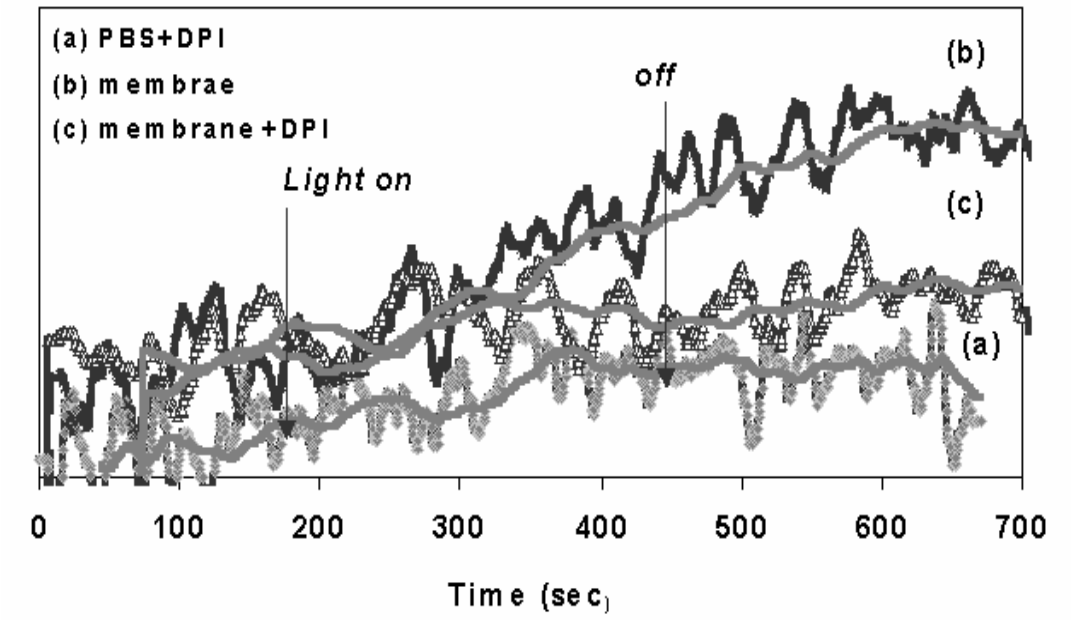

Fig. 5: Intensity changes in the DMPO-OH second peak maximal in isolated 0.36 mg/ml membranes illuminated in the presence of 100 µM DPI and 33 mM DMPO. (a) control-PBS+DPI (b) membrane in PBS (c) membrane in PBS+DPI. Measurements parameters: time constant 1311 ms; resolution: 1024 points; modulation amplitude 1G; modulation frequency 100 KHz; conversion time 655 ms; power 20 mW.

such as, nitrosopyrroline, methylphenyl, uracil radical and more (Buettner, 1987), this finding can only indicate involvement of several radicals in the cell while being illuminated with LEVL. Additionally, it is very interesting to observe a trapped electron in LEVL illuminated cells. Although this result has to be further studied, it can be an indication to an electron transport reaction, which is initiated by LEVL cellular illumination.

The kinetics of DMPO-OH formation in sperm plasma membranes

Although most of the literature considers the mitochondria as the important target for LEVL (Kato, Shinizawa & Yoshikawa, 1981; Yu, Naim, McGowan, Ippolito & Lanzafame, 1997), because it contains a high concentration of chromophores absorbing in the visible region. We claim that the production of ROS in illuminated cells does not have a specific target and thereby are generated in all cell compartments relative to the compartment's photosensitizers concentration. The plasma membranes of a variety of animals contain photosensitizers, which function as a part of the membrane reduction systems. The best-characterized reducing system is the transmembranal NADPH oxidase system. This system is especially active in phagocytes where it produces superoxide ions ($O_2^{\bullet-}$), used for destroying invading pathogens. Non-phagocytic cells have also been reported to express NADPH oxidase systems in their membrane, for example, membranes of endothelial cells (Cross, Jones, Harper & Segal, 1981), fibroblasts (Meier, Radeke, Selle, Younes, Sies, Resch & Habermehl, 1989), and sperm cells (Aitken, Fisher, Fulton, Gomez, Knox, Lewis & Irvine, 1997). In these cells, the NADPHoxidase system is shown to generate low and non-toxic levels of $O_2^{\bullet-}$. This system consists of a membrane catalytic core, flavocytochrome b, which confines cytochrome b and FAD, and a binding site for NADPH. The other components of this complex are water-soluble proteins of cytosolic origin which are assembled upon activation of the phagocytes cells (Vignais, 2002). As we anticipate the membrane catalytic core to serve as a target for light, we isolated bull sperm membranes and used the DMPO spin trap to measure ROS in response to light. To this end, we measured the DMPO spin adduct signal in illuminated sperm membranes. An illumination of 3 min resulted in an increase of the DMPO-OH adduct quartet signal with $a_N = a_H = 14.7$ G. Following the intensity of the first DMPO-OH low-field line peak during membrane illumination (Fig. 5), it shows that DMPO-OH production begins immediately upon the onset of illumination (Fig. 5b) and increases gradually, during the illumination. Turning the illumination off resulted in a plateau. Additionally, when the membranes were illuminated in the presence of diphenyleneiodonium (DPI), which is a

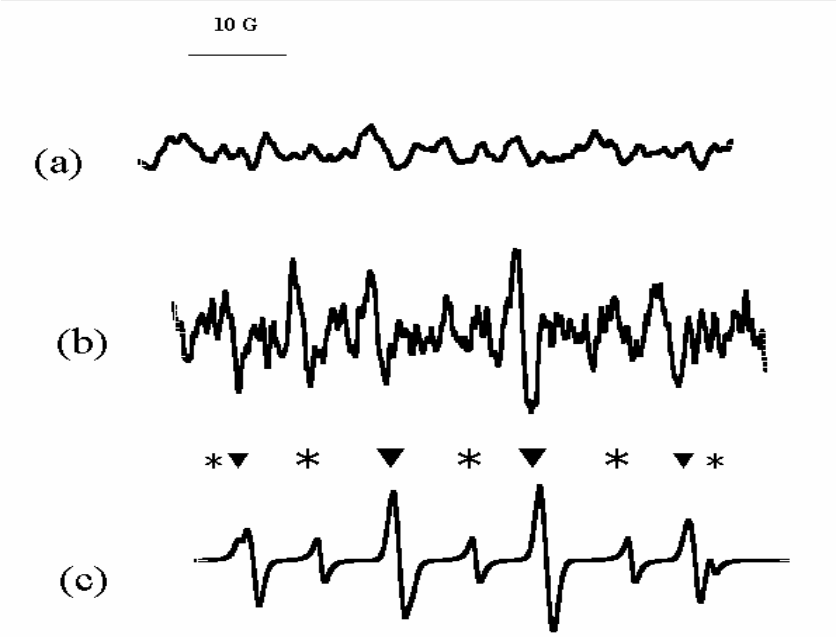

Fig. 6: Illumination of 0.36 mg/ml membrane with 33 mM reveals the appearance of DMPO-H+e spin adduct signal (a) sperm membrane+DMPO; (b) sperm membrane+DMPO after 5 min illumination; (c) simulated spectra: 57 % DMPO-OH (4 lines, ▼) ($a_H = a_N$ =14.9G) + 43 % DMPO-H+e (due to overlapping only 5 of 9 lines appears, ✱) (a_N =15.7G, a_H =22.3 G (x2)). Measurements parameters: time constant 1311 ms; resolution: 1024 points; modulation amplitude 1G; modulation frequency 100 KHz; conversion time164 ms; power 20 mW

NADPHoxidase inhibitor, suggested to react with flavin (O'Donnell, Tew, Jones & England, 1993), an inhibition of the DMPO-OH was noticed (Fig. 5c). These results prove that the plasma membrane can function as a good photosensitizer target for LEVL and that NADPHoxidase might participate in the mechanism of LEVL-cell interaction. Increasing the ROS concentration in the membrane can cause a general increase in membrane oxidation reactions. For example, an oxidation of thiol groups, can stimulate cell signaling (Nakashima, Kato, Akhand, Suzuki, Takeda, Hossain & Kawamoto, 2002). Of a particular interest is the increase of ROS to sperm membrane capacitation reaction. Previously we showed that HeNe laser treatment increases sperm capacitation and acrosome reaction, in a mechanism which involves ROS production (Cohen *et al*., 1998). From these results, we speculate that it involves light-induced activation of NADPH oxidase. Obviously, further experiments are needed to sustain this speculation.

Production of free electrons by illuminated membranes

Careful inspection the DMPO spin adduct spectrum obtained from illuminated membranes reveals that, like in the illuminated cells spectra (Fig. 4), other spin adduct signals are evident in addition to that of the DMPO-OH adduct (Fig. 6b). A simulation of the experimentally observed spectrum (Fig. 6c) demonstrates that the signal represents 57% DMPO-OH ($a_N = a_H = 14.9$ G) and 43% of an additional species ($a_N = 16.0$ G $a_H = 21.7$ G (×2)). This additional signal can be assigned to a protonated DMPO electron adduct (DMPO-e^- + H^+) (Buettner, 1987; Madden & Taniguchi, 2001). Although the rate constant of DMPO-e^-+H^+ formation is very high (1.5×10^{10} $M^{-1}S^{-1}$), its decomposition is also very rapid (2.44×10^2 $M^{-1}S^{-1}$ (Madden & Taniguchi, 2001), and therefore its detection is limited. This kinetic can explain the decrease in DMPO-e^-+H^+ signal during the measurements along the field. Looking at the three central high intensity peaks of DMPO- e^-+H^+ (Fig. 6b, the centered three stars), it can be seen that the intensity of the first low field peak is much higher than that of the following two higher field peaks appearing with time. Since there is a superposition between the DMPO-e^-+H^+ peaks and that of the DMPO-OH peaks, a twisting of the intensity and the proportion of the DMPO-OH quartet occurs.

From this result we suggest that illumination of membranes stimulates free electron production which can be an evidence for light-induced electron transport process. If indeed a transport of electron occurs through the NADPHoxidase, it can be assumed to cause an electric flow in the membrane, as was shown in phagocytes (DeCoursey, Morgan & Cherny, 2003) and in human eosinophils (Schrenzel, Serrander, Banfi, Nusse, Fouyouzi, Lew, Demaurex & Krause, 1998). Therefore, it is tempting to hypothesize that illuminated NADPH oxidase can cause electric flow and therefore serve as a solar battery.

CONCLUSION

In this work we show that visible light, 40 mW/cm^2 can produce ROS such as, $^1O_2$, •OH and $O_2^{\bullet-}$. The intensities of the ROS signals are dependent on the cell type and the oxygen concentration. In general, ROS production due to photosensitization is linked to cell damage. In our systems illumination produce small amounts of ROS that play a positive role in the cell function. In addition to the DMPO oxy radicals adducts, we could also observe, phenyl and hydrated free electron radicals in illuminated cells. The generation of electrons might indicate that LEVL causes a type I photosensitization process (i.e. electron transport mechanism). The generation of phenyl radicals can be a result of ROS generation or it can be a consequence of an electrons transfer from endogenous chromophores to other close by amino acid residues having a phenyl ring. We also suggest here that the NADPH oxidase system, which possesses an electron transport chain, can participate in light-cell interaction.

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