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Effects of melatonin administration on oxidative stress and daily locomotor activity patterns in goldfish

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Melatonin has a number of physiological functions in addition to light-dark transduction. In recent years, many *in vivo* and *in vitro* studies in rodents have revealed an important antioxidant activity of melatonin, both directly and indirectly. Nevertheless, the potential effects of melatonin as an antioxidant in fish remain unknown. The aim of this research was to evaluate the capacity of melatonin injections (3 mg/kg) to attenuate oxidative damage after submitting goldfish to oxidative stress caused directly by hydrogen peroxide (H_2O_2) baths and indirectly by hypoxia and subsequent reoxygenation, as well as the locomotor activity. The results revealed that melatonin decreased lipid damage in muscle after hypoxia/reoxygenation (1.22 *vs* 2.27 nmoles lipid peroxides/g tissue), but not in liver. Mortality caused by oxidative stress was not attenuated by melatonin. Surprisingly, melatonin caused an increase of mortality (50 *vs* 95%) when administered before hypoxia. Locomotor activity was also affected by melatonin but not by the administration of the vehicle, suggesting a sedative effect of melatonin in goldfish. In conclusion, melatonin administration provoked slight effects on lipid peroxidation and mortality resulting from oxidative stress, with reduction of locomotor activity in relation to the vehicle.

Key words: Melatonin, Oxidative stress, Hypoxia/Reoxygenation, H_2O_2, Goldfish, Locomotor activity.

Melatonin (N-acetyl-5-methoxytryptamine) is the main secreted product of the pineal gland. Melatonin synthesis is regulated by light and shows a circadian rhythm, with high values during the night and low values during the day (3). However, the role of melatonin and of the

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pineal gland in the circadian system may vary substantially between different species. In fish, the pineal gland acts as an intracellular circadian oscillator and is involved in direct photoreception, while in mammals, the pineal gland has lost its photoreceptive capacity and circadian oscillator (4).

Melatonin has several functions, the main one being the control of circadian and seasonal rhythms, transducing the light information and acting both as a daily clock and a calendar (5). In addition, in recent years melatonin has been described as a potent direct antioxidant (6, 7). Besides these direct effects, melatonin seems to play an indirect role in protection against oxidative stress, promoting gene expression of antioxidant enzymes (8). Furthermore, a number of circadian rhythms of antioxidative enzyme activity and of LMWA have been described, principally in mammals, suggesting an important role for melatonin in regulating these rhythms (9), for a review). Melatonin antioxidant activity has been observed both *in vitro* and *in vivo* (7, 10). Most studies have been carried out in rodents, while melatonin antioxidant activity in fish has not been evaluated. Furthermore, as melatonin production (and its actions) may differ between fish and mammals, the putative role of melatonin as an antioxidant remains to be investigated in the fish model.

Oxygen-derived free radicals are generated in cells by aerobic processes, but oxidative stress can also be generated by external factors in fish. For instance, cultured fish usually faces stressful conditions such as hypoxia/reoxygenation and hydrogen peroxide (H_2O_2) exposure, all of which generate oxidative stress. Low oxygen pressures are frequent in intensive fish farming and during fish handling, and the subsequent reoxygenation after hypoxia generates an increase in free radicals formation (11, 12). On the other hand, H_2O_2 is used in aquaculture as a treatment to control ectopic diseases of cultured fish (13).

The aim of this research was to evaluate the *in vivo* capacity of an intraperitoneal (i.p.) injection of melatonin (3 mg/kg) to attenuate oxidative damage (lipid peroxidation) and mortality caused by submitting goldfish to oxidative stress either indirectly by hypoxia and subsequent reoxygenation or directly by H_2O_2 baths. In addition, the behavioural response of fish after melatonin administration was evaluated. Furthermore, locomotor activity was recorded and the daily activity patterns of fish were analysed.

Materials and Methods

Animals and housing.– Goldfish used in the experiments (11.9 ± 4.1 g. b.w., mean ± SD) were obtained from a local distributor (Jumipez, S.A., Murcia, Spain). Fish were kept in 60-l tanks, well aerated and equipped with biological and mechanical filters. Water temperature was controlled at 22.5 ± 0.5 ºC and the photoperiod was maintained following a 12:12 light-dark cycle, with light onset at 7 a.m. Fish were fed daily ad libitum with a standard goldfish diet (Wardley Premium Goldfish Flakes).

Oxidative damage caused by hypoxia/reoxygenation.– The duration of hypoxia was set at 6 hours, approaching the Lethal Dose 50 (LD50), previously calculated in our laboratory (data not shown). Hypoxia started two hours after lights-on or lights-off, depending on whether the experiment was performed in the middle of the light phase (ML) or in the dark

phase (MD). Each experimental group (n=20) was transferred into a 20-l tank, where the water had been deoxygenated by bubbling nitrogen gas (Air Liquide Spain, S.A.). Nitrogen was continuously bubbled throughout the hypoxia period to keep oxygen levels at 0.35 mg/l. At the end of hypoxia, fish were transferred into 60-l tanks with an oxygen saturation of 8.7 mg/l. Mortality was evaluated twelve hours after reoxygenation; survivors were anaesthetised in ice and sacrificed by decapitation. Tissue samples from liver and white muscle were collected and immediately frozen at –80 °C.

Two ranges of melatonin concentrations were studied: physiological and pharmacological levels. First, two experimental groups were subjected to hypoxia: at mid-light (HML), when endogenous melatonin is lowest; and at mid-dark (HMD), coinciding with the highest levels of circulating melatonin. Second, the influence of pharmacological levels of melatonin was evaluated in three experimental groups: MEL-1, which received i.p. injected melatonin (Sigma Aldrich Chemicals) (3 mg/kg) immediately before the hypoxia period, MEL-2, which were injected (3 mg/kg) immediately after hypoxia, during reoxygenation, and VEH, a group subjected to hypoxia but receiving only the vehicle (saline with 1% ethanol, Panreac Química S.A., Spain). Such vehicle solution was prepared in a way that fish received 0.4 µmole ethanol/kg body weight, an amount much lower than that observed in physiological concentrations in anoxic conditions (3-5 µmol/g) (22).

Oxidative damage caused by exposure to H_2O_2.– Fish were transferred to 20 l tanks, to which H_2O_2 (Panreac Quimica S.A., Spain) was added (6.2 µmol/l water) and after one hour, fish were returned to their initial aquaria. Mortality was evaluated and tissue samples were collected as described above. The experimental groups established were as follows: CTRL, which was only exposed to the treatment of H_2O_2; and MEL, which also received an i.p. administration of melatonin (3 mg/kg) one hour prior to H_2O_2 exposure. In addition, chronic administration of melatonin and repeated exposure to H_2O_2 was tested. This protocol consisted of three baths of H_2O_2 (2.2 µmol/l water), administered every-other-day, as previously described (13). Three experimental groups were designed: CTRL, which only received H_2O_2 treatment; VEH, which received the administration of saline + 1% ethanol; and MEL, which received the administration of melatonin (3 mg/kg). Both vehicle and melatonin were administered six hours prior to H_2O_2 exposure, seven days prior to the experiment and during treatment with H_2O_2.

Analysis of locomotor activity.– It was measured by means of an infrared photocell (Omron, mod E3S-AD62, Japan) placed against the aquarium wall, 6 cm from the bottom and 16 cm from the corner. The number of light beam interruptions was counted and stored every ten minutes by a computer. This study was run in parallel to the experiment of repeated exposure to H_2O_2 where the same fish were studied during the phase prior to H_2O_2 treatment, when VEH and MEL groups only received the daily administration of vehicle or melatonin, respectively.

Melatonin analysis.– Plasma melatonin levels after i.p. administration was measured two, four, eight and twelve hours after injection. Endogenous plasma melatonin at ML and MD were also measured

as controls. Melatonin concentration in plasma was determined using a commercial radioimmunoassay kit (Melatonin direct RIA, RE 293 01, IBL Hamburg). Procedure and validation of the analysis has previously been described by BAYARRI *et al.* (14). Radioactivity was measured using a γ counter (Wallac 1470, Perkin Elmer).

Lipid hydroperoxide assay.– Lipid hydroperoxide levels in liver and white muscle tissues were determined by means of a commercial kit (Bioxytech LPO-560^{TM}, OXIS Health Products, Inc.). Tissue samples were homogenised (20% w/v) in 20 mM phosphate buffer, then 1% (v/v) 0.5 M BHT (Sigma Aldrich Chemicals) was added to prevent sample oxidation. Homogenates were immediately centrifuged (5000 xg for 15 minutes at 4 °C) and the supernatant was collected and stored at –80 °C until analysis.

Data statistical analysis.– It was performed with Excel and SPSS software. Mortality data were subjected to a chi-square test, while the means of lipid peroxidation and waveforms of activity were compared by an analysis of variance (ANOVA), followed by the Duncan post hoc test. The analysis and representation of locomotor activity records were performed with the chronobiology software El Temps (Version 1, 187; Prof. Díez-Noguera, University of Barcelona).

Results

Melatonin levels.– Plasma melatonin in goldfish under normal physiological conditions showed low levels at ML (41.3 pg/ml), rising to 292.1 pg/ml at MD (Fig. 1). Melatonin i.p. injection brought about an increase in its plasma levels,

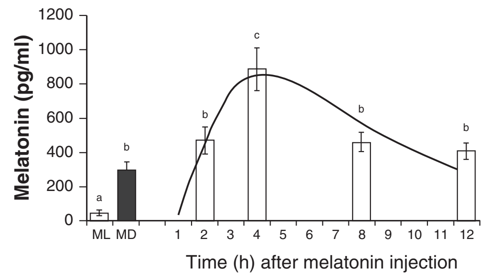

Fig. 1. *Physiological plasma melatonin levels at ML, mid-light; and MD, mid-dark; and pharmacological levels 2, 4, 8 and 12 hours after i.p. administration of 3 mg/kg.*

Values are expressed as mean ± S.E.M. (n=8). Data were subjected to one way ANOVA, $p<0.05$. Different letters indicate significant differences between groups. The black line represents the fitted equation calculated for melatonin kinetics after its administration.

which, after two hours, were higher than nocturnal levels (466.5 *vs* 292.1 pg/ml, respectively). The highest levels were found four hours after melatonin administration (878.1 pg/ml), decreasing significantly thereafter.

Oxidative damage caused by hypoxia/reoxygenation.– Mortality in the HMD (hypoxia during MD) and HML (hypoxia during ML) groups was 50% and 60%, respectively, with no statistically significant differences being observed (chi-square test, $p<0.05$). However, when melatonin was administered before hypoxia (MEL-1), mortality showed significant differences. In this group, mortality reached 95%, but remained around 50% in the other groups (hypoxia time was set at LD50).

Muscle lipid hydroperoxide levels found in HMD, but not in HML, increased compared with their respective control values, CMD (intact animals sampled at mid-dark) (Fig. 2A). Surprisingly, liver lipid hydroperoxide levels (Fig. 2B)

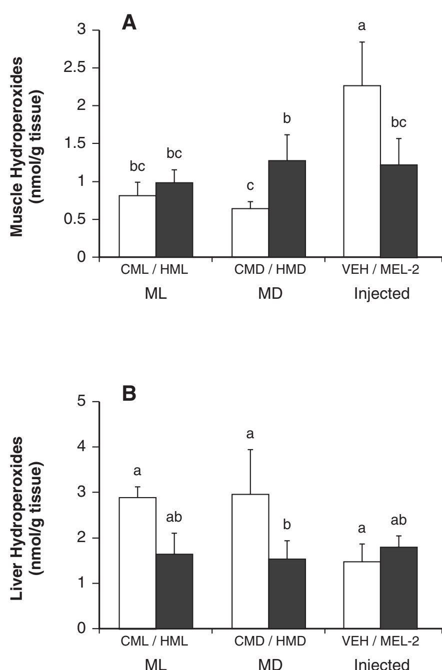

Fig. 2. *Lipid hydroperoxides (nmol/g tissue) in white muscle (A) and liver (B) at ML and MD (physiological groups), and pharmacological groups (administered with vehicle or melatonin). Black bars represent groups subjected to hypoxia (HML, HMD and MEL), and white bars represent their respective controls (CML, CMD and VEH). CML and CMD represent the peroxidation levels in intact animals at ML and MD, respectively.*
Data have been represented as mean ± S.E.M. (n=6). Different letters indicate significant differences between groups (ANOVA, p<0.05). Data corresponding to MEL-1 are not represented due to the high mortality in this group, and thus the lack of available samples.

in HMD decreased in relation to the concentrations observed in its respective control group (ANOVA, p<0.05).

Melatonin administration during reoxygenation (MEL-2) significantly reduced muscle lipid peroxidation compared with the effect that the vehicle alone had (Fig. 2A) (ANOVA, p<0.05). Liver peroxidation when melatonin was administered during reoxygenation did not show differences with the vehicle group (Fig. 2B) (ANOVA, p>0.05).

Oxidative damage caused by H_2O_2.– Mortality caused by acute exposure to H_2O_2 was similarly high in CTRL and MEL (90% and 80% respectively) and was not statistically different (chi-square test, p>0.05). Daily administration of melatonin (chronic exposure to H_2O_2) did not significantly decrease mortality (chi-square test, p>0.05). Surprisingly, administration of vehicle (VEH) during chronic treatment significantly reduced mortality down to 35.7%.

Lipid peroxidation both in muscle and liver showed similar levels in CTRL, VEH and MEL, and did not differ significantly between any groups (ANOVA, p>0.05).

Melatonin and daily activity patterns.– Goldfish showed diurnal behaviour, with most of their activity occurring during the light phase (data not shown). Analysis of the daily activity waveforms over seven days, showed that CTRL fish displayed a stable and regular activity during the light phase (Fig. 3A). In VEH fish, however, an increase of activity (up to 65%) in the hour following manipulation could be observed (Fig. 3B). In contrast, MEL fish did not show an increase in activity (Fig. 3C), but sustained a level similar to that found in CTRL.

Differences between VEH and MEL were further analyse by representing the mean activity of both groups as the increase in activity in relation to the baseline of CTRL group (Fig. 4). VEH fish showed higher activity levels than MEL after manipulation, the latter group showing similar activity levels to the CTRL group. The highest differences between

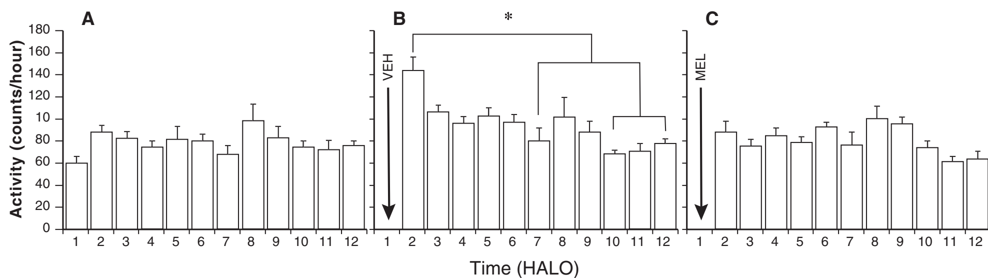

Fig. 3. *Mean locomotor activity of CTRL (A), VEH (B) and MEL (C) group goldfish.* Data were obtained during a seven day period. Activity levels, measured as the number of interruptions of the photocell infrared beam, are represented on the ordinate axis, while the hours after light onset (HALO) are represented on the abscisa axis. Vehicle (saline + 1% ethanol) and melatonin (3 mg/kg dissolved in vehicle) were administered at the first hour after lights onset (indicated by a black arrow). Data have been expressed as mean ± S.E.M. (n=7). Asterisks indicate significant differences between different hours of a same group (ANOVA, $p<0.05$).

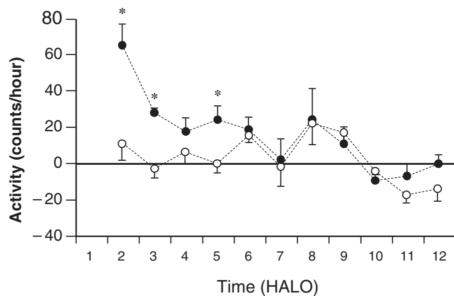

Fig. 4. *Mean locomotor activity during recording period of VEH (black circles) and MEL (open circles).* Values have been calculated as difference of activity in relation to mean activity of control group (baseline y=0). Locomotor activity is represented on the ordenate axis and the hours after light onset (HALO) are represented on the abscisa axis. Data are expressed as mean ± S.E.M. (n=7). Asterisks indicate significant differences between the two groups at that hour. It can be observed that VEH group showed higher activity levels after manipulation than the MEL group, which showed similar activity levels to the CTRL group.

these groups appeared one hour after manipulation, although significant differences still remained four hours after manipulation (*t*-test, $p<0.05$).

Discussion

This is the first study carried out to evaluate melatonin antioxidant activity in fish. Previous studies have related melatonin with an antioxidant effect, protecting against oxidative damage generated by free radicals or reactive oxygen species (ROS) (6, 7). In other studies, no protective effect of melatonin was observed (18, 19), and its administration even resulted in an increase on cellular damage (20, 19). In goldfish, mortality was not attenuated by melatonin in the two protocols of oxidative damage tested: hypoxia/reoxygenation and exposure to H_2O_2. On the other hand, administered melatonin attenuated lipid peroxidation in muscle in hypoxia/reoxygenation group.

Overall mortality results, both in the hypoxia/reoxygenation treatment and H_2O_2 exposure, showed that melatonin failed to protect against oxidative damage. Indeed, melatonin was seen to have a negative effect in the hypoxia/reoxygenation experiment, as mortality drastically increased up to 95%. This result could be explained by the hypoxia resistance strategy of these animals, which are regularly

subjected to short hypoxia and/or anoxia periods, so that anaerobic metabolism plays an important role (21). Goldfish belong to this group of animals, where tissue glycogen is the main source of energy during anoxia (22). As a result, during hypoxia, plasma glucose increases fivefold over normal levels (23), depletion of the glycogen store appearing to be the factor that finally limits anoxic goldfish survival (17). Previous experiments carried out in goldfish demonstrated the hypoglycaemic effect of melatonin (30), which promoted both a decrease in plasma glucose and an increase in liver glycogen stores. Thus melatonin administration before hypoxia resulted in a reduction of the energy available for the fish to deal with oxygen deprivation, reducing survival, which might explain the high mortality rate obtained in MEL-1 group. In conclusion, in the case of goldfish any beneficial effect of melatonin as an antioxidant is counteracted by its interference with glucose metabolism during hypoxia.

In the experiment causing a direct oxidative stress, both by acute and chronic H_2O_2 exposure, melatonin failed to reduce mortality. Surprisingly, mortality of VEH after chronic exposure was significantly lower in relation to CTRL. This lower mortality rate in VEH than in CTRL could be explained by the manipulation that the former received both during the seven days prior to H_2O_2 exposure as well as exposure period. The stress generated by manipulation might have induced mucus secretion and/or stimulated epithelial regeneration, thus increasing protection against H_2O_2. Previous studies have demonstrated that stressful conditions can induce proliferation of mucous cells in the skin of fish (31). However, MEL was manipulated in the same way as VEH but mortality was not reduced. The way melatonin may block such a stimulus after manipulation is unknown.

The results obtained for lipid peroxidation did not show a clear effect of melatonin on oxidative stress. In muscle, melatonin significantly lowered values, coinciding with the effect observed in other antioxidants in fish like vitamin E (16). In contrast, in liver (Fig. 2), lipid peroxidation in MEL-2 showed similar values that VEH. The decrease in lipid peroxidation observed in HML and HMD in liver may seem surprising. However, hypoxia-tolerant animals as goldfish, develop mechanisms of anticipation to the oxidative damage generated by reoxygenation (32), so that, during anoxia and before reoxygenation, activity of antioxidant enzymes and GSH levels increase (33). In goldfish an increase in activity of liver catalase and glutathion reductase has been found, which could explain the decrease obtained in liver peroxidation in our study. However, LUSHCHAK *et al.* (33) found an inverse pattern in tissue peroxidation after hypoxia/reoxygenation, where lipid hydroperoxides decreased in muscle and increased in liver. These differences could be explained by differences in the protocol used to expose goldfish to oxygen deprivation, the levels of oxygen during hypoxia and the duration of this period.

Melatonin has also been described as inducing behavioural changes, promoting sleep in diurnal species (3). The effect of melatonin on locomotor activity in fish has only been studied to date in zebrafish, a diurnal species (35), in which melatonin administration reduced locomotor activity and induced a sleep-like state. This action seemed to be mediated by melatonin specific receptors because the effects on locomotor activity disappeared when both luzindole and melatonin were administered (35). In our study in goldfish, melatonin did not reduce locomotor

activity, but blocked the increase in activity associated with manipulation (VEH, Fig. 3). However, no increase in locomotor activity was observed in the MEL group, which was manipulated in the same way as the VEH group. The effect of melatonin on activity in goldfish has not been reported before, although PINILLOS *et al.* (36) observed a dose-dependent reduction in food intake by melatonin, an inhibition that persisted eight hours after administration, but was suppressed when both melatonin and luzindole were administered. However, in our study, differences in activity between VEH and MEL were observed only for four hours, which may indicate the existence of different pathways of melatonin action on food intake and locomotor activity. Summarising, these results provide the first insight into a sedative effect of melatonin in goldfish.

In conclusion, although melatonin demonstrated antioxidant properties in many model systems, in our study melatonin appeared to have little effect, if any, as an antioxidant in the two protocols of oxidative stress investigated. Indeed, the possible effect of melatonin in attenuating oxidative damage was counteracted by its effect on glucose metabolism, thus increasing mortality caused by hypoxia. In addition to the above metabolic effects, melatonin also had behavioural effects, as its administration avoid the increase in activity following fish manipulation.

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J. F. LÓPEZ-OLMEDA, M. J. BAYARRI, M. A. ROL DE LAMA, J. A. MADRID and F. J. SÁNCHEZ-VÁZQUEZ. *Efectos de la administración de melatonina en el daño oxidativo y los patrones diarios de actividad motora del carpín.* J. Physiol. Biochem., **62** (1), 17-26, 2006).

La melatonina presenta varias funciones fisiológicas además de la transducción de la señal lumínica. Estudios *in vitro* e *in vivo* en roedores han puesto de manifiesto una importante actividad antioxidante de la melatonina. No obstante, todavía se desconocen los efectos antioxidantes potenciales de la melatonina en peces. El objetivo de este trabajo fue estudiar la capacidad de la melatonina (3 mg/kg) para atenuar el daño oxidativo después de someter a carpines a estrés oxidativo causado directamente por baños con peróxido de hidrógeno (H_2O_2) e indirectamente por hipoxia y posterior reoxigenación, así como evaluar su capacidad de alterar los ritmos diarios de actividad. Los resultados revelan que la melatonina disminuyó el daño oxidativo después de la hipoxia/reoxigenación en músculo (1.22 *vs* 2.27 nmoles peróxidos lipídicos/g tejido), pero no en hígado. Sin embargo, la melatonina no atenuó la mortalidad causada por el estrés oxidativo. Además, la administración de melatonina antes de la hipoxia causó un incremento de mortalidad (50 *vs* 95%). La administración de melatonina, pero no de vehículo, también afectó a la actividad motora, sugiriendo un efecto sedante de la melatonina en el carpín. En conclusión, la administración de melatonina parece mostrar un débil efecto protector sobre la peroxidación lipídica y la mortalidad generadas por el estrés oxidativo, con reducción de la actividad motora.

Palabras clave: Melatonina, Estrés oxidativo, Hipoxia/Reoxigenación, H_2O_2, Carpín, Actividad motora.

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