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Photodynamic toxicity and its prevention by antioxidative agents in *Bufo arenarum* embryos

Juan C. Stockert [a,b], Jorge Herkovits [a,*]

[a] *Instituto de Ciencias Ambientales y Salud, Fundación PROSAMA, Paysandu 752-760, 1405 Buenos Aires, Argentina*
[b] *Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*

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Abstract

In this work we describe an experimental model to evaluate the photodynamic toxicity on amphibian embryos, as well as the protective effect of antioxidants against the lethal oxidative stress induced by photosensitization. *Bufo arenarum* embryos were treated with 10 mg/l methylene blue (MB) in AMPHITOX solution for 72 h and then irradiated with a red laser or white light for variable times. Both light sources affected the survival of MB-treated animals and lethal effects occurred within the initial 12 h post-irradiation. For white light irradiation, the most effective phototoxic condition in our study, the LD10, 50 and 90 at 6 h post-irradiation corresponded to 13.57, 19.87 and 29.10 J/cm^2, respectively. To explore the action of antioxidants against the photogenerated oxidative stress, MB-treated embryos were incubated with 1 mM glutathione (GSH) or ascorbic acid (AA) during 48 h before irradiation. For GSH and 21.6 J/cm^2 irradiation, the survival increased from 20 to 90%, whereas 100% survival was achieved with AA even after 43.2 J/cm^2 irradiation. These results indicate that both the lethal photodynamic effect and its prevention by antioxidants can be evaluated by means of a simple toxicity test employing amphibian embryos.

Keywords: Amphibian embryo; AMPHITOX; Antioxidants; Methylene blue; Oxidative stress; Photodynamic action; Phototoxicity

1. Introduction

The photodynamic action corresponds to a toxic effect caused by photogenerated reactive oxygen species (ROS, and particularly singlet oxygen, $^1O_2$), which are produced after cellular accumulation of a suitable photosensitizer (PS) and irradiation with visible light in the presence of molecular oxygen. This effect is now applied in the photodynamic therapy (PDT) of cancer, which represents a promising therapeutic modality for the treatment of some tumours (Dougherty et al., 1998; McCaughan, 1999), as well as dermatological (Kalka et al., 2000) and ophthalmologic diseases (Harding, 2001). In addition to medical applications of PDT, other biological and environmental outcomes may be obtained by photodynamic action, examples being the use of PSs as photoactive herbicides (Gupta and Tripathy, 2000), insecticides (Mangan and Moreno,

Abbreviations: AA, ascorbic acid; AS, AMPHITOX solution; GSH, glutathione; i.p., intraperitoneal; LD, lethal dose; MB, methylene blue; mM, millimolar; $^1O_2$, singlet oxygen; PDT, photodynamic therapy; PS, photosensitizer; ROS, reactive oxygen species; UV, ultraviolet

* Corresponding author. Tel.: +54-11-4432-1111; fax: +54-11-4431-2445.
E-mail address: herkovit@mail.retina.ar (J. Herkovits).

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2001), and compounds for photosterilization of water contaminated with pathogenic bacteria (Kussovski et al., 2001). On the other hand, the oxidative stress induced by ROS is also a common mechanism of biochemical damage provoked by many toxic agents and environmental pollutants.

In order to analyse the photodynamic toxicity induced by PSs, several test systems have been designed. Examples are the use of *Paramecium*, *Candida*, and other microorganisms (Daniels, 1965; Knudsen, 1985; Joshi and Misra, 1986), *Artemia salina* (Matthews, 1995), *Allium cepa* roots (Molero et al., 1985), sister chromatid exchanges (Hazen et al., 1987), photohemolysis (Hetherington and Johnson, 1984), K^+ efflux from erythrocytes (Cardenas et al., 1992), intradermal assays (Kaidbey and Kligman, 1978; Wagai and Tawara, 1991), and sea urchin eggs and embryos (Marthy et al., 1990). Cell cultures are also a widely used test system to evaluate photokilling properties and mechanisms of new PSs (Duffy et al., 1987; Lasarow et al., 1992; Merlin et al., 1992; Villanueva et al., 1999; Cañete et al., 2000).

Methylene blue (MB), which shows light absorption between 550 and 700 nm with an absorption peak at 661 nm (Gurr, 1971; Green, 1990), is a well known PS with a considerable quantum yield of $^1O_2$ production ($\Phi_\Delta = 0.5$, Wilkinson et al., 1993). MB has been used in basic studies on photosensitization (Singh and Vadasz, 1977; Yao and Zhang, 1996; Saitow and Nakaoka, 1997), phototoxicity of cell cultures (Gill et al., 1987; Fowler et al., 1990; Stockert et al., 1996; Trindade et al., 2000; Mellish et al., 2002), and experimental and clinic PDT (Konig et al., 1987; Williams et al., 1989). MB has also high photobactericidal (Millson et al., 1996; Usacheva et al., 2001) and photovirucidal activity (Wagner, 2002).

On account of their high sensitivity to toxic agents, amphibian embryos are increasingly employed to assess the adverse effects of physico-chemical stressors (e.g. Herkovits and Pérez-Coll, 1990; Herkovits et al., 1997, 2000; Hall and Henry, 1992) including complex mixtures of contaminants in the environment (Herkovits et al., 1996; Burkhart et al., 2000). For instance, even the UV-B component of sunlight could produce both malformation and lethality in amphibians (Tietge et al., 2001; Ankley et al., 2002). By means of AMPHITOX, a standardised test customised for acute, short-term chronic, chronic and early life stages toxicity assessment, the hazard of a single agent or complex mixtures can be reported according to the more convenient end point in each case (Herkovits and Pérez-Coll, 1990; Herkovits et al., 2002). In this work we describe the photodynamic effects on MB-treated *Bufo arenarum* embryos based on lethality as end point, and the protection achieved against phototoxicity by means of antioxidative agents. According to the results obtained, the AMPHITOX toxicity test could be adapted as a very simple, precise, sensitive and inexpensive bioassay conducted with amphibian embryos for oxidative stress-dependent phototoxicity studies.

2. Materials and methods

Bufo arenarum adult females weighing around 200–250 g were collected in Lobos (Buenos Aires Province). Ovulation was induced by i.p. injection of a suspension of one homologous hypophysis in 2 ml of AMPHITOX solution (AS) (Herkovits et al., 2002). Oocytes were fertilized in vitro with a sperm suspension and embryos were maintained in AS until complete operculum, the last stage of embryonic development (S25). At that stage, batches of 50 animals in 200 ml of AS were treated with methylene blue (Color Index 52015; Sigma) using concentrations of 10, 20, 40 and 60 mg/l for variable times. Taking into account that an incubation time of 72 h with the lowest MB concentration did not exerted lethality or any other visible adverse effects along the following 6 days post-treatment, the photodynamic experiments were performed on groups of 10 embryos (by duplicate) treated with 10 mg/l MB for 72 h.

In order to explore the possibility to protect by means of natural antioxidants against the oxidative stress induced by photodynamic action, glutathione (GSH) or ascorbic acid (AA) (both from Sigma) were used at a final concentration of 1 mM for 48 h. This concentration was selected taking into account that the tissue concentration of these compounds is in the mM range (Behrens and Madere, 1987; Perego et al., 1997). Embryos were treated with GSH or AA alone, or with GSH or AA in the presence of MB (during the last 48 h of 72 h MB-treatments), washed by several changes of fresh AS, and either subjected to irradiation or left as controls.

Two visible light sources were employed for irradiation of the experimental embryos and their controls. First, irradiation with a semiconductor red laser diode CPS182 (Thorlabs, Newton, NJ, USA) (emission wavelength: 635 nm, optical output power: 3 mW, as measured with a Broadband Power Energymeter 13 PEM 001, Melles Griot, Laser Optics, Buenos Aires) was used. As the laser beam corresponded to a surface of 1 mm × 3 mm, only one embryo each time could be subjected to irradiation. In this case, the embryo was placed on a wet filter paper to avoid motility and position changes during irradiation. The distance between the embryo and the red laser diode was 10 cm, the light intensity at this site being 100 mW/cm^2. The light dose for different irradiation times were: 2.5 min: 15 J/cm^2; 5 min: 30 J/cm^2;10 min: 60 J/cm^2.

The second irradiation procedure was by means of white light from an Agfa-Gevaert slide projector (Diamator 1500) equipped with a 150 W lamp. In this case, 10 animals placed into plastic dishes (3.5 cm in diameter) containing 4 ml of fresh AS were irradiated simultaneously. The light beam was filtered through a 4.5 cm water layer to absorb heat. The projector was placed at a vertical distance of 6 cm from the embryos, and the light intensity at this site was 72 mW/cm^2, as measured with the previously mentioned energymeter. The irradiation times corresponded to the following light doses: 2.5 min: 10.8 J/cm^2; 5 min: 21.6 J/cm^2; 7.5 min: 32.4 J/cm^2; 10 min: 43.2 J/cm^2. The survival of the irradiated embryos was registered at different times between 0.5 and 48 h, that is within the acute period following the irradiation exposure. The statistical analysis was conducted by means of PROBIT (US EPA).

3. Results

No effects on the survival of animals were observed in control experiments using MB, GSH, or AA alone. Likewise, the irradiation with red laser or white light for 30 min in the absence of MB had no effects on the embryos.

The photodynamic toxicity was evaluated at several time intervals within the acute period following the irradiation process. A typical alteration of embryos, either during irradiation or few hours later, was their reduced or absent motility. As some embryos recovered from this "narcotic" state, lethality was confirmed as complete absence of motility at the following day.

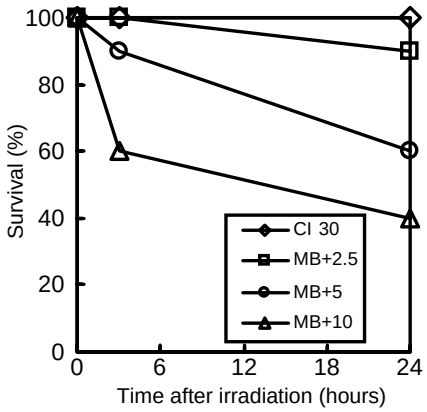

Fig. 1. Survival of *B. arenarum* embryos treated with 10 mg/l MB for 72 h followed by irradiation with red laser light for 2.5, 5, and 10 min. Control of irradiation (CI) corresponds to irradiation alone for 30 min. Abscissa: time after irradiation (h).

Irradiation with red laser light provoked considerable lethality in animals treated with MB (Fig. 1), which was proportional to the light dose and resulted in 60% of lethality within 24 h after exposure to 10 min irradiation. No additional adverse effects were registered along the following 6 days post-irradiation. Irradiation with white light resulted also in lethality proportional to the light dose employed but with a more profound adverse effect on the embryo. For instance, within the first hour after 15 min irradiation lethality resulted in 100% of the experimental embryos and the same result was achieved with 5 min irradiation at 12 h post-exposure. Thus, as recorded at 12 h post-exposure, white light irradiation for 2.5, 5, 7.5 and 10 min resulted in lethality of MB-treated embryos of 0, 80, 100 and 100%, respectively (Fig. 2). Lethality of the experimental embryos did not increase from 12 h after irradiation onwards. Light doses expressed as J/cm^2 exerting LD10, 50 and 90 at 6 h post-irradiation were 13.57 (6.95 and 17.16), 19.87 (14.86 and 23.99) and 29.10 (24.09 and 44.18), respectively (in parenthesis the lower and upper confidence limits). A comparative analysis among white and red light exerting lethal effects on MB-treated embryos can be observed in Fig. 3. It is noteworthy that red light induced less

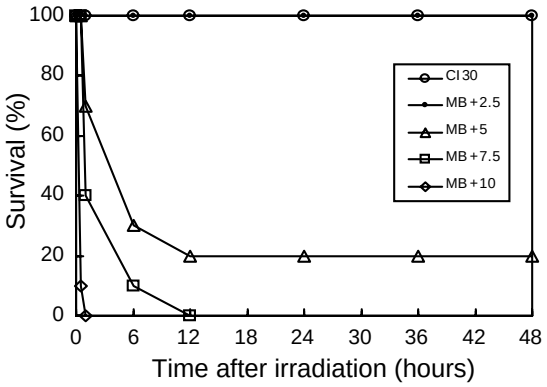

Fig. 2. Survival of *B. arenarum* embryos treated with 10 mg/l MB for 72 h followed by irradiation with white light for 2.5, 5, 7.5 and 10 min. Control of irradiation (CI) corresponds to irradiation alone for 30 min. Abscissa: time after irradiation (h).

adverse effects at the same light dose than white light.

The action of antioxidants as possible protective agents against the oxidative stress induced by the photodynamic action of MB was studied on embryos treated with 1 mM GSH or AA for 48 h before irradiation. Both agents revealed an efficient protective activity. Using GSH in the case of 5 min irradiation, the survival of animals increased from 20 to 90% but no significant beneficial effect was observed in the case of higher irradiation doses, whereas 100% survival was achieved with AA even after 10 min irradiation (Fig. 4).

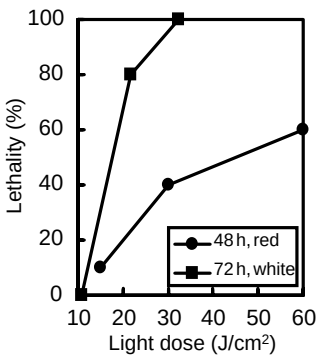

Fig. 3. The light dose–effect curves for red and white light on MB-treated *B. arenarum* embryos.

4. Discussion

In this work we describe efficient photosensitization protocols with lethal effects on a complex and whole organism such as the amphibian embryo employing MB as a well known PS. Although the adverse effects due to the photodynamic action was observed with both light sources, red laser and white light, the results point out that in the last case a more profound adverse effect was obtained, possibly because to the fact that, although somewhat more intense, the laser source only provided a very narrow excitation band for MB. Lethality was always proportional to light doses, higher doses reducing the time required for adverse effects as well as increasing the number of organisms affected. It is noteworthy that the lethal effect on *B. arenarum* embryos due to photodynamic toxicity occurs within the initial 12 h post-irradiation.

The oxidative stress is triggered by the occurrence of ROS ($^1O_2$, superoxide and hydroxyl radicals, hydrogen peroxide), which induce mitochondrial damage, disturbance in Ca^{2+} homeostasis, and apoptosis (Orrenius et al., 1989; Hermes-Lima, 1995). Signal transmission in the central nervous system, as well as ligand binding to membrane receptors and coupling of receptors to proteins and effector enzymes are severely affected by oxidative stress (Van der Vliet and Bast, 1992; Beal, 1995; Sastry and Subba Rao, 2000), a fact which could explain the “narcotic” as well as lethal effect observed by the photodynamic action on *B. arenarum* embryos. In this respect, it is known that extreme phototoxic reactions may produce the death of organisms. This is the case for the rapidly lethal photosensitization of mice induced by systemic hypericin and high light doses (Kamuhabwa et al., 1999). From the results obtained with amphibian embryos at 6 h post-irradiation, PROBIT analysis shows that the LD50 is 19.87 J/cm^2 of white light exposure.

It is worth to note that the occurrence of skin melanin did not prevent the photodynamic action on the embryos. The spectral absorption of melanin is very high at UV wavelengths (Parrish et al., 1983), but the absorption intensity at 661 nm (the absorption maximum of MB) is reduced to a 10% of the value at 300 nm, thus allowing considerable excitation of MB. Although low molecular weight melanin fractions can also generate ROS upon irradiation at wavelengths shorter than 400 nm (Simon et al., 2002), no evidence

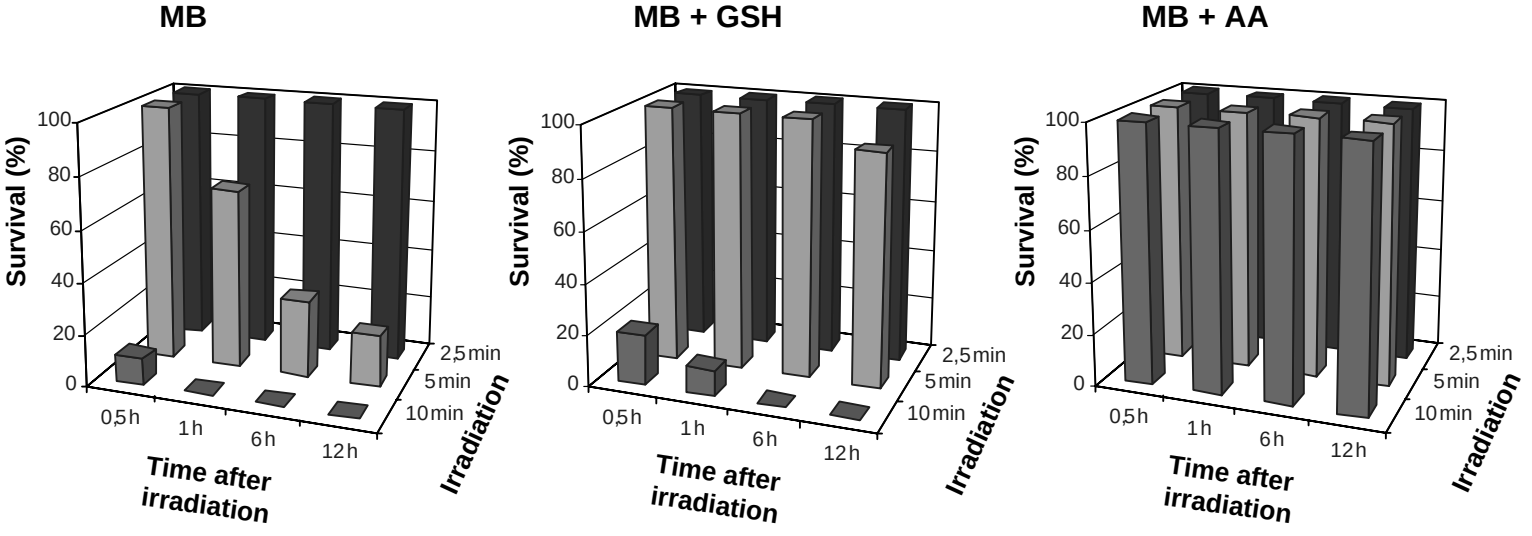

Fig. 4. Survival of *B. arenarum* embryos treated with 10 mg/l MB for 72 h and then subjected to different irradiation times with white light, either without antioxidant agents (MB) or in the presence of glutathione (MB + GSH) or ascorbic acid (MB + AA). The survival was recorded at different observation times after irradiation.

of intrinsic photodynamic effects was observed in embryos irradiated with white light.

GSH and AA play an important role in physiological functions related to their antioxidant properties, including modulation of the intracellular redox status, detoxication processes, and particularly, protection from oxidative stress (Meister, 1994). Although the protective effect exerted by AA is greater than that of GSH, it seems logical to assume that in both cases protection is based on the prevention of the oxidative stress induced by the phototoxic treatment. In this context, exogenous antioxidants are considered promising chemoprotectors against neuro- and nefrotoxicity produced by antitumour drugs (Zunino et al., 1989; Hamers et al., 1993).

Amphibian embryos by means of the AMPHITOX test were employed successfully for standardised hazard assessment studies (e.g. Herkovits et al., 2002; Herkovits and Pérez-Coll, 2003). Present results on phototoxic effects induced by MB on whole *B. arenarum* embryos show that the photodynamic action can be easy and conveniently studied in this experimental model. Thus AMPHITOX test is expanded as a standard procedure for photodynamic studies. Our results also shows that the lethal oxidative stress induced by the photodynamic action can be prevented using AA and GHS, allowing to determine the efficiency of both PSs and antioxidants in a rapid and precise way.

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