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The effects of UV radiation on the visual system of the crab *Neohelice granulata*: A protective role of melatonin

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ABSTRACT

The first and main target-structure of ultraviolet (UV) radiation in animals is the body surface, including the skin and eyes. Here, we investigated cell damage in the visual system of the crab *Neohelice granulata* acclimated to constant light and exposed to UVA or UVB at 12:00 h for 30 min. The reactive oxygen species (ROS) production, antioxidant capacity against peroxyl radicals (ACAP), lipid peroxidation (LPO) damage, catalase (CAT) activity, and the melatonin immunohistochemical reactivity in the eyestalks were evaluated. The animals that received melatonin and were exposed to UVA and UVB radiation showed a decreased ROS concentration ($p<0.05$).The ACAP test showed a decrease ($p<0.05$) in their values when the animals received 2 pmol/crab of melatonin (physiological dose) before the exposure to UVA radiation. The animals exposed to UVB radiation after receiving the same dose of melatonin showed an increase ($p<0.05$) in the ACAP test compared with the animals exposed to UVB radiation after receiving only crab physiological saline. The CAT activity increased ($p<0.05$) in the animals that received melatonin and were exposed to UVA and UVB radiation. Animals exposed to UVA and UVB displayed an increase ($p<0.05$) in the LPO levels, whereas animals treated with melatonin showed lower ($p<0.05$) LPO levels when irradiated. The results indicate that the specific oxidative parameters altered by UV radiation can be modulated by a physiological dose of melatonin. Moreover, the melatonin regularly produced by virtually all eyestalk cells suggests that it may function to modulate the noxious effects of radiation, at least in the crab *N. granulata*.

1. Introduction

In recent decades, the increasing incidence of ultraviolet (UV) radiation on Earth has received more attention by scientists, since it may produce biological changes and some impact on biodiversity (Kirchhoff et al., 2000; Casiccia et al., 2003; Bertagnolli et al., 2007; McKenzie et al., 2007). UV radiation includes electromagnetic wavelengths between 200 and 400 nm, and in order to analyze its capacity to cause damage in cellular targets, UV has been divided into UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). The wavelengths of UV that include UVA and UVB are also called Solar UV (Diffey, 2002).

The first and main target-structure of UV radiation in animals is the body surface, including the skin and eyes. The influence of UV radiation on the skin has been thoroughly investigated: in general, the effects of UV radiation on the epidermis are noxious (Thanh et al., 2008), although a well-known exception is the stimulation of vitamin D synthesis in vertebrates (Holick, 2008). In vertebrates, the adverse effects of UV radiation include immunosuppression (Timares et al., 2008), production of reactive oxygen species (ROS) (Heck et al., 2003), photoaging, DNA mutation, and cancer (Albert and Ostheimer, 2003). In the eye of vertebrates, damage has been reported in the cornea, characterized by inflammation; while in the lens, cataract is the most common pathology (Sliney, 2001). Moreover, in the retina, especially in the photoreceptors, apoptosis was observed (Miguel et al., 2003). The mechanism underlying these types of damage is suggested to be oxidative stress.

Oxidative stress is the result of an imbalance between ROS, mainly the hydroxyl radical (HO•), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), and the antioxidant defense system (ADS). In other words, oxidative stress occurs when ROS production exceeds the capacity of the ADS, reacting with protein, lipids, and DNA and/or disrupting the redox state of the cell (Sies, 1991; Jones, 2006). The ADS is composed of enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), and glutathione reductase (GR), as well as non-enzymatic molecules, such as vitamin A, vitamin E, and the tripeptide glutathione (GSH) (Storey, 1996; Gavin and Sies, 2001).

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Although the adverse effects of UV radiation have been relatively well studied, in invertebrates the efforts in this regard are still in the initial stages. To our knowledge, few studies have evaluated the effects of UV radiation on crustaceans. Miguel et al. (2002) reported morphological damages in the retina and *lamina ganglionaris* cells in the mangrove crab *Ucides cordatus* exposed to UVC and UVB; Gouveia et al. (2005) reported oxidative and DNA damage in the cephalothorax and pereiopod epithelia of *Neohelice granulata* crabs exposed to UVA and UVB; Nazari et al. (2010) revealed important effects induced by UVB on embryos of the freshwater prawn *Macrobrachium olfersi*; and Vargas et al. (2010) showed changes of the oxidative status in the eyestalks of the crab *N. granulata* induced by UVA and UVB.

Melatonin is most widely known as a product of the pineal gland in vertebrates, although it is also synthesized in extra-pineal sites. The pineal gland shares a common embryonic origin with the eye, and these highly specialized organs have many structural similarities (Ung and Molteno, 2004). Melatonin is synthesized by the eyes of vertebrates and modulates the photoreceptor outer segment shedding rate (Grace et al., 1999) and light sensitivity (Djamgoz et al., 1997). Also, the role of melatonin in scavenging destructive ROS has been shown by Tan et al. (1993), and other authors have suggested that melatonin plays a role as an antioxidant (Reiter et al., 1996; Tan et al., 1998; Reiter et al., 2002; Tan et al., 2002; Anisimov, 2003). In crustaceans, melatonin has been found in the hemolymph, head, muscle, optic lobes, and the whole eyestalk (for review see Maciel et al., 2010). Recently, it has been suggested that melatonin can also modulate ADS in crustaceans (Geihs et al., 2010).

Vargas et al. (2010) showed changes in the oxidative status in the eyestalks of the crab *N. granulata* exposed to UVA or UVB radiation, and interestingly, the effects were more evident in animals acclimated in a 12L:12D photoperiod than in animals acclimated to constant light. Since melatonin has a biphasic profile in crabs acclimated to constant darkness, as well as a basal level in animals acclimated to constant light (Maciel et al., 2008), we hypothesized that melatonin may have a protective role against UV radiation in the eyestalks of the crab *N. granulata*. Therefore, in this study we investigated cell damage in the visual system of *N. granulata* acclimated to constant light and exposed to UVA or UVB at 12:00 h (noon) for 30 min, by evaluating ROS production, antioxidant capacity against peroxyl radicals (ACAP), lipid peroxidation (LPO) damage, and CAT activity in the eyestalks. In addition, we evaluated the presence of melatonin in the eyestalk of this crustacean by immunohistochemistry.

2. Material and methods

2.1. Animals

Adult male crabs *N. granulata* (previously known as *Chasmagnathus granulata/granulatus* — see Sakai et al., 2006) weighing 7.0 ± 0.5 g (mean ± S.E.M) were collected in salt marshes around Rio Grande City, Brazil. The crabs were transferred to the laboratory for an acclimation period of at least 10 days in tanks under constant conditions of temperature (20 °C) and salinity (20°/00), and in constant light. All procedures adopted in this study were performed after approval by the National Environmental Committee (IBAMA document number 1.637.714), and every effort was made to minimize animal suffering.

2.2. Assays

After the acclimation period, the animals (five samples of five animals each) received an injection of 100 μL of melatonin in the hemolymph through the articulation of the fifth pair of pereopods (0.02; 2 or 200 pmol/crab: infraphysiological, physiological, and supraphysiological doses, respectively) or of 100 μL of crab physiological saline (control group), which was composed of, in mmol L^{-1}: $MgCl_2$, 0.01; NaCl, 0.355; $CaCl_2$, 0.016; H_3BO_3, 0.005; $KHCO_3$, 0.010; $Na_3C_6H_5O_7$, 0.008; pH 7.6, 15 min prior to the exposure to UV radiation. This period of time is the necessary and reliable time for melatonin to circulate in the hemolymph and to arrive in the target tissues. The animals were then irradiated for 30 min at 12:00 h (noon) with 1.575 J/cm^2 of UVA or 1.294 J/cm^2 of UVB doses. The UVA (VL: 115 L, 30 W) or UVB (VL: 115 C, 30 W; Vilber Lourmat, Marne Lavalee, France) lamps were monitored using a radiometer/photometer (model IL 1400A, International Light, Newburyport, MA, USA). The UVA lamp irradiation was 1.39 mW/cm^2 UVA, with contamination of 0.006 mW/cm^2 of UVB and 0.001 mW/cm^2 of visible light. The UVB lamp irradiation was 1.195 mW/cm^2, with contamination of 0.493 mW/cm^2 of UVA and 0.0001 mW/cm^2 of visible light. Both lamps showed no contamination with UVC. An extra group (n = 5) was injected with 100 μL of crab saline or melatonin (0.02; 2 or 200 pmol/crab) maintained under fluorescent lamps (Philips TLT 40 W/75, São Paulo, Brazil) irradiating 96.0 mW/cm^2 visible light, instead of UV irradiation. Saline was injected in order to exclude the possible stressful effects of the injection on the animals, and melatonin was injected in the extra group in order to check its effect on the oxidative status, with no intervention of UV radiation. After the exposure, the animals were killed by severing the supra-esophageal ganglion and the eyestalks were removed for further analyses. This entire procedure was conducted 3 times, due to the differences in sample preparation for quantification of ROS/antioxidant capacity, CAT activity, and LPO, as described below.

2.2.1. ROS production

Eyestalks were weighed and homogenized (1:20 w/v) in a cold (4 °C) buffer solution containing sucrose (250 mM), phenylmethanesulfonyl fluoride (PMSF — 1 mM), and ethylenediamine tetraacetic acid (EDTA — 5 mM), with pH adjusted to 7.6. The samples were centrifuged twice (2000 g, 4 °C for 20 min) and the supernatant was collected and centrifuged again (10,000 g, 4 °C for 45 min). The supernatant resulting from this last centrifugation was used for the determination of ROS (Viarengo et al., 1999). For ROS detection, we used 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA, Molecular Probes). This molecule in the presence of ROS generates a fluorochrome, detected at 488 and 525 nm wavelengths for excitement and emission, respectively. The analyses were carried out in a fluorescence microplate reader (Victor 2 Perkin) with readings every 5 min for 60 min. The total fluorescence production was calculated by integrating the fluorescence units (FU) over the time of the measurement, after adjusting FU data to a second-order polynomial function. ROS concentration was referred to the total protein content present in the biological sample and expressed in FU (mg of protein)$^{-1}$.

2.2.2. Antioxidant capacity against peroxyl radical analysis (ACAP)

Eyestalks were weighed, homogenized and centrifuged as described above (Section 2.2.1). The supernatant of the last centrifugation was used for the analyses of ACAP according to the method of Amado et al. (2009). Briefly, 10 μL of the supernatant prepared for the enzymatic analysis was pipetted into a white 96-well microplate, six wells per sample. The reaction buffer (127.5 μL) containing 30 mM HEPES (pH 7.2), 200 mM KCl, and 1 mM $MgCl_2$ was added to the wells with the samples. In three of the six wells of each sample, 7.5 μL of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) was added, while the same volume of ultrapure water was pipetted into the other three wells. The microplate was inserted into a fluorescence microplate reader (Victor 2, Perkin-Elmer), at a programmed temperature of 35 °C, and the peroxyl radicals were produced by thermal decomposition of ABAP. Immediately before reading, 10 μL of the fluorescent probe 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA) was added to the wells at a final concentration of 40 μM (Ferreira-Cravo et al., 2007). H_2DCF-DA is cleaved by esterases according to their presence in the samples, and the non-

fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound DCF, which is detected at wavelengths of 488 and 525 nm, for excitation and emission, respectively. The thermal decomposition of ABAP and ROS formation were monitored with readings every 5 min for 60 min. According to Regoli and Winston (1999) and Regoli (2000), non-enzymatic low-molecular-weight scavengers (GSH, ascorbic acid, uric acid, vitamin E) generally account for 70% of the total scavenging capacity for peroxyl radicals. Therefore, if enzymatic inhibition occurs due to the high temperature (since crabs are poikilotherms) needed for ABAP decomposition in peroxyl radical (35 °C), the decrease of the antioxidant capacity should be a minor problem. The total fluorescence production was calculated by integrating the fluorescence units (FU) over the period of the measurement, after adjusting the FU data to a second-order polynomial function. The results were calculated as the difference in area of the FU × min in the same sample, with and without ABAP addition, and standardized to the ROS area without ABAP (background area). The inverse of the relative difference between ROS area with and without ABAP was considered as a measure of the antioxidant capacity: the greater the difference in area, the higher the antioxidant capacity, since high fluorescence levels were obtained after adding ABAP, meaning low competence to neutralize peroxyl radicals.

2.2.3. Catalase (CAT) activity

The optic ganglia (not the whole eyestalk, because of the presence of pigments in the retina that interfere with the color of the reaction) were weighed and homogenized (1:20 w/v) in a cold (4 °C) buffer solution containing Tris base (20 mM), EDTA (1 mM), dithiothreitol (1 mM, Sigma), KCl (150 mM), and PMSF (0.1 mM), with pH adjusted to 7.6. Homogenates were centrifuged at 9000 g, 4 °C, for 30 min, and the supernatant was then used for the analyses. The catalase (EC 1.11.1.6) activity was analyzed according to Beutler (1975), determining the initial rate of H_2O_2 (50 mM) decomposition at 240 nm. The results were expressed in CAT units: one unit is the amount of enzyme that hydrolyzes 1 μmol H_2O_2 per minute and per mg of protein, at 25 °C and pH 8.0. This procedure was performed using a digital spectrophotometer (Biomatte 3).

2.2.4. Lipid peroxidation (LPO)

Eyestalks were weighed and homogenized (1:20 w/v) in cold (4 °C) methanol and centrifuged at 1000 g, 4 °C, for 10 min. The supernatant was used for the analyses. The methodology, termed FOX (Hermes-Lima et al., 1995; Monserrat et al., 2003), is based on the oxidation of Fe(II) under acidic conditions and measures the quantity of lipid peroxides. For the lipid peroxidation (LPO) measurements, $FeSO_4$ (1 mM), H_2SO_4 (0.25 M), xylenol orange (1 mM, Sigma), and MilliQ water were added sequentially. Samples (30 μL) or methanol (blanks) were added and incubated for 450 min. Thereafter, absorbance (550 nm) was determined using a microplate reader (Victor 2; Perkin-Elmer), and cumene hydroperoxide (CHP; Sigma) was employed as a standard. LPO was expressed in cumene hydroperoxide (CHP) equivalents per gram of wet mass.

2.3. Histology and immunohistochemistry

The eyestalks of the crabs were dissected at 12:30 h, fixed in 4% paraformaldehyde for 4 h in 0.1 M phosphate-buffered saline (PBS), and dehydrated in a graded ethanol series. The material was embedded in paraffin wax, and 12 μm sections, obtained using a rotatory microtome, were collected on slides. Some sections were stained with hematoxylin and eosin for general observation of the structures. The following procedure using the ProTaqs® Stain ABC-POD kit was performed for other sections. To block the endogenous peroxidase, the sections were incubated with 3% H_2O_2 and washed in PBS. Blocking of unspecific binding was performed with the blocking reagent. Next, the slides were washed in PBS and incubated with the primary antibody (rabbit anti-melatonin; SIGMA) overnight at 4 °C. The next day, the sections were washed in PBS, incubated with the secondary antibody, and washed again in PBS. The sections were then covered with Streptavidin-Complex and incubated for 60 min. After they were washed in PBS, the sections were covered with fresh DAB-chromogen solution and incubated until the color intensity was satisfactory. The sections were then washed in distilled water, counterstained with hematoxylin, and the slides were mounted with Proataqs® (PARAmount). Negative controls for the reaction were prepared by omitting the primary antibody (no counterstaining was used). The positive control was prepared by using rat pineal gland sections (also, no counterstaining was used).

2.4. Statistical analyses

The statistical analyses were carried out by analysis of variance (ANOVA) followed by Newman–Keuls test ($\alpha = 0.05$). Normality and variance homogeneity were verified as ANOVA assumptions. Mathematical transformations were performed when necessary (Zar, 1984). The results of all experiments were expressed as a percentage compared to the extra control group (the crabs that did not receive melatonin, only saline, and were not exposed to UV radiation).

3. Results

3.1. ROS concentration

The crabs that were not exposed to UV radiation and that received the three melatonin doses did not show any significant ($p > 0.05$) difference from those that received physiological saline (Fig. 1A). The animals exposed to UVA showed no significant ($p > 0.05$) difference when compared with the control group. On the other hand, the animals that received a melatonin dose of 200 pmol/crab (supraphysiological dose) showed a decrease in ROS concentration (Fig. 1B). The animals injected with crab saline and exposed to UVB radiation showed an increase ($p < 0.05$) in ROS concentration. However, in animals treated with melatonin, the ROS concentration was similar ($p > 0.05$) to that of non-irradiated crabs (Fig. 1C).

3.2. ACAP quantification

The animals not exposed to UV radiation and that received melatonin did not show any significant ($p > 0.05$) difference from the animals that received crab saline (Fig. 2A). In animals exposed to UVA radiation, there was no significant ($p > 0.05$) difference from the controls. However, the animals that received a dose of 2 pmol/crab of melatonin showed a decrease ($p < 0.05$) in the ACAP levels (Fig. 2B). When the animals were exposed to UVB radiation, the ACAP levels were lower ($p < 0.05$) than in the controls; nevertheless, the animals that received doses of melatonin showed ACAP levels similar ($p > 0.05$) to the control group (Fig. 2C).

3.3. CAT activity

CAT activity in animals that were not exposed to UV radiation and that received the three melatonin doses displayed no significant ($p > 0.05$) difference from the activity in animals that received physiological crab saline (Fig. 3A). When the animals were exposed to UVA radiation, they showed no significant ($p > 0.05$) difference from the control group; the same ($p > 0.05$) was observed in animals that received a dose of 0.02 pmol/crab of melatonin. However, in animals that received doses of 2 or 200 pmol/crab, CAT activity increased ($p < 0.05$, a tendency to higher levels) (Fig. 3B). When the animals were exposed to UVB radiation, no significant ($p > 0.05$) difference from the control group was observed; however, in animals

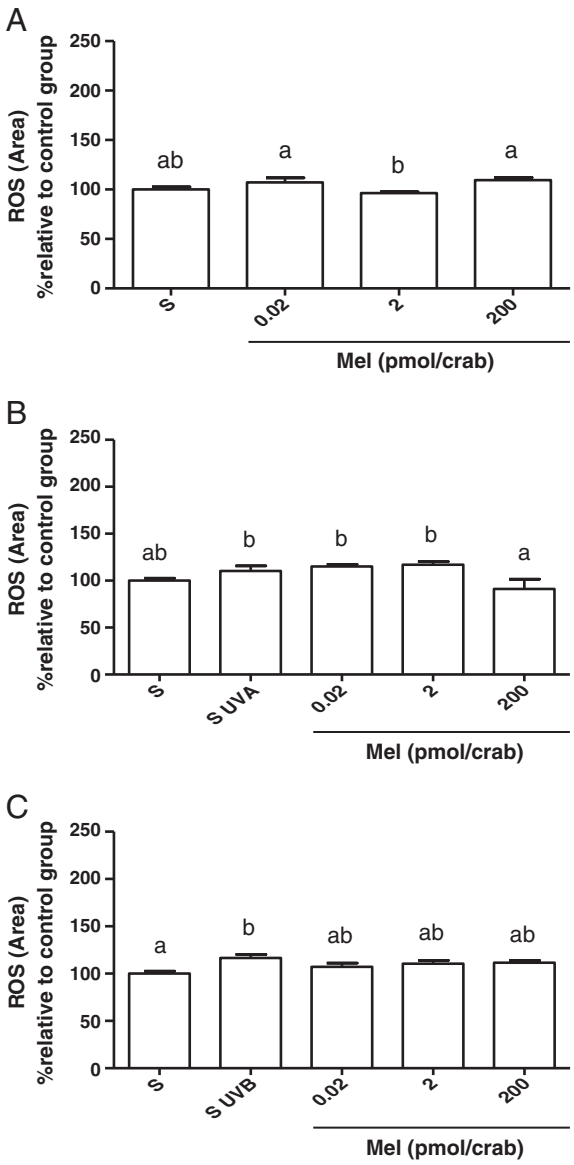

Fig. 1. Noon (12:00 h) concentration of reactive oxygen species (ROS) in eyestalks of animals acclimated to constant light: (A) not exposed to UV radiation, (B) exposed to UVA radiation, (C) exposed to UVB radiation. Each point represents a percentage of the mean ± S.E.M. (n = 5). S = control group.

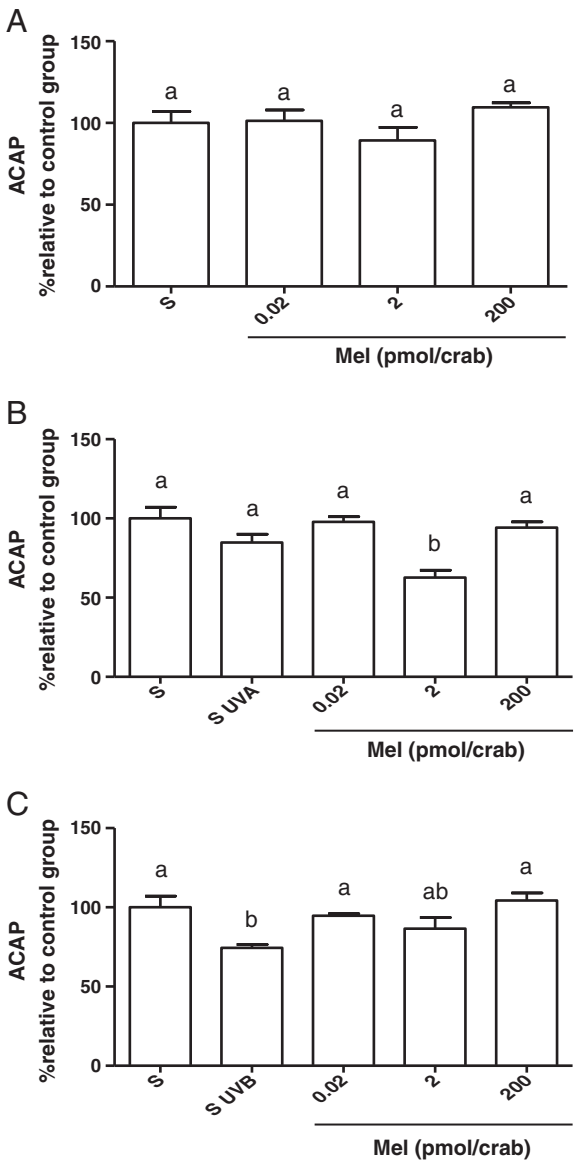

Fig. 2. Antioxidant competence against peroxyl radical (ACAP) in eyestalks of animals acclimated to constant light at 12:00 h: (A) not exposed to UV radiation, (B) exposed to UVA radiation, (C) exposed to UVB radiation. Each point represents percentage of mean ± S.E.M. (n = 5). S = control group.

that received doses of 0.02 or 2 pmol/crab of melatonin, an increase ($p<0.05$) of CAT activity was observed. Interestingly, animals that received a dose of 200 pmol/crab showed a decrease ($p<0.05$) in this enzyme activity compared to the other melatonin-treated groups (Fig. 3C).

3.4. LPO quantification

Regarding LPO, the animals that were not exposed to UV radiation and that received the three melatonin doses showed no significant ($p>0.05$) difference from the animals that received physiological saline (Fig. 4A). Animals exposed to UVA radiation showed no significant ($p>0.05$) difference from the control group; the same ($p>0.05$) was observed in animals that received melatonin doses of 0.02 or 2 pmol/crab. We noted only a trend toward increase in the LPO levels. However, the animals that received a melatonin dose of 200 pmol/crab showed a decrease ($p<0.05$) in LPO levels when compared with the other melatonin-treated groups, but did not differ ($p>0.05$) from the control (Fig. 4B). Animals exposed to UVB radiation showed an increase ($p<0.05$) in the LPO levels; however, the crabs that received melatonin doses of 0.02 or 2 pmol/crab showed a decrease ($p<0.05$) in the LPO levels. In animals that received a melatonin dose of 200 pmol/crab, only a tendency ($p>0.05$) toward a decrease was observed (Fig. 4C).

3.5. Histology and immunohistochemistry

Fig. 5A shows the general structure of the eyestalk of the crab, stained by hematoxylin and eosin. The retina is constituted by photoreceptors and pigment glial cells. The photoreceptors project through the *zona fasciculata* to a group of three successively arranged optic ganglia: the *lamina ganglionaris*, the external medulla, and the internal medulla. Two chiasms, the external and the internal, connect the *lamina ganglionaris* to the external medulla, and the external medulla to the internal medulla, respectively. The basement membrane limits the retina proximally. This system was well described by Grassé et al. (1976) and by Allodi et al. (1995), and also briefly described for the crab *N. granulata* by Vargas et al. (2010).

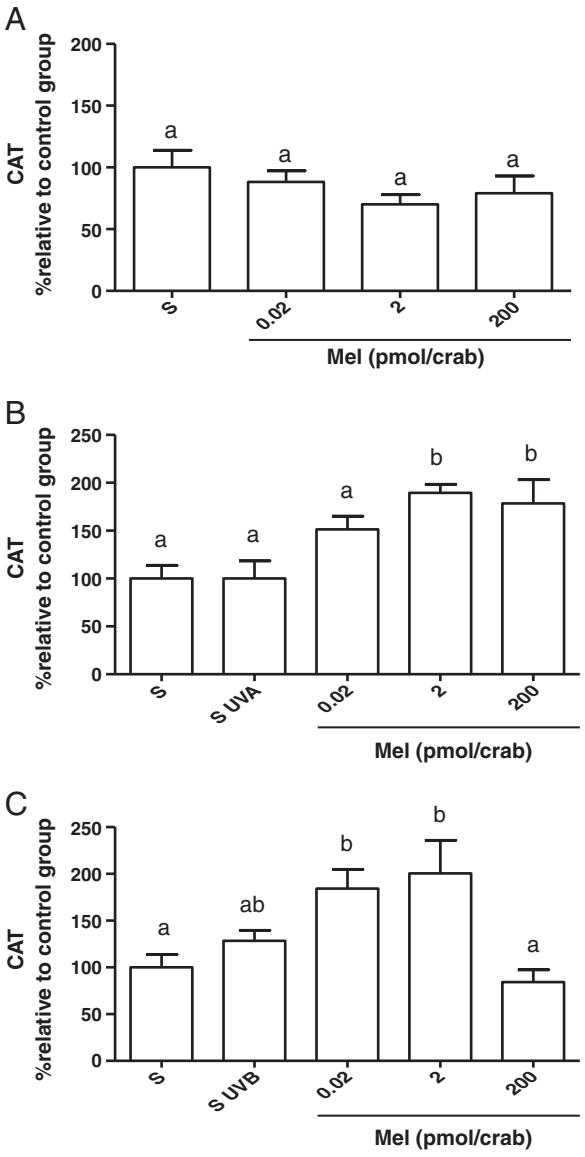

Fig. 3. Catalase activity in eyestalks of animals acclimated to constant light at 12:00 h: (A) not exposed to UV radiation, (B) exposed to UVA radiation, (C) exposed to UVB radiation. Each point represents a percentage of the mean ± S.E.M. (n = 5). S = control group.

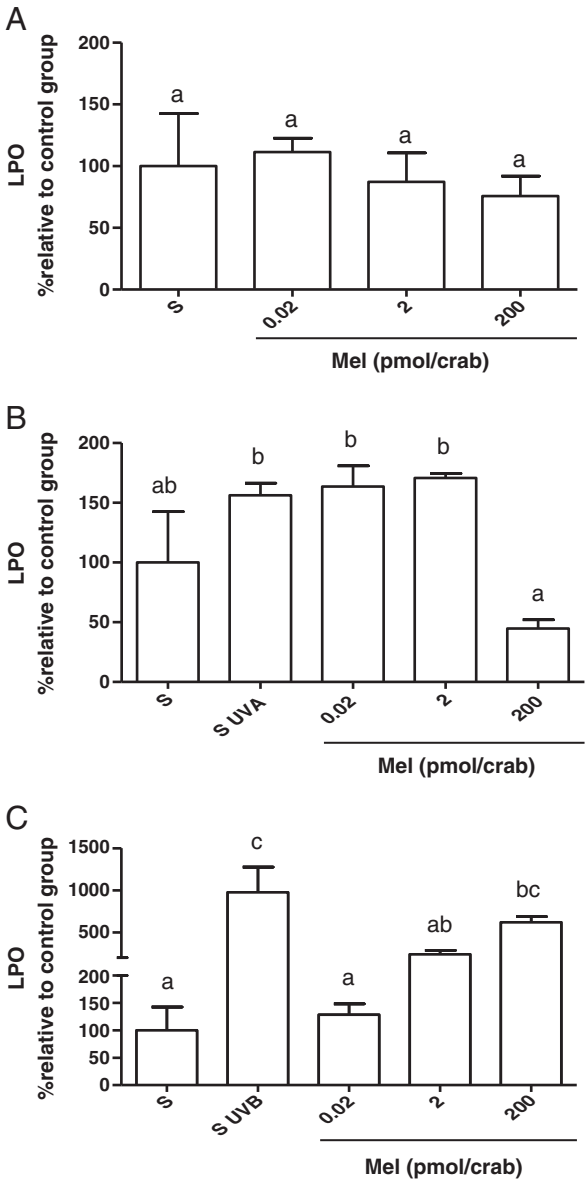

Fig. 4. Lipid peroxidation (LPO) in eyestalks of animals acclimated to constant light at 12:00 h: (A) not exposed to UV radiation, (B) exposed to UVA radiation, (C) exposed to UVB radiation. Each point represents a percentage of the mean ± S.E.M. (n = 5). S = control group.

A histological section of a crab eyestalk not immunoreacted with anti-melatonin (negative control of the reaction) is shown in Fig. 5B. Fig. 5C shows the positive control of the reaction (rat pineal gland). The immunohistochemistry for melatonin showed a strong reaction in virtually all cell types of the retina (Fig. 5D) and of the other structures of the eyestalk, as is apparent in the *lamina ganglionaris* and external chiasm (Fig. 5E).

4. Discussion

In the beginning of the 1990s, Tan et al. (1993) demonstrated that melatonin is a scavenger for ROS. Since then, many studies have confirmed that melatonin is a potent antioxidant agent (Hardeland et al., 1995; Reiter et al., 1997; Reiter, 1998; Pandi-Perumal et al., 2006; Peyrot and Ducrocq, 2008; Hardeland et al., 2009; Belforte et al., 2010). However, in invertebrates, especially in crustaceans, reports on this subject are quite scarce. Recently, Maciel et al. (2010) showed that melatonin may act to decrease the aerobic metabolism and ADS in the gills of the crab *N. granulata*, whereas in muscle (Geihs et al., 2010) melatonin in the ADS acts in a time- and dosage-dependent manner. In the present study, we explored the possibility that melatonin decreases the effects of UV radiation in the eyestalk of *N. granulata*.

When the crabs were exposed to UVA radiation, the ROS concentration tended to increase; nevertheless, when the animals received a dose of 200 pmol/crab of melatonin this effect was not observed (Fig. 1B). Additionally, when the animals were exposed to UVB radiation, an increase of ROS concentration was observed; however, the crabs that received injections of melatonin showed a decreased ROS that was similar both to the control and to the exposed animals (Fig. 1C). These results are comparable to findings in rabbit cornea cell cultures exposed to UV radiation: corneas incubated with melatonin show lower ROS concentrations when exposed to UVB (Ciuffi et al., 2003). This confirms the antioxidant potential of melatonin in the crab, and demonstrates that melatonin is a scavenger for ROS (Peyrot and Ducrocq, 2008; Hardeland et al., 2009; Belforte et al., 2010).

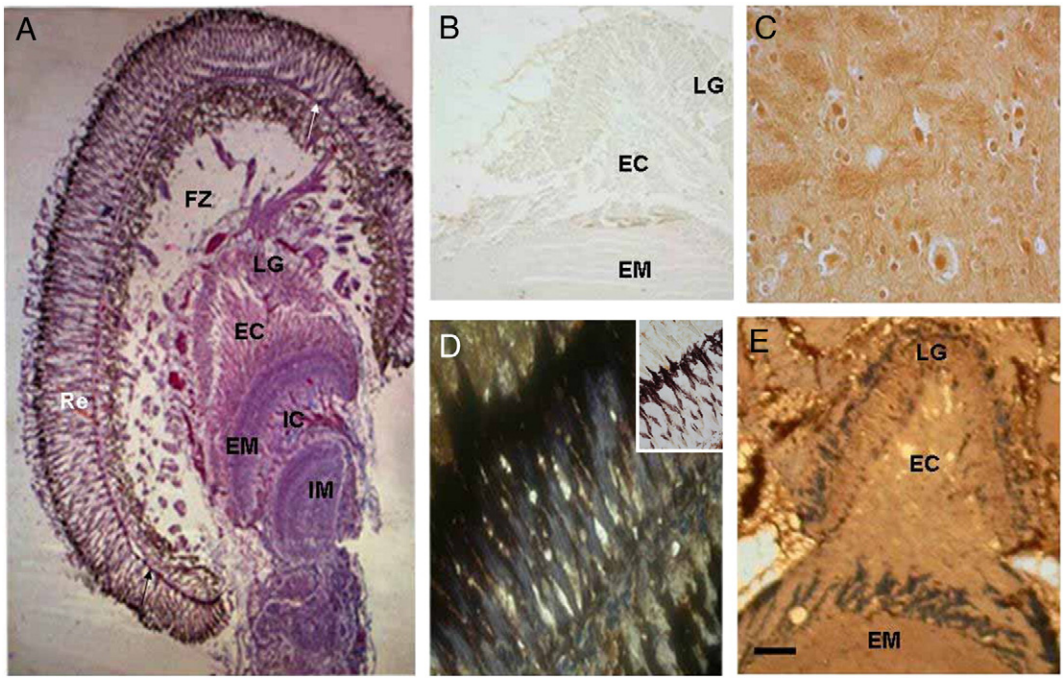

Fig. 5. Structure of the crab eyestalk and immunohistochemical reaction using an antibody against melatonin. (A) Low magnification of an eyestalk stained with hematoxylin and eosin, showing the retina (RE), basement membrane (white arrow), fasciculated zone (FZ), *lamina ganglionaris* (LG), external chiasm (EC), external medulla (EM), internal chiasm (IC), and internal medulla (IM). (B) Negative control in the eyestalk of the crab *Neohelice granulata*. Abbreviations as in A. (C) Positive control in the pineal gland of a rat. (D) Immunohistochemistry for melatonin in the retina. Insert: Negative control of the reaction. (E) Immunohistochemistry with anti-melatonin in the *lamina ganglionaris* (LG), external chiasm (EC) and part of the external medulla. Scale bar: A — 500 μm; B — 150 μm; C — 25 μm; D — 40 μm, Insert — 120 μm; E — 180 μm.

Increased ROS concentration can produce cell damage. To help avoid this noxious effect, ACAP must also increase, thus preventing cell damage triggered by the increase of ROS concentration. Interestingly, in the present study we observed that crabs exposed to UVA radiation (not injected with melatonin) did not show any changes in the ACAP levels. Because the optimum conditions were maintained in the ACAP assays, thus favoring the maximization of the antioxidants, we do not rule out a certain decrease of the ACAP levels in biological conditions. In the crabs exposed to UVA radiation and injected with 2 pmol of melatonin/crab, the ACAP levels decreased compared with the other groups (Fig. 2B). This melatonin effect is similar to that observed in rats, where ocular damage was induced by light (Bubenik and Purtill, 1980; Wiechmann and O'Steen, 1992). However, under UVB radiation, the same dose (2 pmol/crab) injected in the crabs did not produce the same effect. The ACAP decrease in *N. granulata* acclimated to constant light compared with that in crabs acclimated to a 12L:12D photoperiod (Vargas et al., 2010) may be attributed to the absence of melatonin rhythm (Maciel et al., 2008). Therefore, the exogenous melatonin might act in a dosage- and time-dependent manner, thus changing the profile of ACAP, as we presume occurred in the muscle of non-stressed crabs (Geihs et al., 2010). Hence, this situation seems to be also occurring in the eyestalk of the crab *N. granulata,* suggesting that not only the dose, but also the kind of stressor (UVA or UVB, in this case) is important to determine the response to melatonin.

Melatonin is known to be an antioxidant, not only a scavenger of ROS, but also a modulator of the antioxidant enzymes (for review, see Rodriguez et al., 2004 and Hardeland, 2008). Concerning UV radiation, Cejková et al. (2000) showed that in the rat corneal epithelium the activities of some antioxidant enzymes, such as CAT, SOD, and GPx are decreased after UVB radiation. Similar effects, reduction in SOD and GPx activities, were also observed in the rat lens (Anwar and Moustafa, 2001). However, increases of SOD and GPx activities in rat lenses were observed when the animals were treated with melatonin. In the present study, crabs exposed to UVA radiation and injected with melatonin (either 2 or 200 pmol/crab) showed increased CAT activity compared to the control group (Fig. 3B). A similar trend was observed in crabs exposed to UVB radiation (Fig. 3C); however, the highest dose of melatonin reduced CAT activity. In rats, the UV radiation seemed to induce CAT when endogenous melatonin was not sufficiently available to, for example, scavenge the increasing ROS production; whereas exogenous melatonin could have up-regulated CAT activity in this organ. Interestingly, no effect of melatonin on CAT activity was observed. However, melatonin has been shown significantly to increase SOD activity and GSH levels in the rat eye (Belforte et al., 2010).

The peroxidation of lipids is considered to be one of the most important types of ROS damage generated by UV radiation (Fig. 4A). We showed here that melatonin did not produce any alteration in LPO levels in animals not exposed to UV radiation. However, when the animals were exposed to UVA, a bias towards an increase in LPO levels occurred (Fig. 4B), although animals that received 200 pmol/crab of melatonin showed a decrease in the LPO levels, indicating that melatonin is protecting the tissues from damage caused by UV radiation. In animals exposed to UVB and not injected with melatonin, the intense increase in LPO levels may suggest that the dose of UVB radiation used in our experiments caused serious damage in the visual system of the crab *N. granulata* (Fig. 4C). In this regard, our experiments revealed a remarkable result: both the doses of 0.02 and 2 pmol/crab of melatonin produced a higher capacity of reducing LPO levels, compared with the dose of 200 pmol/crab (Fig. 4C). A similar result was observed when rat lenses were exposed to both UVA and UVB radiation: there was an increase in LPO levels. Nevertheless, when the rats were injected with melatonin, they showed lower levels of LPO (Anwar and Moustafa, 2001). Moreover, melatonin decreased the LPO levels in the eye tissues of rats (Belforte et al., 2010). Therefore, based on the results of other authors as well as on our own, we suggest that melatonin is capable of decreasing the noxious effects of UV radiation in the visual system of both mammals and crustaceans.

Many studies are concerned with demonstrating melatonin in the eyestalk of crustaceans: for example, in *Macrobrachium rosenbergii*

(Withyachumnarnkul et al., 1992a,b), *Penaeus monodon* (Withyachumnarnkul et al., 1995), *Procambarus clarkii* (Agapito et al., 1995; Balzer et al., 1997), *Astacus fluviatilis* (Meyer-Rochow, 2001), *Saduria entomon* (Meyer-Rochow, 2001), *Uca pugilator* (Tilden et al., 1997), and *Euphausia superba* (Pape et al., 2008). In the crab *N. granulata,* the presence of melatonin in the eyestalk (Maciel et al., 2008) and recently in the muscle (Geihs et al., 2010) was shown by mass spectrometry. However, none of these studies investigated melatonin using immunohistochemistry. This procedure is important to accurately characterize *in situ* the exact structure where melatonin appears. We revealed the presence of melatonin in the whole eyestalk, suggesting that virtually every cell type located in this structure is either capable of producing this amine or of being directly influenced by this molecule. This result is also reinforced by the lipophilic nature of melatonin (Wiechmann and Summers, 2008), which may therefore, diffuse freely through neighbor cells within the eyestalk.

In conclusion, this study emphasizes the importance of the relationship between the effects of UV radiation and the protective role attributed to melatonin. Since UV radiation was capable of increasing ROS concentration and LPO damage and of decreasing the ACAP, as a consequence, melatonin was capable of decreasing both ROS concentration and LPO damage, in addition to increasing CAT activity. Thus, melatonin appears to be an effective antioxidant against the high production of ROS induced by UV radiation, at least in the eyestalk of the crab *N. granulata.*

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