Research Article

Melatonin supplementation protects against the benzo(e)pyrene cytotoxicity and optic cup formation disruption in chicken embryos

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ABSTRACT

Benzo(e)pyrene is a cytotoxic chemical to the eyes, while neurohormone melatonin may exhibit protective effects on this cytotoxicity. In the current study, we have investigated the cytotoxic effects of benzo(e)pyrene on the chicken embryonic optic cups formation and whether melatonin supplementation protects chicken embryos against this xenobiotic toxicity. Fertilized chicken eggs were incubated for 48 h and then, they were divided into different groups. These groups included basal (without any treatment), control (distilled water), benzo(e)pyrene, melatonin and benzo(e)pyrene + melatonin groups, respectively. The 10 µl of distilled water or same volume of solution containing treatment compounds were injected into the air sac of the chicken egg. After an additional 18 h of incubation, the chicken embryos were excised and analyzed. The cytotoxicity was measured by a colorimetric whole chick embryo trypan blue assay. In embryos from basal, control and melatonin (0.01, 1 and 100 µM) groups, the frequency of the embryos with normal optic cups was 100% and had no increase in the embryonic cell death observed in post excision. In contrast, the frequency of normal optic cups in the benzo(e)pyrene (0.02 to 1200 µM) groups was significantly reduced (log IC50= -4.24 ± 0.02, R^2= 0.98) with concentration-responsive manner. In addition, an increase in the embryonic cell death was also observed (log IC50 = -7.23 ± 0.28; R^2 = 0.63). Melatonin treatment dose-responsively inhibited the benzo(e)pyrene-induced optic cups abnormality by 22.35 ± 4.06, 76.38 ± 3.30 and 100 % at the concentrations of 0.01, 1 and 100 µM, respectively. This same phenomenon was also observed in benzo(e)pyrene-induced embryonic cell death, i.e., melatonin suppressed the embryonic cell death by 16.67 ± 4.17, 54.17 ± 4.17 and 100 % with the abovementioned concentrations, respectively. Thus, melatonin supplementation injected into the chicken eggs protected against the benzo(e)pyrene embryotoxicity. Different pathways can be involved in melatonin's protective effects.

Keywords: benzo(e)pyrene, melatonin, melatonin receptors, optic cup, cytotoxicity, chicken embryo, trypan blue assay.

1. INTRODUCTION

Melatonin is the ancient molecule secreted majorly by pineal gland of vertebrates nocturnally and it serves as a signal of biological clocks with the environmental illumination (1). Besides of its chronobiological functions, in cells, melatonin hampers apoptosis caused by enzymatic cascade via scavenging free radicals, enhancing phagocytosis, increasing the gene expression of the antioxidant enzymes [superoxide dismutase (SOD)], and reducing the gene expression of prooxidative enzymes of nitric oxide synthases (2-9). Melatonin membrane receptors are coupled to G proteins. The melatonin receptors 1a, 1b, and 1c (Mel1a, Mel1b and Mel1c) were cloned from animals (10). Their corresponding membrane receptors are usually referred as MT1 and MT2. A truncated Mel1c ortholog named GPR50 (Melatonin-related receptor or G protein-coupled receptor 50) was cloned from the mammals (10). Several melatonin biding sites are also studied including the calcium binding protein calmodulin and NRH: quinone reductase enzyme 2 (QR2), the previously classified melatonin receptor 3 (MT3) (10). The melatonin synthetic machinery and the mRNA transcripts of melatonin receptors were identified in the fertilized avian egg, which also content substantial amounts of the melatonin (11-13). Regarding to melatonin membrane receptors, Mel1a was localized in mammalian blastocysts (14), in the embryos of the reptiles (15), and in the avian embryos (16).

Benzo(e)pyrene, an environmental air pollutant, is among the polycyclic aromatic hydrocarbons (PAHs) (17). It is an isomer of the benzo(a)pyrene, which is used as a parameter to reflect the environmental PHA pollution (18, 19). These kinds of pollutants are present in agriculture environment with a large scale, affecting humans and wild animals, especially birds that taking up large volumes of the air containing particles (20). These compounds are found in soil (21), water (22), vegetable oils (23), and processed foods (24). The benzo(e)pyrene biotransformation machinery is critical for its cytotoxicity (25, 26), and this machinery was found in the avian embryos (27).

Benzo(e)pyrene induces oxidative stress, inflammation (28), and apoptosis (29) in the eye of the many species, including human (29-32). Melatonin has protective effects against the cytotoxicity of the isomer benzo(a)pyrene via antioxidant and antiapoptotic pathways (33-36) and also by inhibition of the PHA metabolizing P450 enzymes (37). Benzo(e)pyrene also inhibited QR2, which is a catechol quinone reductase in the chick retina (38), and influenced the melatonin metabolism in fish (39).

In chicken embryos, the rudimentary retina begins to develop at 48 h of incubation and forms the optic cups with the calcium-calmodulin-dependent invagination of the optic vesicles (40). Remarkably, melatonin in the eggs activates calmodulin pathway and melatonin receptors to regulate the formation of the optic cup (16). In the present study, the cytotoxicity of benzo(e)pyrene on formation of the optic cup in chicken embryos and the potential protective effects of melatonin were investigated by visually yes/no observation method, using a simple microscope. The chicken embryo cell death post-excision was also evaluated by a trypan blue whole chicken embryo colorimetric assay, which was read by a microplate read.

2. MATERIALS AND METHODS

2.1. Animal care.

The embryos were used according to the international organizations of ethics in animal experimentation in chicken embryonic stages. The project had the approval of the local Ethics Committee (CEPAE-UFPA) and the approved protocol number is 229-14. A local hatchery (Makarú, PA, BR) provided the fertilized chicken eggs (Gallus gallus domesticus). At sunset of the incubation day 0, the eggs were placed in a vertical position (air sac facing up) in an

automatic incubator (Dove Factories, Vinhedo, São Paulo, Brazil), at 37.8 °C and humid atmosphere (55-60% relative humidity). The incubator had a transparent acrylic lid that allowed the light and dark cycle of about 12:00 in each cycle (Equatorial line).

2.2. Chemicals.

Benzo(e)pyrene and melatonin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The benzo(e)pyrene concentration range was selected from a previous chicken retina study (38), with alterations to use a higher spectrum of the concentrations since there were no pharmacological data available regarding the toxic benzo(e)pyrene levels on chicken embryo and to make sure that the compound will cross the yolk sac penetrating into the embryo. Benzo(e)pyrene was firstly diluted in ethanol/distilled water vol/vol under vortex to attain a 1200 µM stock solution. A serial dilution with distilled water to make the 0.02 to 800 µM working solutions was done. The given volume was 10 µl for an egg. Melatonin concentrations were chosen from a previous study (16). Melatonin stock solution (1 mM) was prepared in ethanol/distilled water vol/vol under agitation, and the work solutions (0.01 to 100 µM) were prepared by serial dilution with distilled water. The concentrations of melatonin work solutions were 0.01 to 100 µM.

2.3 Chicken embryo "*in ovo*" assay.

The chicken embryos were staged following Hamburger & Hamilton (1951) (41). The fertilized chicken eggs were placed in the incubator for 48 h (HH12), once the pre-lens ectoderm contacts with the evaginated optic vesicles at this incubation time (42, 43), then the eggs were used for chemical challenges. These groups included basal (without treatment), control (10 µl of distilled water), benzo(e)pyrene serial concentrations (0.054 to 1200 µM), melatonin serial concentrations (0.01, 1 and 100 µM), and benzo(e)pyrene plus melatonin. Treatments were inoculated through a small hole (2 mm), which was made with a clamping tip in the eggshell, above the air sac (10 µl per egg). After the hole have been closed with a microporous tape, the eggs were incubated for additional 18 h, the time-frame in which the invagination of the optic vesicle forms the optic cups (primitive retinas) (43). Then each eggshell was carefully opened in the region above of the air sac, and the area vasculosa was visually analyzed. After a total 66 h of incubation, the chicken embryo was excised and washed in phosphate-buffered saline (PBS) (4 °C, pH 7.4). The embryo's external morphological characters were analyzed using a common optical microscopy (Bioval L-1000B, São Paulo, SP, Brazil). The normal chicken embryos (HH18/19- chicken embryo at stage 18/19, as described by Hamburger and Hamilton, 1951) (41) were used to optic cups analysis via yes/no method (16, 44), the percent of the normal chicken embryos from each group was calculated, and a representative embryo with or without optic cup from each group was imaged using a digital color camera (Nikon Coolpix 2300, Tokyo, Japan) coupled to the microscope. Data were presented as the frequency of the normal chicken embryo per group, i.e., the percentage of the 66 h of incubation chicken embryos presenting normal external morphology into basal group, control group, and each treatment group; the assays were repeated five times until each group have been completed by a total of 20 embryos (N = 20).

2.4. Colorimetric trypan blue assay.

Whole chicken embryos' trypan blue assay was previously applied to 66 h of the incubation chick embryos by other authors (45), and the present microtiter plate protocol was developed from a previous study (46). Embryos from basal (6 embryos), control (10 µl of distilled water)

(6 embryos), melatonin (0.01 or 1 or 100 µM) (6 embryos each concentration), benzo(e)pyrene (0.02 to 1200 µM) (6 embryos each concentration), and benzo(e)pyrene plus melatonin (6 embryos each concentration) were excised and washed in PBS (4° C; pH 7.4). The embryos were put in a calcium and magnesium free saline solution (CMF, pH 7.4) to remove the extraembryonic tissues. Then, the embryos were incubated in a solution containing 875 µl of calcium-and magnesium-free Hank's balanced salt solution (HBSS CMF) and 125 µl of trypan blue-Hanks solution 0.4 % (SIGMA), one embryo per well (20 wells plate). The plate was shaken (45 rpm) two minutes, followed by 10 min of rest. Subsequently, the embryos were removed from the plate containing trypan blue-Hanks solution, passed through three PBS-washing cycles, and moved to a well containing 240 µl of the PBS (96 wells microtiter plate). The trypan blue absorbance was read at 630 nm (reference value 590 nm) by the BioTekTM Absorbance Microplate Reader ELx800 (Fisher Scientific). Then, the embryos were imaged using a digital color camera (Nikon Coolpix 2300, Tokyo, Japan) coupled to the microscope.

2.5. Calculation of the optic density of the imaged embryos.

The optic density (OD) of the embryos reacted with trypan blue was calculated based on the account of trypan blue taken by embryos, thus it is discriminated over a background (47). Mean of the image intensity (I) and its respective background (k) was obtained using the resources offered by the Image J with "IHC Profiler" plugin (48). The OD was calculated through the equation OD = log10 (I - k) using the resources of the Graph Pad Prism software. The area analyzed in each embryonic region was 192 mm^2, including the center of the optic region. The OD of the whole optic region was measured, the areas were between 270 mm^2 and 360 mm^2.

2.6. Statistical analyses.

Curves adjusting and statistical analyses were done using resources offered by the program Graph Pad Prism 6.00 for Windows, GraphPad Software, La Jolla, California, USA (www.graphpad.com, accessed in June 2018). The adjust of the concentration-response curves was by nonlinear regression (http://www.graphpad.com/guides/prism/6/curve-fitting/, accessed in June 2018). One-way ANOVA followed by Dunn's multiple comparisons test were used to compare the differences between the groups. Two-way ANOVA followed by Multiple comparisons test were used to evaluate the variations in function of the treatment and of the embryonic region. Results were considered significant at $P < 0.05$ (http://www.graphpad.com/guides/prism/6/statistics/, accessed in June 2018).

3. RESULTS

3.1. Effects of benzo(e)pyrene and melatonin on morphogenesis of optic cup in chicken embryo.

The 66h-incubation eggs were carefully opened above the air sac for morphological analysis. Macroscopically, the area vasculosa exhibited normal morphology in basal, control and all melatonin treated groups. In contrast, in benzo(e)pyrene 800 and 1200 µM treated groups, the area vasculosa lacked the translucent feature, which was observed in the basal and other groups (Table 1). The morphology of chicken embryos after 66 h of incubation in basal, control, and all melatonin groups were in accordance with the HH18 stage chicken embryo, as it was described by Hamburger and Hamilton, 1951 (41); however, the morphology of embryos treated with benzo(e)pyrene starting from 400 µM group did not match well with the normal HH18 stage chicken embryo since they did not have optic cups. The normality was restored by cotreated with melatonin 100 µM. (Table 1) (Figure 1).

Table 1. Summary of variable concentrations of benzo(e)pyrene and melatonin on the morphology of chicken embryos.

Groups	Embryos/ Group	Number of Assays	Area Vasculosa	% Normal Embryos
Basal	20	5	Normal	100
Distilled water 10 µl	20	5	Normal	100
Mel 0.01 µM	20	5	Normal	100
Mel 1 µM	20	5	Normal	100
Mel 100 µM	20	5	Normal	100
Benzo(e) 0.54 µM	20	5	Normal	100
Benzo(e) 1.64 µM	20	5	Normal	100
Benzo(e) 4.94 µM	20	5	Normal	100
Benzo(e) 14.81 µM	20	5	Normal	100
Benzo(e) 44.44 µM	20	5	Normal	050
Benzo(e) 133.30 µM	20	5	Normal	030
Benzo(e) 400 µM	20	5	Normal	000
Benzo(e) 800 µM	20	5	Abnormal	000
Benzo(e) 1200 µM	20	5	Abnormal	000
Mel 0.01 + Benzo(e) 0.54 µM	20	5	Normal	100
Mel 0.01 + Benzo(e) 1.64 µM	20	5	Normal	100
Mel 0.01 + Benzo(e) 44.44 µM	20	5	Normal	035
Mel 0.01 + Benzo(e) 133.30 µM	20	5	Normal	000
Mel 0.01 + Benzo(e) 400 µM	20	5	Normal	035
Mel 0.01 + Benzo(e) 800 µM	20	5	Normal	035
Mel 1 + Benzo(e) 0.54 µM	20	5	Normal	100
Mel 1 + Benzo(e) 1.64 µM	20	5	Normal	100
Mel 1 + Benzo(e) 44.44 µM	20	5	Normal	070
Mel 1 + Benzo(e) 133.30 µM	20	5	Normal	100
Mel 1 + Benzo(e) 400 µM	20	5	Normal	070
Mel 1 + Benzo(e) 800 µM	20	5	Normal	070
Mel 100 + Benzo(e) 0.54 µM	20	5	Normal	100
Mel 100 + Benzo(e) 1.64 µM	20	5	Normal	100
Mel 100 + Benzo(e) 44.44 µM	20	5	Normal	100
Mel 100 + Benzo(e) 133.30 µM	20	5	Normal	100
Mel 100 + Benzo(e) 400 µM	20	5	Normal	100
Mel 100 + Benzo(e) 800 µM	20	5	Normal	100

Mel: melatonin, Benzo(e): benzo(e)pyrene.

The frequency of the chicken embryos with optic cups was reduced by benzo(e)pyrene treatment at concentration dependent manner (-4.24 ± 0.02, R2 = 0.98) (N = 20) (Figure 2a). The injection of the melatonin into the air sac of chicken egg 10 min before benzo(e)pyrene, inhibited the benzo(e)pyrene toxic responsive curve. Melatonin at the concentrations of 0.01, 1.0 and 100 µM respectively suppressed by 22.35 (± 4.06), 76.38 (± 3.30) and 100 %, of the maximal benzo(e)pyrene effect ($P<0.05$). Melatonin did not significantly modify the logIC50 of benzo(e)pyrene concentration responsive curve (alpha = 0.05), suggesting a non-competitive inhibition (N = 20) (Figure 2b).

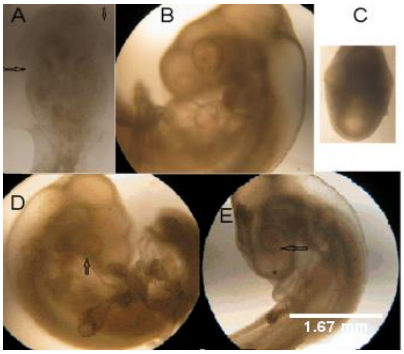

Fig. 1. Representative photos of chick embryos with different treatments.

The embryos were freshly prepared. A. Dorsal view of the 48h-incubated chicken embryo, with an arrow pointing to the optic vesicles. B. Lateral view of a normal 66h-incubated chicken embryo. C. Dorsal view of the head of a 66h-incubated chicken embryo treated with benzo(e)pyrene (400 µM). It lacked the optic cups (OC) but presented an upper plane ledge. D. Lateral view of a 66h-incubated chicken embryo treated with benzo(e)pyrene (400 µM), with an arrow pointing to the optic region. E. Lateral view of a 66h-incubated chicken embryo cotreated with benzo(e)pyrene (400 µM) and melatonin (100 µM), with an arrow pointing to an optic cup.

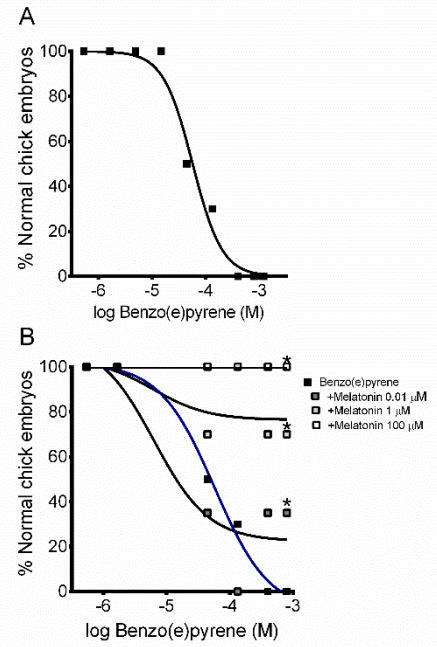

Fig. 2. Concentration-responsive curves of benzo(e)pyrene or benzo(e)pyrene plus melatonin on the morphology of chicken embryos.

*A. A concentration-responsive curve of benzo(e)pyrene on the morphology of chicken embryos. The presence of optic cups is referred as the normal embryos. B. Effects of different concentrations of melatonin on the embryotoxicity induced by benzo(e)pyrene. (N = 20). *P < 0.05.*

The frequency of the chicken embryos with optic cups was reduced by benzo(e)pyrene treatment at concentration dependent manner (-4.24 ± 0.02, R2 = 0.98) (N = 20) (Figure 2a). The injection of the melatonin into the air sac of chicken egg 10 min before benzo(e)pyrene, inhibited the benzo(e)pyrene toxic responsive curve. Melatonin at the concentrations of 0.01, 1.0 and 100 µM respectively suppressed by 22.35 ± 4.06, 76.38 ± 3.30 and 100 %, of the maximal benzo(e)pyrene effect (P<0.05). Melatonin did not significantly modify the logIC50

of benzo(e)pyrene concentration responsive curve (alpha = 0.05), suggesting a non-competitive inhibition (N = 20) (Figure 2b).

3.2. Effects of benzo(e)pyrene and melatonin on cell death of the post-excision chicken embryos.

The post-excision embryonic cell death was evaluated by a trypan blue colorimetric assay, which was developed to measure the cell death that occurs immediately after entire chicken embryo excision from the egg. The absorbance values, the indication of the cell death in the excised chicken embryos, among basal, control and different melatonin concentrations groups were not statistically different ($P > 0.05$) (N = 6). Therefore, the cells death in chicken embryos under these treatments was not detectable. On other hand, an increase in embryonic cell death was observed in benzo(e)pyrene treated groups and the cell death was in the function of benzo(e)pyrene concentrations (log IC50 = -7.23 ± 0.28; $R^2 = 0.63$) (Figure 3a). However, the maximal benzo(e)pyrene effect on cell death was significantly inhibited by different concentrations of melatonin indicated by the suppressed concentration-effective corves (Figure 3b). Melatonin at the concentrations of 0.01, 1 and 100 µM suppressed the post-excision embryonic cell death induced by the benzo(e)pyrene by 16.67 ± 4.17, 54.17 ± 4.17 and 100 %, respectively ($P < 0.05$) (N = 6) (Figure 3c).

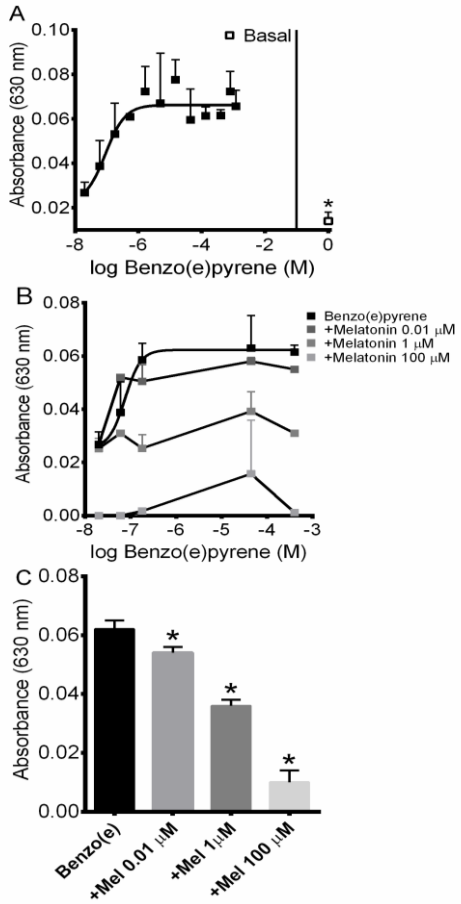

Fig. 3. Effects of the benzo(e)pyrene and melatonin on the embryonic cell death.

*The test was performed with a whole chick embryo trypan blue assay in excised embryos. A. Benzo(e)pyrene concentration-dependent cells death curve (N = 20). B. Effects of the melatonin at the different concentrations on benzo(e)pyrene induced embryonic cell death (N = 6). C. Highlight of the maximal benzo(e)pyrene (benzo(e)) effect alone and in the presence of the melatonin (Mel) (N = 6). *P < 0.05.*

To analyze the embryonic image, the excised embryos were imaged after trypan blue assay. No apparent variations were observed in the trypan blue staining distribution in benzo(e)pyrene groups compared to the basal group. However, the natural color of fresh chicken embryo certainly masked the results of trypan blue assayed. To correct this shortcoming, images of the trypan blue stained embryos were analyzed by Image J software with IHC plugin (48) and the same was done for a basal group embryo, which was not stained with trypan blue (NTB). The optic density was measured at a selected embryonic region around 192 mm^2 including the center of the optic region, and the extraembryonic epithelium which are attached to the embryo. The whole optic region areas were between 270 mm^2 and 360 mm^2. Two-way ANOVA statistical analyses indicated optic density variation in function of the embryo's regions ($p < 0.0001$) and of the different treatment groups ($p < 0.0001$). The optic density at the center of the optic region and at the metencephalon region in embryos from different benzo(e)pyrene concentration groups were not statistically significant when compared with the NTB. At some benzo(e)pyrene concentrations, the others embryonic regions, including the whole optic region, presented significant optic density differences when compared with NTB. However, regarding the analyses of the image of the basal embryos reacted with trypan blue, significant differences with NTB were found only in the extraembryonic epithelium region (Figure 4). This indicated that the natural cell death immediately occurred after the embryo excision, which was detected by the trypan blue reaction. Thus, the cell death in the absence of the benzo(e)pyrene treatment mainly occurred at extraembryonic epithelium level.

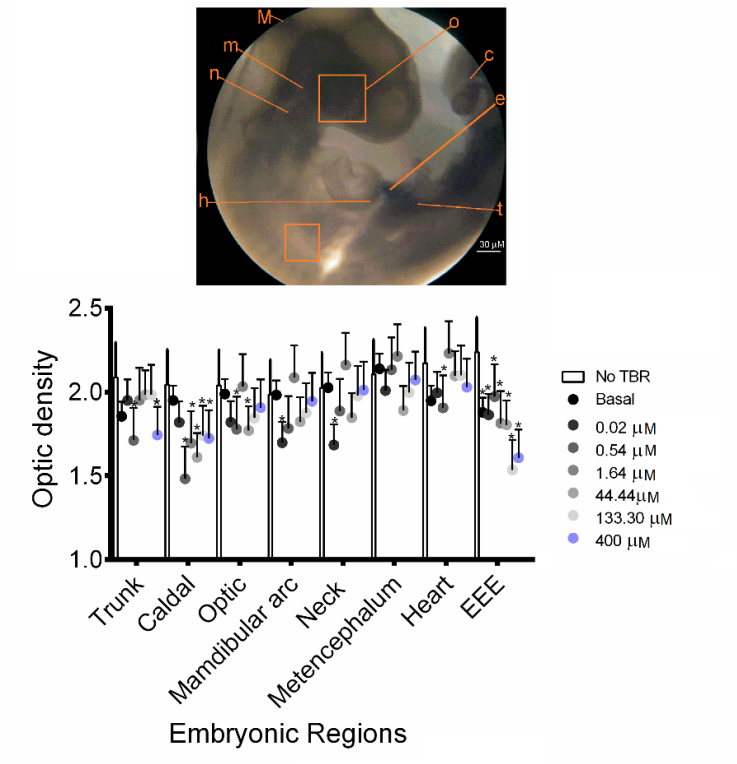

Fig. 4. Effects of different treatments on optic densities in different regions of excised chicken embryos.

Up panel, image of the 66h-incubated chicken embryo in lateral view. A photo of the representative embryo treated with benzo(e)pyrene 44.44 µM assayed with trypan blue microtiter assay. Optic (o), mandibular (m), neck (n), metencephalon (M), heart (h), trunk (t), caudal (c) and extraembryonic epithelium - EEE (e). Squares show the area to be measured the optic density of the all optic region (270 mm^2) and the area used to estimate the optic density of the others highlighted embryonic regions (192 mm^2). Lower panel. Statistical

analyses of the variations of the optic density in the images of the embryonic regions depicted in up panel, in function of the benzo(e)pyrene concentrations and comparation with images of the embryos in the absence of the trypan blue reaction (NTB). Basal corresponds to a chicken embryo that developed without benzo(e)pyrene treatment. Two-way ANOVA statistical analyses showed variation of the optic density in different embryonic regions ($p < 0.0001$) of the embryos submitted to different treatments ($p < 0.0001$). Stars correspond to the variation in the optic density when compared with an image of the 66 h of incubation chick embryo that was not assayed with trypan blue (NTB) into each embryonic region ($p < 0.05$). Error bars correspond to SD. N = 3.

4. DISCUSSION

Benzo(e)pyrene has been chosen instead of its isomer benzo(a)pyrene in this study because it is a potent cytotoxic agent with a limited carcinogenic activity compared to benzo(a)pyrene, which is a powerful carcinogen (49-52). As the best knowledge of ours, little was known about the embryotoxicities of benzo(e)pyrene, while the embryotoxicities of benzo(a)pyrene have well been documented in avian species (53-55). For example, benzo(a)pyrene application at 72h-incubated mallard duck embryo caused eye development deformation after the hatch (53).

The optic cup formation is a calcium-calmodulin mediated process that is more dependent on the extracellular than the intracellular calcium levels (40). Benzo(e)pyrene increases the cytosolic calcium levels in the human T cells and endothelial HMEC-1 cells, but a reduction in the extracellular calcium levels was not observed in these cells in the *in vitro* systems (56-58). In mouse Sertoli cell lines (TM4) benzo(a)pyrene (0.1-100 μmol l^{-1}) triggered apoptosis by increase the intracellular calcium levels and calmodulin expression (59). It seems that the disturbance of optic cup formation caused by benzo(e)pyrene may not associate to a direct reduction of the extracellular calcium levels or the calmodulin inhibition. On the other hand, an increased cytosolic calcium and expression of calmodulin protein induced by benzo(e)pyrene trigger the post excision embryonic cells death and by this has been observed in other animal studies (59-60). In porcine retinal arterioles, benzo(e)pyrene had a vasodilator effect caused by superoxide production and endoplasmic reticulum stress (61). The alterations in the area vasculosa caused by benzo(e)pyrene in the current study can also be explained by this observation (61). It can be suggested that the benzo(e)pyrene could act as a vasodilator in the chicken embryo, even in the absence of the cardiac affections at the concentrations used in the study. Benzo(e)pyrene may mimic the prazosin, a Mel1c antagonist (62), which caused cardiovascular disturbance and optic cups deformation also in the 66h-incubated chicken embryos. These adverse effects of prazosin were inhibited by melatonin treatment depending on concentrations, in a mixed manner, suggesting that prazosin not only inhibited the Mel1c receptor, but also induced oxidative stress. Melatonin supplementation also reduced the frequency of the chicken embryos without optic cups via the blockage of the Mel1a and the calmodulin (16). *In vitro* studies and *in vivo* studies showed that prazosin also suppressed the activity of QR2 (63-64), which was also inhibited by benzo(e)pyrene in chicken retina (38). Up to now, there is little evidence to show that benzo(e)pyrene inhibits the binding of melatonin to Mel1c or Mel1a. In addition, melatonin supplementation counteracted the adverse effects of benzo(e)pyrene by decreasing the frequency of the abnormal chicken embryos in a non-competitive manner, suggesting that melatonin acts at the different sites with benzo(e)pyrene.

Previous studies have shown that the benzo(e)pyrene hydroxylation by the cytochrome P450 is critical to its cytotoxicity (25-26). The P450 genes (CYP genes) locate in a locus positively modulated by the cytosolic aryl hydrocarbon receptor (AhR) (65). Benzo(e)pyrene binds to AhR with low affinity (66) to form AhR-PHA complex which is translocated into the nucleus, where controls a gene battery related with oxidative stress response, cell cycle control,

and apoptosis (67-68). Benzo(e)pyrene is a low-affinity ligand to AhR but also a bad substrate of the P450 enzyme (69). The study in rainbow trout also showed that AHR2α might not directly involve the benzo(e)pyrene oxidative stress (70). The AhR can be activated by a benzo(e)pyrene complex with the dimeric form of the glycine N-methyltransferase named 4S polycyclic hydrocarbon-binding protein. The AhR activation by the complex of benzo(e)pyrene triggers the CYP1A1 gene expression (65 -71). The CYP1A1 protein is also known as aryl hydrocarbon hydroxylase (AHH), an enzyme that solubilizes PHA for further elimination (27), and the PHA binding affinity to avian binding site is higher than that to mammalian one (69). In chicken embryo development, the 4S polycyclic aromatic hydrocarbon-protein was found in the 14-day-old chick embryo liver (72). The AHH (CYP1A1) basal activity was found since the third day of incubation in the whole chicken embryo, and in the rudimentary liver since the fourth day of incubation. The AHH basal and induced activities were also observed in the brain, muscle, allantoid sac, yolk sac membrane, heart, and digestive tract. Chicken embryo has hepatic mensurable PHA liver microsomal hydroxylation capability (AHH induced activity) only after the its fifth embryonic day, when the rudimentary liver appears (27). Here, study focused on the cytotoxicity induced by benzo(e)pyrene at the optic cup development time-frame. Thus, the 48 h fertilized chicken eggs were given benzo(e)pyrene into the yolk sac, and the embryos were excised after additional 18 h incubation; consequently, the effects were observed in the 3-day old chick embryo that had basal, but not measurable inducible AHH activity (27). As a result, the benzo(e)pyrene hydroxylation machinery was represented by the inducible AHH of the mature extraembryonic tissues in the chicken embryos used in this study.

In current study, only the effects of benzo(e)pyrene on the surface of the whole chick embryos were studied, by a trypan blue microtiter colorimetric assay which was developed to exclude the potential contamination by the internal tissues. Other studies showed a variation in the cell viability with trypan blue and the mechanisms related with PAHs apoptosis. For example, when rainbow trout feed with PAHs at different time intervals, the oxidative stress occurred at tissue-specific benzo(e)pyrene concentration responsive manner and with an increase in CYP1A protein cytosolic levels (70). The similar results were observed in the human tissues. The human vascular epithelium was more susceptible to caspase-dependent apoptosis triggered by benzo(e)pyrene then retinal cells. The mature human retinal cells treated with benzo(e)pyrene at the concentrations of 400 and 1000, but not 100 µM, their viability was reduced when measured by the trypan blue colorimetric assay (29, 30). In these studies, the apoptosis was also evaluated by the measurement of the caspase enzymes activity (73). In the retinal neurosensory cells (R28 cells), the activity of the hallmark of apoptosis effectors caspases 3/7 and of the initiator caspase 12 were only observed at benzo(e)pyrene concentrations excessive 200 µM , while the initiator caspase 8 was only at the level of 200 µM, and the initiator caspase 9 was observed at less concentrations (29). A similar caspase activity spectrum was obtained from experiments using human retinal pigment epithelial cells (ARPE-19) (30). However, for human microvascular endothelial cells (HMVEC) benzo(e)pyrene starting at 100 µM reduced the cell viability and increased the caspase 3/7 activity (29). Thus, the vascular epithelium was more susceptible to caspase-dependent apoptosis triggered by benzo(e)pyrene then retinal cells. This tissue-specificity was also observed in the present study, where the trypan blue optic density distribution in the surface of the embryos was in function of the embryonic region and of the benzo(e)pyrene concentrations. Pharmacologically, the morphological results were reinforced by observation that benzo(e)pyrene has less potent to reduce the frequency of the normal chicken embryos than to increase the post excision embryonic cells death. In fact, the half maximal dose to increase the embryonic cells death was about 0.06 µM, while to reduce to a half the frequency of the normal chicken embryos the dose was about 65 µM.

In human RPE cells, melatonin at the level of 100 µM inhibited H_2O_2-induced cell damage, decreased the apoptotic rate, and increased the mitochondrial membrane potential (9) and this is the concentration also used in the current study. Melatonin protects against benzo(a)pyrene cytotoxicity in rats mainly through its free scavenger action activity (35). A study in rodents showed that melatonin inhibited the P450 enzyme at a similar concentration range (3 - 300 µM) (37) used herein. It is obvious, that melatonin at the concentrations of 1 - 100 µM had the capacity to suppress benzo(e)pyrene induced embryonic cells death. Melatonin at the concentration of 1 µM was more potent to increase the frequency of normal chicken embryos (73.75 ± 3.67 %) than to reduce the embryonic cells death (54.17 ± 4.17 %), suggesting melatonin counteracts benzo(e)pyrene cytotoxicity mainly by its free scavenging activity, the same was observed when melatonin was tested against the oxidative stress triggered by the isomer benzo(a)pyrene (35). Benzo(e)pyrene triggers free radical production which can deplete the melatonin content in avian embryos or eggs (11-13) by consumption to scavenging free radicals. This in rodents results in melatonin reduction by formation of a cascade of the metabolites with antioxidant activity, but less melatonin can bind to its receptors or binding sites (74), reducing its function to modulate optic cups formation (16). In brief, the modulating function of melatonin on optic cup formation in chicken embryos are jeopardized by benzo(e)pyrene treatment. A potential mechanism is that melatonin is depleted by free radical formation induced by benzo(e)pyrene, i.e. melatonin is exhausted by its interaction with free radical. Thus, the lowered melatonin level cannot functionally modulate a normal optic cup formation. To overcome this melatonin deficiency, exogenously applied melatonin is required as proved by the current study.

In conclusion, the present pharmacological results suggest that benzo(e)pyrene can induce an increased embryonic cells death and a reduction in the frequency of the embryos with normal optic cups. Additionally, melatonin is an anti-apoptotic molecule. Its supplementation can restore the frequency of the embryos with normal optic cups under the oxidative stress induced by benzo(e)pyrene or other toxic agents. Melatonin supplementation can be used as a preventive therapy for pregnant human or animals inhabiting under a potentially PHA polluted environment.

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AUTHORSHIP

Felipe Teixeira Soares and Antonilde Marcelina Arruda de Sá: acquisition of data and analysis/interpretation by Yes/No method, and drafting of the manuscript. Hiroyuki Otsuki Guimarães and Paulo Marcelo Silva da Silveira: acquisition of data by Colorimetric trypan blue assay, data analysis/interpretation, and drafting of the manuscript. Lucia de Fatima Sobral Sampaio: conception of the Project, experiments design, data analysis/interpretation, drafting the manuscript, writing and revising the final version of the manuscript. The final version to be published was approved by all authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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