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ORIGINAL ARTICLE

Acute Bisphenol-A Exposure Triggers Superoxide-Nitric Oxide Imbalance and Immunocompetence Impairment of Eisenia Fetida Earthworm

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Highlights:

1. BPA in concentrations considered tolerable had a negative effect in E.fetida.
2. BPA induces oxidative stress causing DNA damage and compromises the immune competence.
3. DNA and innate immune metabolism damage may be associated with NCDs.

ABSTRACT

Bisphenol-A (BPA) is an endocrine-disrupting molecule associated with the risk of several non-transmissible chronic diseases. We postulated that BPA triggers oxidative alterations, altering immunocompetence and contributing to physiological dysfunction. To evaluate the effects of BPA on the oxidative and immune system, Californian earthworms were reared in a culture medium containing different BPA concentrations for 24 and 72 h. Coelomocytes were used to evaluate the effects of BPA on oxidative markers, cellular proliferation, and apoptosis, and immunocompetence effects were investigated by yeast-exposure assay and the modulation of genes related to immune response. Low BPA concentrations induced coelomocyte proliferation, imbalanced superoxide/NO levels, higher micronucleus frequency, and apoptosis. BPA also induced Amp1 gene overexpression and a low efficiency of dead yeast capture. The association between DNA damage and changes in innate immune metabolism could be related to the action of BPA, which is associated with the risk of physiological disturbances and non-transmissible chronic diseases.

Keywords: endocrine disruptors; genotoxicity; oxidative stress; inflammation; apoptosis; immunocompetence.

EXPOSIÇÃO AGUDA AO BISFENOL-A DESENCADEIA UM DESEQUILÍBRIO SUPERÓXIDO-ÓXIDO NÍTRICO E COMPROMETIMENTO DA IMUNOCOMPETÊNCIA NA MINHOCA *EISENIA FETIDA*

RESUMO

O bisfenol-A (BPA) é uma molécula desreguladora endócrina associada ao risco de diversas doenças crônicas não transmissíveis. Postulamos que o BPA desencadeia alterações oxidativas, alterando a imunocompetência e contribuindo para a disfunção fisiológica. Para avaliar os efeitos do BPA no sistema oxidativo e imunológico, minhocas californianas foram criadas em meio de cultura contendo diferentes concentrações de BPA por 24 horas e 72 horas. Celomócitos foram utilizados para avaliar os efeitos do BPA em marcadores oxidativos, proliferação celular e apoptose, e efeitos de imunocompetência foram investigados por ensaio de exposição a leveduras e modulação de genes relacionados à resposta imune. Baixas concentrações de BPA induziram proliferação de celomócitos, níveis desequilibrados de superóxido/NO, maior frequência de micronúcleos e apoptose. O BPA também induziu a superexpressão do gene Amp1 e uma baixa eficiência de captura de levedura morta. A associação entre danos no DNA e alterações no metabolismo imune inato pode estar relacionada à ação do BPA, que está associado ao risco de distúrbios fisiológicos e doenças crônicas não transmissíveis.

Palavras-chave: desreguladores endócrinos; genotoxicidade; estresse oxidativo; inflamação; apoptose; imunocompetência.

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INTRODUCTION

Many chemicals derived from industrially processed materials have undesirable biological activities, such as bisphenol A-[4,4'-(propane-2,2-diyl)-diphenol] (BPA), one of the most widely used synthetic compounds on Earth. As BPA is an endocrine-disrupting molecule, several lines of evidence have suggested that it has a relevant impact on human health[1]. Regarding the effects of BPA on the immune system, some studies have shown that this molecule could harm neutrophil function, the most abundant population of peripheral blood leukocytes, which represents the primary defense against microorganisms, tissue injuries, and cancer cells[2-3-4-5]. Furthermore, BPA appears to worsen the prognosis of some non-transmissible chronic diseases (NCDs) through alterations that contribute to the establishment, maintenance, or intensification of chronic inflammatory states or a decrease in immunocompetence related to the recognition of microorganisms and procarcinogenic cells[6-7].

However, contradictory aspects related to the potential concomitant effect of BPA on immune function have been reported[8]. These inconsistencies are related to the complexity of the factors involved in the effects of BPA on the immune system. BPA may generate oxidative stress with pleiotropic consequences on cells, including: (1) DNA damage triggering cellular dysfunction and (2) high concentration of derived residues, mainly from the oxidation of macromolecules, associated with mitochondrial dysfunction, by an increase in superoxide ($O_2^{\bullet-}$) and nitric oxide (NO) reactions, generating peroxynitrite ($ONOO^-$), which form hydroxyl radicals ($OH^\bullet$) and nitrogen dioxide ($NO_2^\bullet$). These reactive oxygen species (ROS) cause extensive lipoperoxidation, protein carbonylation, and DNA damage, leading to cellular dysfunction, such as autophagy impairment by lysosomes. The increase in the concentration of intra- and extracellular immunogenic metabolic residues (damage-associated molecular patterns, DAMPs) triggers low-grade chronic inflammatory states in several NCDs[9-10].

To test the association between oxidative stress states and concomitant alterations caused by BPA on immunocompetence, mainly involving inflammatory response, we performed an *in vivo* study using the red Californian earthworm *Eisenia fetida (E. fetida*) as an experimental model. The choice of this model was based on the following reasons: previous evidence suggests that BPA has a toxic effect on earthworms, triggering hyperplasia of the epidermis, increased body wall thickness, ovarian atrophy[11], and the differential modulation of genes related to detoxification (metallothionein), stress response (HSC704), and genotoxicity (PARP1)[12]. Furthermore, earthworm immune cells, named coelomocytes, can be easily and abundantly accessed from coelomic body fluid, presenting similarities with the human innate immune response. Coelomocytes represent the first line of immune defense against microorganisms and pollutants, including physiological processes, such as migration, phagocytosis, cellular aggregation, and earthworm neutrophil extracellular trap (eNET) production[13]. Like humans, earthworm innate metabolism is responsive to environmental components, such as antioxidant and anti-inflammatory foods and pollutants. In this case, these components seem to improve or worsen immune efficiency in capturing and destroying pathogens[14-15].

In this context, we evaluated the simultaneous impact of acute exposure to different concentrations of BPA on immune *E. fetida* metabolism by analyzing oxidative markers (NO, superoxide anion (S), lipoperoxidation (LPX), protein carbonylation (PCarb), genotoxicity by identification of micronucleus (MN) formation, apoptosis, and immunocompetence markers.

METHODS

Earthworm rearing conditions and ethics issues

The earthworms used in this study were commercially obtained from Minhocas Japi (Jundiaí, São Paulo, Brazil) and maintained under standardized laboratory conditions, in which sterilized humus

enriched with yerba mate powder or coffee residues that are used as food by these organisms were used. Humidity was maintained at 60-70% and temperature between 20–22°C. For the experiments, the earthworms were placed in a Biochemical Oxygen Demand (BOD) incubator in the dark. Like many other countries, Brazil does not require approval for studies involving earthworms and other invertebrate organisms by animal research ethics committees. For this reason, this study did not receive formal approval from an ethical board. The authors declare that the research was performed according to the EU Directive 2010/63/EU for animal experiments.

General experimental design and earthworms reared conditions

A total of 102 earthworms were used in the experiments with 54 earthworms to conduct toxicity tests considering the control group and the groups exposed to five different concentrations of BPA. The tests were carried out in triplicate with three earthworms per plate. A total of 54 earthworms were also used to carry out laboratory tests, repeating the same sampling distribution used in toxicity tests. In all assays, we used clitellate earthworms transferred to a vial containing 3.5 g of starch thickener (Nutilis, Danone, São Paulo), 37.50 mL of water, and 12.5 mL of a solution with BPA (Sigma Aldrich¸ San Luis, Missouri, USA) previously diluted in 0.1% dimethylsulfoxide (DMSO) at concentrations of 0.1, 0.3, 1, 10, and 30 µM. DMSO was used as a solvent because the dilution of BPA in water is very low. Although this solvent has some toxicity depending on the concentration used, the dilution of BPA in DMSO has been carried out in studies with other chemicals molecules using *E. fetida* as experimental model[17]. Even so, a pilot test conducted (data not shown) from the experimental conditions that will be described below indicated that the concentration of DMSO used in the study had no toxic effect on earthworms.

The starch medium was used to guarantee the ingestion of the substrate by the earthworm BPA-contaminated because it does not require heating the medium, which could cause some interference in the BPA molecule. Pilot tests showed that this commercial thickener, commonly used by patients with swallowing difficulties, was not toxic or aversive to earthworms. To prevent earthworms from leaving the containers, they were covered with perforated plastic to allow for gas exchange. Earthworm coelomic fluid was collected for most analyses conducted in this study. The earthworms were euthanized with cold alcohol 70% and the posterior segments were cut, fixed, and histologically stained for further analysis of the earthworm immune response.

The inflammatory response to an antigen involves the activation of the immune system, mainly the production of nitric oxide and cell proliferation as part of a series of complex events to combat infections and maintain the body's homeostasis. In a previous study, it was observed that the exposure of earthworms to rotenone induced an inflammatory response observed through changes occurring after 24h and 72h.[15] Therefore, this study was used as a reference to evaluate the effect of BPA on earthworms.

Three analyses were performed by flow cytometry. Initially, we evaluated whether BPA induced immunological activation in earthworms 72h BPA-exposed through analysis of alterations in the cell proliferation rate, apoptotic events, and distribution of amoebocytes and eleocytes in the coelomic fluid. To confirm that BPA was inducing an inflammatory activation in the earthworms, a subsequent analysis was carried out on the distribution pattern of cells throughout the cell cycle. It was considered that the increase in the percentage of cells in S and G2 phase in earthworms exposed to BPA for 24 hours would confirm the influence of this substance on the immune system. The levels of the following oxidative markers were also evaluated in the coelomic fluid supernatant (S, NO, LPX, and PCarb) of earthworms 24h BPA-exposed, since the differential modulation of oxidative metabolism is part of the inflammatory activation process, mainly considering the levels of NO and S molecules.[16]

On the other hand, micronucleus assay (MN), one of the most popular methods to assess genotoxicity of different chemical and physical factors, must be conducted in mitotic cells, which would be more easily obtained in earthworms exposed to BPA for 72 hours. In this test it is possible to detect rounded bodies of chromatin visible in the cytoplasm of cells, generally produced by DNA damage or genomic instability. The effect of BPA on DNA damage was evaluated via MN assay formation in two types of coelomocytes (amoebocytes and eleocytes) present in the coelomic fluid.

To evaluate the effect of BPA on earthworm immunocompetence markers, the expression of two genes related to immune response was analyzed: Toll-like receptor gene (eaTLR) (NCBI: JX898685) and antimicrobial peptide (AMP1) gene (AF060552), also known as "lumbricin 1"[18]. In earthworms, both genes are modulated by microorganisms, tissue injury, or the presence of some toxicants[19,20]. In addition to gene expression, the effect of BPA on the immunocompetence of earthworms has been evaluated using a yeast capture assay[14].

The micronucleus, the structures formed in the yeast capture assay, and the storage of brown bodies (BBs) in the posterior segments of the earthworm were also analyzed by optical microscopy using the Digimizer® image analysis software package, which allows for precise manual measurement as well as automatic object detection with measurements of object characteristics.

Coelomic fluid collection

The coelomic fluid was obtained using a protocol previously described by Alves et al.[14] with some modifications (Figure 1A). After treatment, the earthworms were removed from the medium, quickly washed with distilled water, dried on filter paper, and placed individually or up to three earthworms in six-well culture plates. Next, the six-well plate was covered with a lid containing filter paper soaked in 2 mL of ethyl ether. Within two minutes, the earthworms released the coelomic fluid without this process, triggering the death of the organisms. The earthworms were removed from the culture plate and phosphate buffer (PBS) was added. When necessary, the coelomic fluid was centrifuged to conduct laboratory analyses. The concomitant collection of coelomic fluid from at least six earthworms reduced the time required to obtain biological material, thus improving the experimental conditions.

Cell proliferation and apoptosis by flow cytometry assay

Pollutants can trigger the activation of the inflammatory response, which leads to an increase in the rate of proliferation of immune cells. In contrast, oxidative events can trigger DNA damage that induces apoptosis. Both conditions can reduce the immunocompetence of organisms in the face of pathogenic challenges. For this reason, the effect of BPA on the cell cycle of coelomocytes, on the proportion of cells associated with the immune response of earthworms (amoebocytes) and on the induction of apoptosis was evaluated using flow cytometry, as previously described by Alves et al.[14] and Jung et al.[15]. Briefly, flow cytometry was performed using a FACS Canto™ II Flow Cytometer (BD Biosciences®, San Diego, CA, USA), and data acquisition and cell content analysis were performed using FlowJo vX.0.7 software® (Tree Star, Inc., Ashland, OR, USA). The coelomic fluid was collected and added to 2 mL of PBS containing 5 mM ethylenediamine tetraacetic acid (EDTA) to decrease cell aggregation events and subsequently centrifuged at 250 × *g* for 2 min. The supernatant was removed and coelomocytes were used to perform three flow cytometry analyses. The effect of BPA on coelomocyte proliferation was determined by cell cycle analysis as follows: cells were washed with cold PBS, resuspended in cold 70% ethanol, and centrifuged at 250 × *g* for 2 min. The cells were then resuspended in a staining solution containing 50 µg/mL propidium iodide (PI) solution, 100 µg/mL RNase A, and PBS solution with 0.05% Triton X-100, and incubated for 40 min at 37°C. The two main coelomocyte populations were identified by granulometry and size, with cells sorted according to their forward scatter/side scatter (FSC/SSC) patterns.

Apoptosis events were identified using the FITC Annexin V Apoptosis Detection Kit® according to the manufacturer's instructions. Briefly, the cells were washed twice with cold PBS and resuspended in 1X binding buffer at a concentration of 1×10^6 cells/mL. Next, 100 µL of each sample was transferred to a culture tube, with the addition of FITC Annexin V (5 µL) and PI (5 µL) staining solution. Before analysis, the cells were gently vortexed and incubated for 15 min in the dark at room temperature. Then, 400 µL of 1X binding buffer was added to each tube, and cell fluorescence was determined by flow cytometry.

Biochemical quantification of oxidative markers

The oxidative markers present in the supernatants of the coelomic bodies were quantified using spectrophotometric techniques. Initially, total proteins were quantified using the biuret method (total monoagent proteins kit; Bioclin, Minas Gerais, Brazil) to normalize all other biochemical analyses performed. The S-anion was quantified by analysis of the formazan concentration triggered by the Nitroblue Tetrazolium Test (NBT-reaction)[21] and NO by determination of nitrate levels using the Griess reagent[22]. The LPX concentration was estimated by the formation of thiobarbituric acid-reactive substances (TBARS) measured spectrophotometrically and read at 532 nm[23]. PCarb was determined by the Levine et al.[24] method and read at 370 nm.

Micronucleus assay

After the extrusion of the coelomic fluid, the coelomocytes became highly adherent, and MN analysis was performed in six-well plates, instead of a part of the sample being transferred to a histological slide, as is usually done. This strategy increased the number of whole cells available for analysis. The coelomic fluid was gently spread to the bottom of the well to help spread the cells, as this fluid was dense because of the large amount of mucus. Coelomocyte staining was performed using the Rapid Panoptic Hematologic Dye® (Laborclin Co., São Paulo) based on the principle of haematological staining established by Romanowsky, which has three reagents: a fixative solution composed of a 0.1% triarylmethane solution, 0.1% xanthene solution, and 0.1% thiazine solution.

After drying, all the samples were fixed and stained according to the manufacturer's instructions. Panoptic staining allowed for the visualization of nuclear and cytoplasmic structures, which is a critical aspect for the analysis of MN in coelomic fluid, as most cells fuse their cytoplasm, making the analysis difficult. Slides were analyzed following the criteria proposed by Holland et al.[25], and the number of micronuclei in 1000 cells per sample was counted under a light microscope at a magnification of 400×.

Immunocompetence assays

Yeast capture test

The effects of BPA on the immunocompetence of earthworms were evaluated using three complementary analyses. The first assay evaluated the *ex vivo* response of coelomocytes exposed to dead yeasts, involving migration, formation of cell aggregates, NETs, and BBs, as previously described by Alves et al.[14]. Briefly, the yeasts were inactivated with boiling water at a concentration of 10^3/mL, added to 1000 µL of coelomic fluid, and placed in six-well plates.

Figure 1B shows the main immune events triggered by fluid celomic extrusion from the controls and earthworms treated with BPA: (1–3) coelomocyte migration, fragmentation, and fusion of eleocytes, which form a polynucleated cytoplasmic mass; (4–5) attraction of amoebocytes (cells more cuboidal, granular, and nucleus proportionately larger than the cytoplasm) to this cytoplasmic polynucleated mass, forming cell aggregates; (6) production of eNETs from cell aggregates forming a network, which often links one cell aggregate to another, allowing the capture and imprisonment of microorganisms and other substances; (7-8) aggregates form BBs. Thus, within 1 h, it was possible to

analyze and compare the immune responses of coelomocytes from control earthworms and those exposed to BPA. Further, the six-well plates were stained with the Panoptic kit as described in the preparation and analysis of MNs, followed by analysis by optical microscopy.

Histological analysis of brown bodies (BBs) deposition

As the formed BBs detached and remained floating in the middle, the posterior segments of the earthworm were histologically analyzed to evaluate the formation of these structures. The earthworms were euthanized with cold 70% alcohol for 5 min, and 10 posterior segments in the tail direction were cut with the aid of a scalpel and fixed in 4% buffered formaldehyde for further preparation and histological analysis. The samples were dehydrated using a 15-min dehydration battery per step with a Kline shaker (GT-20IBSU®) with 70% alcohol, 80% alcohol, 90% alcohol, two passages in anhydrous alcohol, alcohol, and xylol, and two more passages in xylol. Two hours later, the samples were kept in xylol with paraffin, then paraffin 1 and paraffin 2, and subsequently blocked and dried overnight at room temperature. Histological sections were obtained using a semiautomatic microtome (Yd-335) at a thickness of 7 µM. The slides were mounted and dried overnight at room temperature for subsequent staining. For histological staining, the slides were subjected to a standard dewaxing process, with two passages in xylol heated to 50°C (5 min each), anhydrous alcohol (5 min), 90% alcohol (3 min), 70% alcohol (3 min), running water (3 min), and distilled water (1 min).

The histological sections were stained with the Masson-Goldner trichrome (MGT) staining technique, which identifies components of connective and muscle tissues selectively visualized by using a combination of three different staining solutions: azophloxine and tungstophosphoric acid orange G solutions stain components such as muscle and cytoplasm, and light green SF solution then counter-stains connective tissue. A Sigma-Aldrich kit was used to perform the MGT staining (100485). Photographic images were analyzed using Digimizer® software, which allowed for the counting of cell aggregates (CA) per field (200× magnification), measuring the perimeter of the CAs (µm), identifying the presence of NETs, and the deposition of BBs in the posterior region of the body of the earthworm. Ten optical fields were evaluated per treatment.

Analysis of the expression of genes associated with immune response

The effect of BPA on the expression of eaTLR and AMP1 genes was determined as previously described by Jung et al[15]. Briefly, the earthworms were rinsed with distilled water, dried, and ground. At least three earthworms from each treatment were used to extract RNA using Quick-Zol® (TriZol; Ludwig Biotech Co, Alvorada, Brazil), in accordance with the manufacturer's instructions. The extracted RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

qRT-PCR was performed using a thermocycler (Axygen® MaxyGene II Thermal Cycler; Corning Life Sciences, Tewksbury, MA, EUA) with RNA samples treated with 0.2 µL of DNase (Invitrogen Life Technologies®, Carlsbad, CA, USA) at 37°C for 5 min to digest any DNA contamination, and at 65°C for 10 min. Reverse transcription was performed using the following primers: EATLR (forward: 5'-GAGACATC-GCTGAAAACATC-3'; reverse: 5'-CTGCATCTGAATCTGGAGTC-3')[20] and AMP (forward: 5'-CATACTCGGAACG-CAAGAACC-3'; reverse: 5'-TTTGATGACCTTCTGCGGTG-3')[19]. RNA was reverse-transcribed to cDNA in the presence of 1 µL of iScript reverse transcriptase and 4 µL of iScript Mix (Bio-Rad Laboratories, Hercules, CA, USA) using the following reaction steps: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and 5°C for 60 min.

qRT-PCR analysis was conducted using a 1× QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) and 20 µL containing 1 µL of cDNA sample (1:20 dilution). The amplification of both genes was performed using the following cycling conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a melting gradient from 50°C to 90°C (1°C increments every 5 s). Reactions were performed in duplicate, and β-actin (Y09623) was used as the housekeeping gene

(forward: 5'-CGCCTCTTCATCGTCCCTC-3'; reverse: 5'-GAACATGGTCGTGCCTCCG-3'). The amplification curves, melting curves, and standard curve slope were checked to ensure that non-specific amplification and the relative quantification of Ct values were determined[19] and compared among BPA treatments.

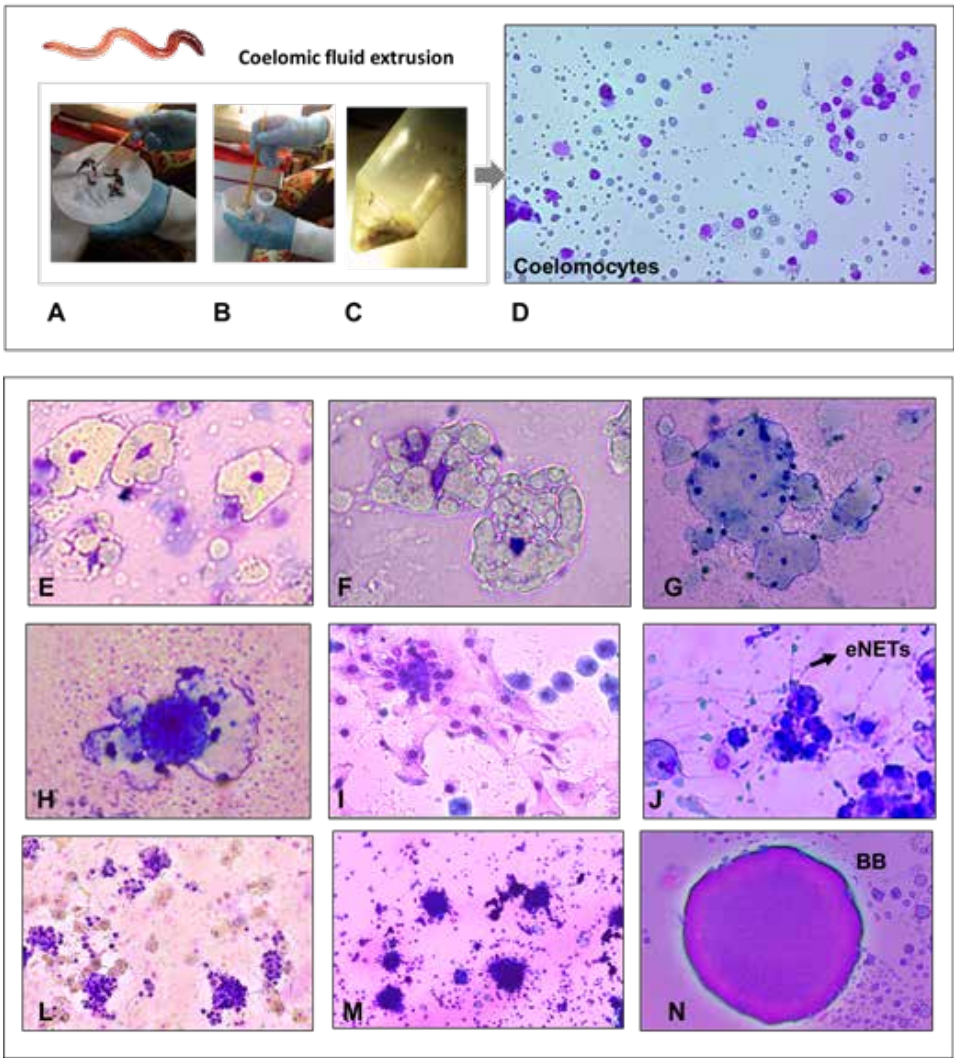

Figure 1 – Schematic for obtaining the coelomic fluid used in the experiments, immune mechanisms involving in the pathogens capture by ***E. fetida*** earthworms, and general outline of the study. (A) Three earthworms were selected, washed and dried to obtain coelomic fluid; (B) They were then transferred to a Falcon tube covered with a cotton swab to which 1 ml of ethyl ether was added, which is an irritating substance for the earthworm; (C) After 1 to 2 minutes, the earthworms expelled the yellowish coelomic fluid. Similar to human leucocytes, coelomocytes represent a group of heterogeneous cells, mainly eleocytes and amoebocytes. Mainly amoebocytes play a relevant role in the immune response induced by exposure to pathogens or antigenic substances. (B) The study postulated that bisphenol-A (BPA) exposure could impact earthworm immunocompetence in capture and kill pathogens. This process involves the main phases, which were compared among treatments: (D,E,F) cellular migration and fusion forming a polynucleated cytoplasmic mass, that exert chemotaxis in other coelomocytes; (G,H) production of cellular aggregates (I,J) forming polynucleate structures; (L,M) CAs interconnection formed by DNA and protein networks similar to human neutrophil extracellular traps eNETs; (8–9) CA retraction forming 1–2 mm wide nodules, with cells on its edge flatten out, that quickly darkens via melanization (brown bodies, BBs), stored in the posterior segments. The BBs are then deposited inside pockets of the worm's posterior segments. As BBs are not reabsorbed, the excess of these structures containing cell debris and dead pathogens induces an autotomy process, by which the earthworm cuts and eliminates these posterior parts, quickly regenerating its tail. The increase in BB deposition may indicate that the earthworm is trying to eliminate toxic or pathogenic components. On the other hand, the decrease in the formation of BBs in the presence of a chemical agent, such as BPA, may indicate a decrease in the earthworm's immunocompetence.

Statistical analysis

Data were statistically analyzed using the Prism GraphPad program. Quantitative variables were compared using one-way analysis of variance followed by Tukey's *post-hoc* test. Quantitative variables were analyzed using chi-square or Fisher's exact tests. All tests were considered to be statistically significant at $p < 0.05$.

RESULTS

Effects of BPA on coelomocytes population and apoptosis induction

Initially, it was verified whether 24-hour exposure to BPA at different concentrations could have a cytotoxic effect on the main groups of coelomocytes by analyzing the proportion of amoebocytes and eleocytes and the rate of cells killed by apoptosis or apoptotic cells. As can see in Figure 2, eleocytes are more complex and larger cells than amoebocytes, which can be detected via granulometric analysis (green cloud of cells). In the presence of BPA, the proportion of eleocytes increased while that of amoebocytes decreased when compared to control earthworms.

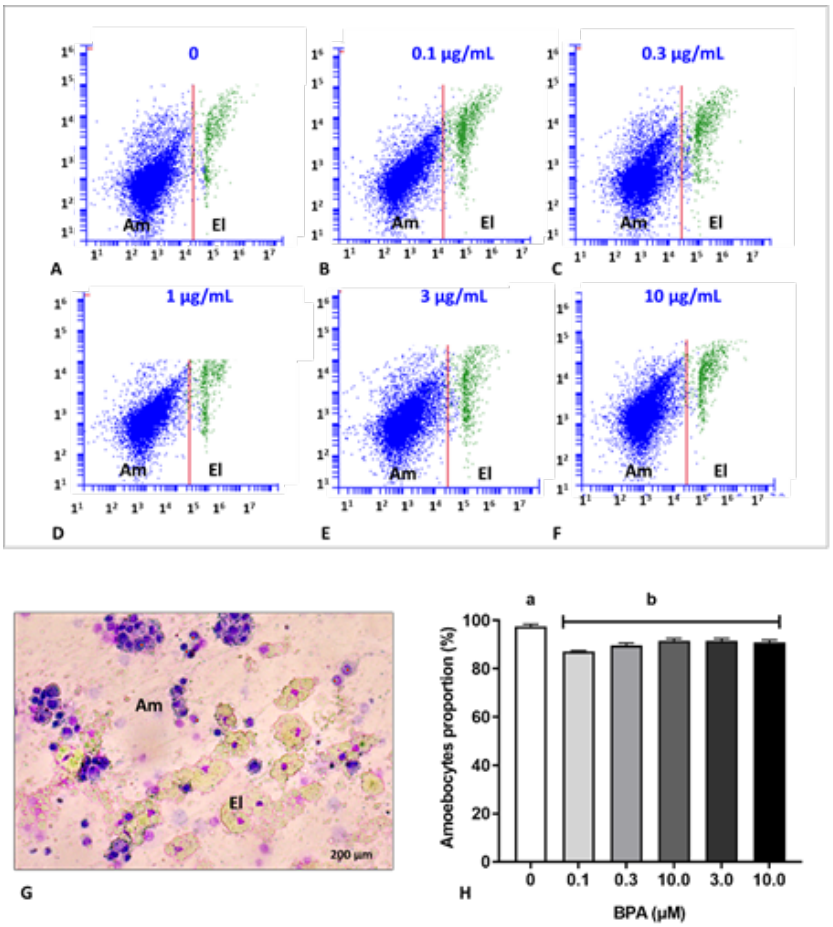

Figure 2 – BPA effects on ***E. fetida*** coelomocyte populations. (A-F) Representative flow cytometry of amoebocytes and eleocytes frequency identified by cell granulometry of earthworm BPA exposed at different concentrations. Am = amoebocytes; El=eleocytes. (G) microphotograph with amoebocytes which include smaller cells, generally blue, that come together to form spheroids, and eleocytes, which are larger cells with a cytomorphology similar to macrophages; (H) Comparison of amoebocytes proportion (%) in coelomocytes from earthworms BPA-exposed at different concentrations. Different letters indicated statistical differences (***p <0.05***) determined by One-Way variance analysis followed by Tukey ***post hoc*** test.

Next, it was assessed whether exposure to BPA could increase the proportion of apoptotic events by flow cytometry analysis. The results showed a significant increase in apoptosis rates in the immune cells of earthworms exposed to lower concentrations of BPA (0.1-1 ug/mL), while higher concentrations showed a proportion of cells killed by apoptosis or apoptotic similar to the control (Figure 3).

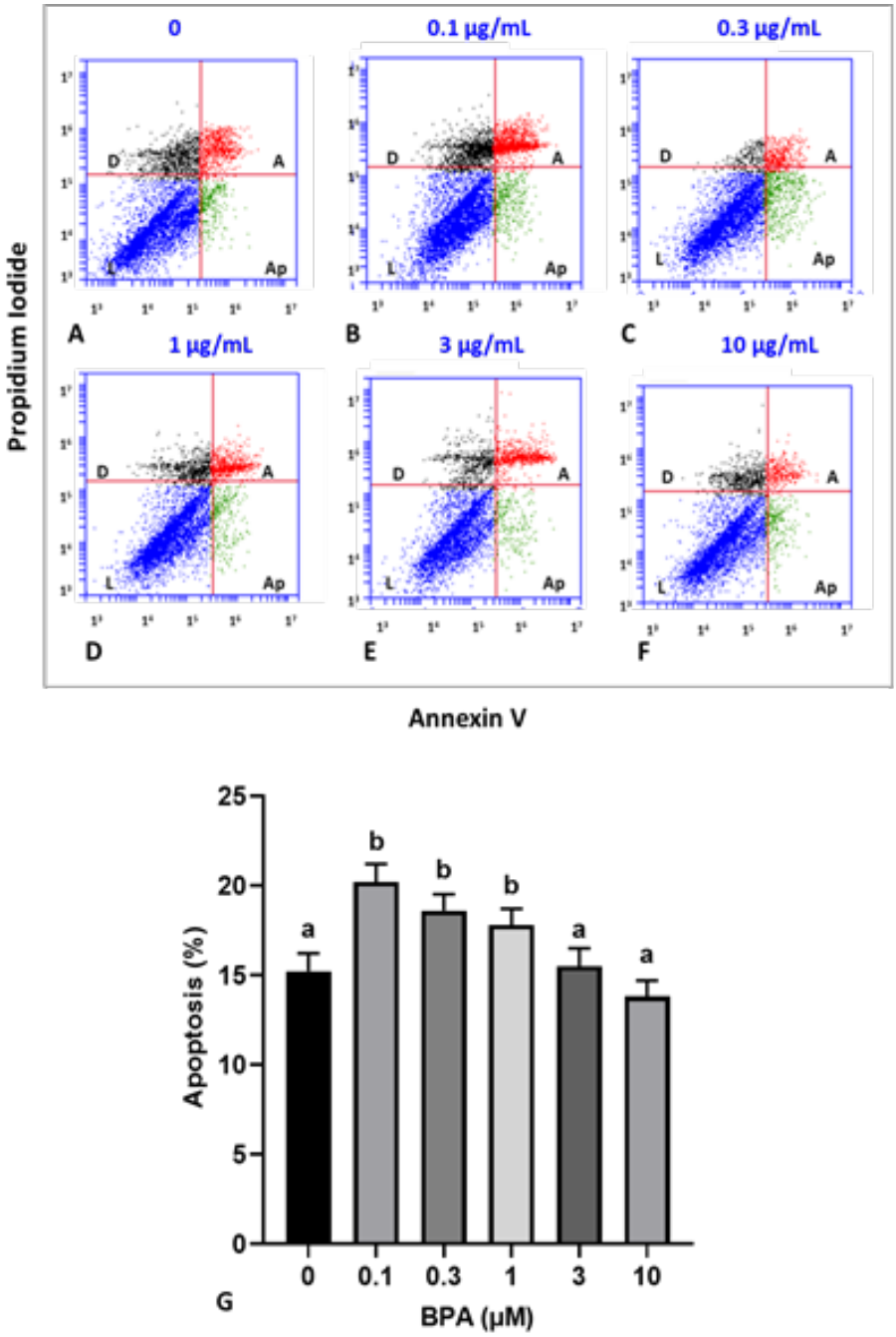

Figure 3 – Cytotoxic BPA effect on ***E.fetida*** coelomocytes. (A-F) representative flow cytometry chart showing general distribution of live cells (L), apoptotic cells (Ap), dead cells for other causes and cells dead by apoptosis (A) identified by Propidium Iodide (Y axis) and Annexin V (X axis) markers. Comparison in the proportion of apoptosis events among control and BPA at different concentrations was performed by One Way analysis of variance followed by Tukey post hoc test. Different letters indicated statistical differences among treatments with $p < 0.05$.

Effects of BPA on coelomocytes cellular proliferation

This analysis evaluated the impact of BPA on earthworm coelomocyte proliferation using cell cycle flow cytometry. After 24 h and 72h, BPA exposure triggered an increase in the frequency of cells in the S and G2 phases, indicating the stimulation of mitotic events. However, BPA at lower concentrations occurred more intense mitotic induction than at higher concentrations (Figure 4).

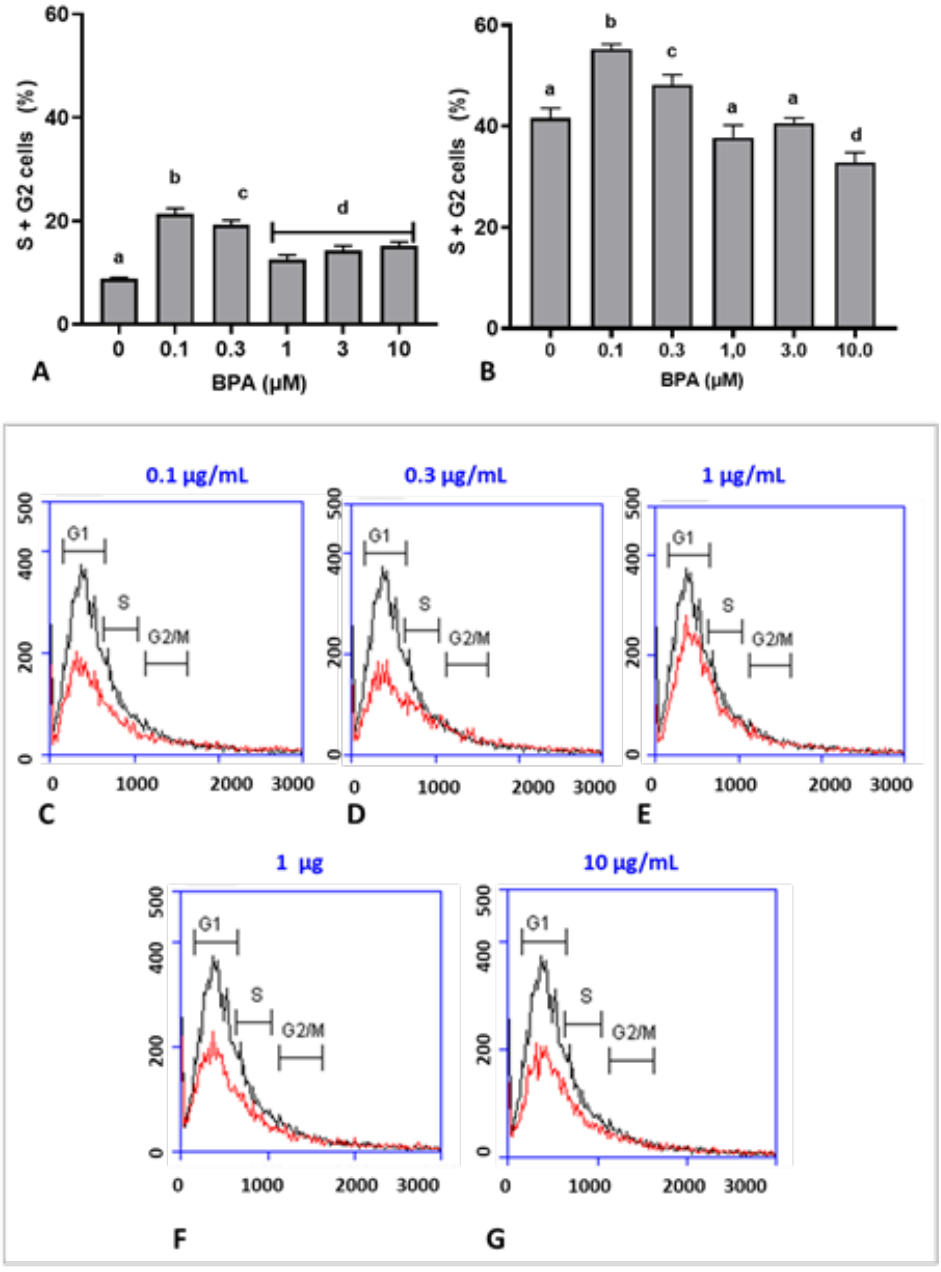

Figure 4 – Effect of Bisphenol-A (BPA) at different concentrations on ***E. fetida*** coelomocytes cell cycle. (A) Comparison among S+G2 frequency in coelomocytes from earthworms 24h BPA-exposed. (B) Comparison among S+G2 frequency in coelomocytes from earthworms 72h BPA-exposed. Statistical analysis of A and B figures was performed with One Way analysis of variance followed by Tukey ***post hoc*** test. Different letters represent significant differences at $p < 0.05$. (C-G) Representative charts of flow cytometry of 72 h treatment showing cells frequency in G1, S and G2/M phases. The black graph represents the frequency of cell cycle phases in the control group, while the red graph represents the treated group of earthworms treated with BPA.

Effects of BPA on coelomocytes oxidative markers

Initially, the amount of total protein was calculated and compared between the control and earthworms exposed to different BPA concentrations, showing no significant alterations (Figure 5A). Contrary to our expectations, BPA induced a significant decrease in superoxide anion levels (Figure 5B). However, an elevation in the NO levels was observed in earthworms exposed at lower concentrations of this pollutant (0.1 to 1 µM) (Figure 5C).

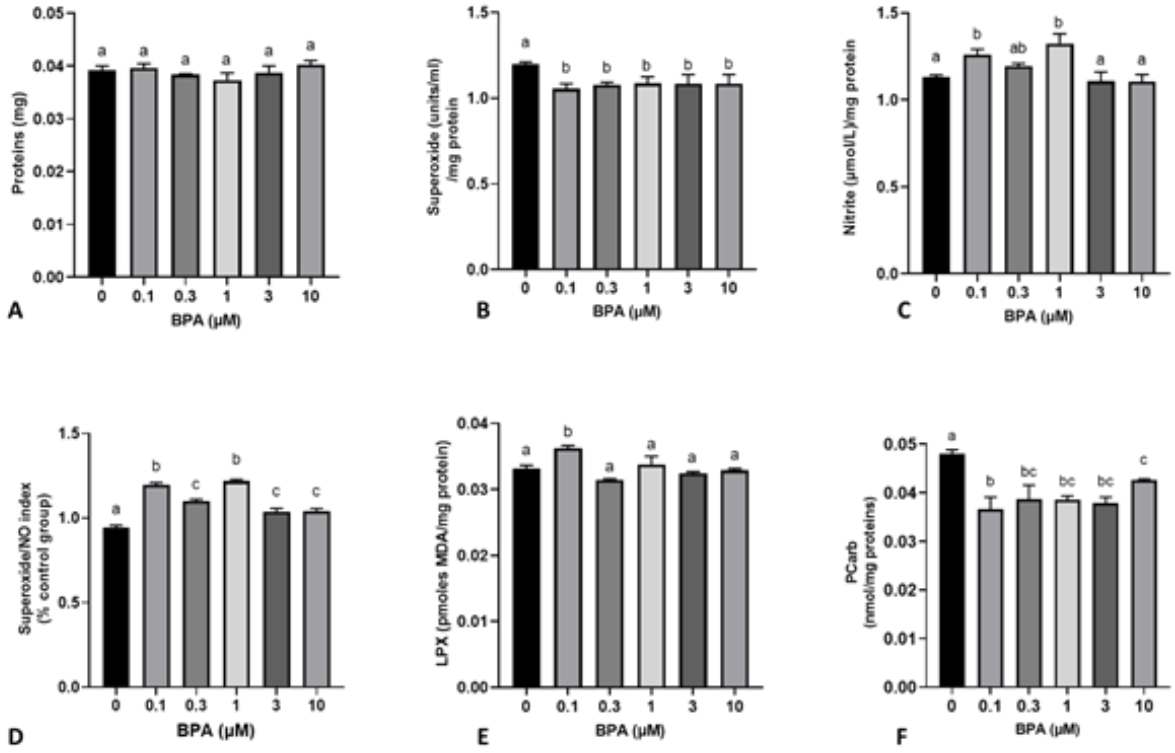

Figure 5 – Comparison of total protein and oxidative markers in earthworms exposed to BPA at different concentrations (0.1–10 µM), 24 h. (A) Total protein. (B) Superoxide anion (S). (C) Nitric oxide (NO) estimated by nitrite concentrations. (D) S/NO index. (E) Lipoperoxidation quantified by TBARS-MDA assay. (F) Protein carbonylation. Treatments were statistically compared to the control by one-way analysis of variance followed by Tukey's test, and significant differences at $p < 0.05$ are identified by different letters. Considering the high affinity of the superoxide anion and NO, the ratio of these molecules was calculated and compared by dividing the concentration of the S anion by that of NO. In the control earthworms, the level of NO was slightly higher than that of anion S. However, exposure to BPA changed this index, which increased significantly in earthworms exposed to BP owing to an increase in the concentration of NO (Figure 5D). These results were more pronounced in earthworms treated with the lowest BPA (0.1–1 µM). Except at 0.1 µM BPA, in which an increase in LPX was observed, all other concentrations did not influence this oxidative marker (Figure 5E). In contrast, all BPA concentrations induced a decrease in PCarb levels compared with the control group (Figure 5F).

Effects of BPA on MN formation

The effects of BPA on chromosomal damage in *E. fetida* coelomocytes were evaluated using MN analysis in both amoebocytes and eleocytes. First, MN analysis was difficult because the cells tended to aggregate and fuse their cytoplasm. Therefore, the detection of individual and whole cells is more laborious. Figure 6 shows the representative micrographs of amoebocytes and eleocytes with relevant nuclear alterations. The results obtained by the sum of all types of coelomocytes are presented in Table 1, grouped by MN mitotic catastrophes (MiC) and chromosomal aberrations (Ab.C) frequencies.

Table 1 – Comparison of three main chromosomal alterations of ***E. fetida*** coelomocytes bisphenol-A (BPA) exposed at different concentrations

Alterations	Cells scored	BPA (µM) 0	0.1	0.3	1	3	10	*p*
MNs	1000	17[a]	21[b]	60[c]	104[d]	98[d]	107[d]	0.001
Ab.C	1000	02[a]	05[b]	18[c]	21[c]	19[c]	19[c]	0.001
MiC	1000	03[a]	05[b]	11[c]	13[c]	09[c]	13[c]	0.001

MNs, mincronuclei; Ab.C, aberrant cells (two or more micronuclei); MiC, mitotic catastrophe. Statistical comparisons were performed using the Chi-squared non-parametric test.

MN frequency increased in a dose-dependent manner up to a concentration of 1 µM BPA. Between concentrations of 1 and 30 µM, the MN rate was similar and remained higher than that of the control and lower concentrations of BPA. The increase in Ab.C and MiC followed a pattern similar to that of MNs, with no significant differences in the distribution of these chromosomal alterations between concentrations of 0.3 and 10 µM BPA. It was not possible to accurately account for cells undergoing apoptosis due to the tendency of cytoplasmic fusion and the presence of many intracellular granules, mainly in some types of amoebocytes.

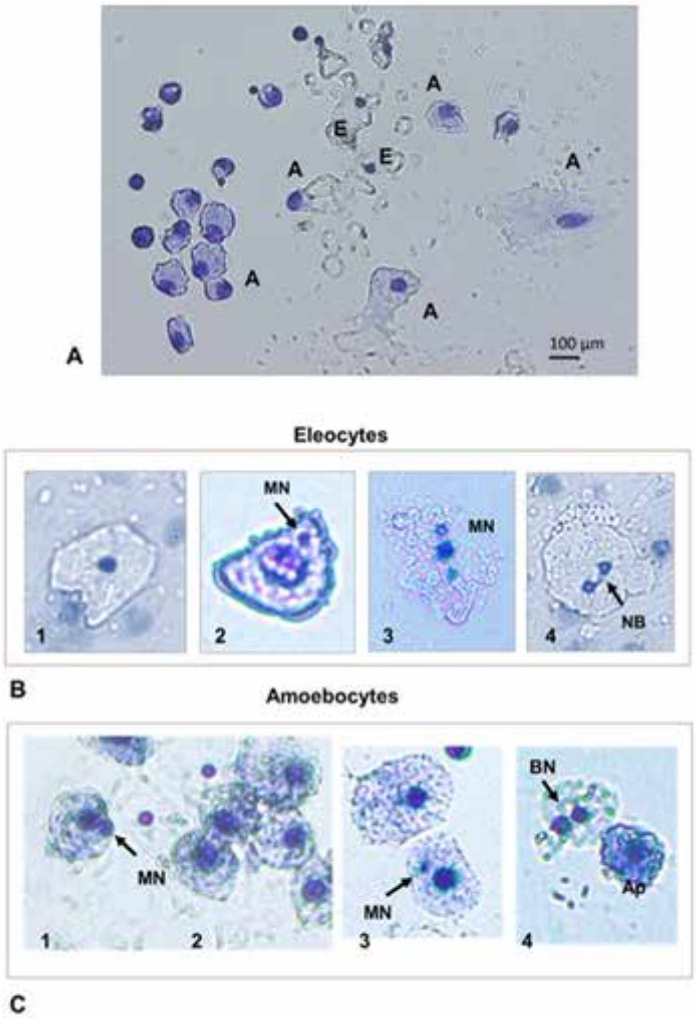

Figure 6 – Representative DNA damage alterations in coelomocytes of ***E. fetida***. (A) Microphotograph presents a representative photo (100 × magnification) showing individualized eleocytes (E) and amoebocytes (A), which were used in the micronucleus (MN) analyses. (B) Microphotograph 1 shows an unaltered eleocytes, with a small eccentric nucleus. Cells with one or more micronuclei (2,3) were observed as well as cells with nucleoplasmic bridge (NB) (4). (C) Amoebocytes include more rounded cells proportionately larger than the cytoplasm. Once the coelomic fluid is expelled, these cells tended to clump together quickly. In some situations, it was possible to detect cells undergoing apoptosis. However, they were not counted as they could be confused with cells undergoing cytoplasmic fusion process. As images from B and C figures are representative, they were extracted and enlarged from microphotographs of different treatments and also of the control group. For this reason, individualized scales are not presented and their original treatment is not identified.

Effects of BPA on immunocompetence markers

The changes in earthworm immunocompetence markers are summarized in Table 2 and Figure 6. The number of CAs per optical field at 200× magnification varied depending on the concentration of BPA to which earthworms were exposed for 24 h. The lowest concentration of BPA doubled the number of CAs compared with that in the control. However, the other concentrations significantly reduced the number of CAs per optical field compared with the control. This pattern indicates that BPA acted as an inhibitor of cell aggregation, which is part of the earthworm's initial immune defense against biotic and abiotic agents.

The perimeter of the CAs was also compared, considering aggregates with four or more amoebocytes, and the results did not show a dose-dependent pattern. While the lowest concentration of BPA had mean CAs perimeter similar to the control, earthworms exposed to 0.3 µM BPA had CAs with greater perimeter. At a concentration of 1 µM BPA, the perimeter of the CAs was significantly reduced compared with that of the control. A notable result was the extensive inhibition of eNET formation in earthworms exposed to BPA at all the concentrations tested. The low cell aggregation was somewhat corroborated by analysis of the storage pattern of BBs in the posterior segments of the earthworm body. The number of segments that also presented this accumulation was significantly lower than that observed in control earthworms.

Table 2 – Comparison of immunocompetence markers of ***E. fetida*** coelomocytes bisphenol-A (BPA) exposed at different concentrations.

Markers	BPA (µM)						*p*
	0	0.1	0.3	1	3	10	
CAs (number/field, 200× magnification)	7.6 ± 2.5[a]	14.1 ± 4.5[b]	2.0 ± 0.8[c]	1.6 ± 0.9[c]	1.8 ± 1.3[c]	1.4 ± .0.6[c]	0.001
CAs (Perimeter, µm)	64.9 ± 17.1[a]	62.7 ± 15.2[a]	133.3 ± 22.3[b]	51.2 ± 12.5[b]	33.4 ± 10.3[c]	31.0 ± 8.2[c]	0.001
eNETs (presence/10 fields, 200 x magnification)	9/10	0/10	0/10	1/10	2/10	0/10	0.001
BBs (presence/10 posterior segments, 200× magnification)	5/10[a]	4/10[a]	3/10[cs]	1/10[d]	1/10[d]	2/10[d]	0.001

CA, cell aggregations in coelomic fluid (>4 amoebocytes); eNETs, ***Eisenia*** neutrophil extracellular traps; BBs, brown bodies counted in histological sections of 10 posterior segments. Statistical analyses of CA number and perimeter were performed using one-way analysis of variance followed by Tukey's post hoc test. eNETs and BBs were statistically compared between each treatment and control using Fisher's exact test. Different letters indicate significance at $p < 0.05$.

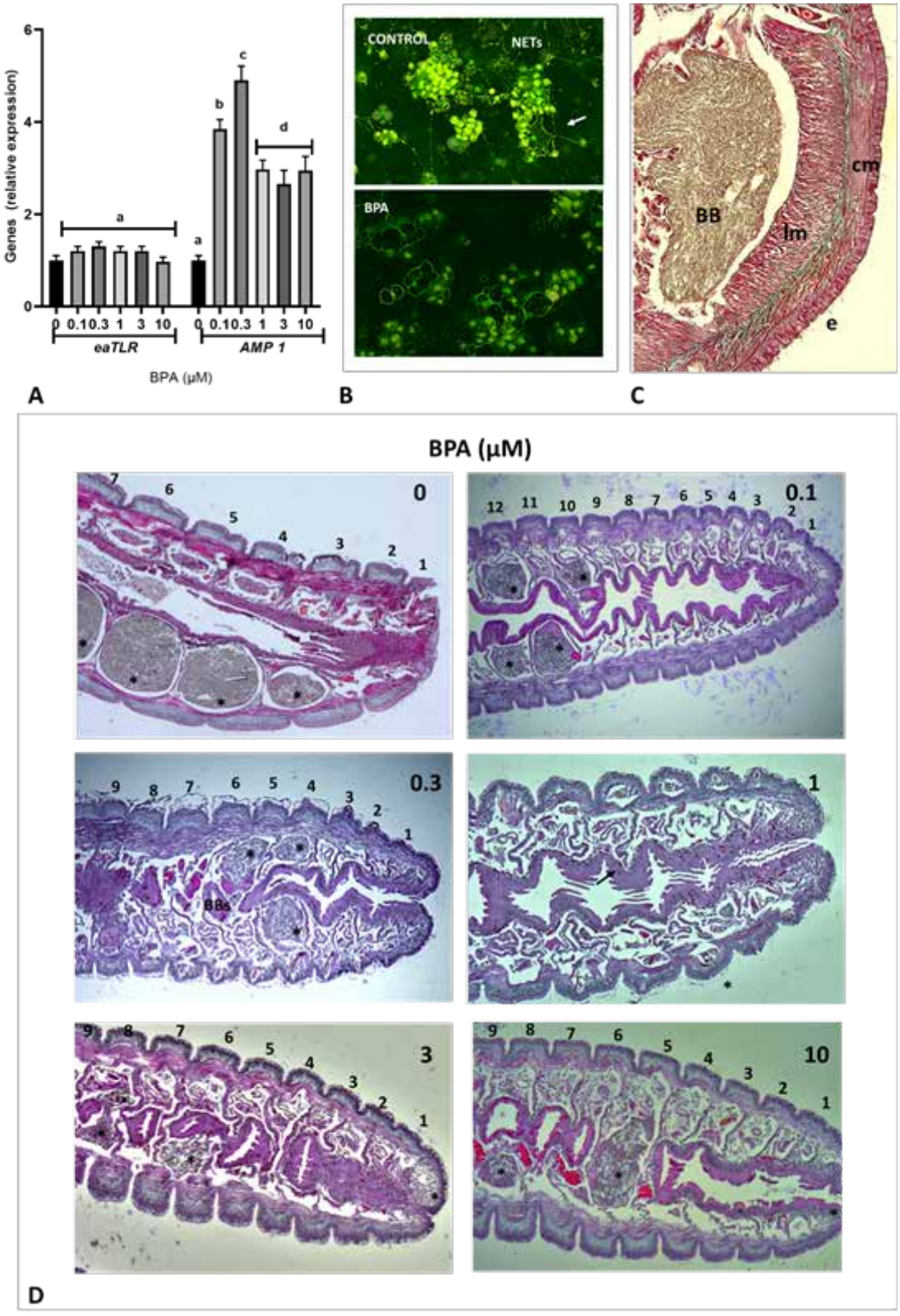

Figure 7 – Bisphenol-A (BPA) effect on ***E. fetida*** immunocompetence markers. (A) Comparison of expression of eaTLR and AMP1 genes among earthworms 24 h exposed to BPA at different concentrations (0.1, 0.3, 3, 1, and 10 µM) by qRT-PCR assay. Genes were normalized by β-actin a housekeeping gene. mRNAs of earthworms without BPA exposure (controls) were considered 1 and used as reference to calculate the relative mRNA expression of other treatments. Comparisons

were performed by one-way analysis of variance followed by Tukey' post-hoc test. Different letters indicate significant differences ($p < 0.05$). (B) Representative microphotographs with inverted staining showing the presence of NETs in coelomic fluid from a control earthworm and absence of NETs in an earthworm exposed to 1 µM of BPA (400× magnification); (C) Detail of a histological section showing the accumulation of brown bodies (BBs) in the coelomic cavity located laterally to the intestines, in the posterior segments of the earthworm. These brown bodies clump together and become very compacted. At a certain moment, which can be triggered by the volume or perhaps by the chemical nature of the residues, the earthworm performs autotomy, eliminating these segments that are quickly regenerated. The microphotograph shows a detail of the body wall with the longitudinal musculature (lm), circular musculature (cm), and the epidermis covered by a cuticle (e). (D) Representative longitudinal histological sections of the last posterior segments of control earthworms and treated for 24 h with different concentrations of BPA. Contrary to expectations, earthworms exposed to BPA presented less compacted brown bodies (BBs *), and with smaller distribution among the segments, mainly in the last five segments close to the anal opening. As images from B and C figures are representative, they were extracted and enlarged from microphotographs of different treatments and also of the control group. For this reason, individualized scales are not presented and their original treatment is not identified.

BPA exposure induced changes in the expression of the eaTLR and AMP1 genes (Figure 6A). AMP1 was significantly overexpressed in earthworms exposed to BPA for 24 h at all concentrations tested, with a greater intensity in earthworms exposed to a concentration of 3 µM. In contrast, the effect of BPA on eaTLR gene expression was greatly reduced. Therefore, BPA triggers an imbalance in the genes directly associated with the immune response.

DISCUSSION

In the present study, red earthworms were used as an experimental model. The results support the hypothesis that a link exists between oxidative stress events and changes in immunocompetence, especially in the inflammatory response triggered by BPA exposure. We chose to carry out an acute effect study based on a previous investigation conducted by Oliveira et al.[11] who reported that exposure to BPA for 48 h significantly decreased the survival of *E. fetida*. Acute BPA exposure has a significant impact on the earthworm immune system, mainly at lower concentrations. It is possible that the effect of BPA on the earthworm immune system also occurs in humans. In fact, many components of the innate immune system have been conserved during metazoan evolution, and for this reason, some species, such as the fruit fly *Drosophila melanogaster*[26] and the nematode *Caenorhabditis elegans*, have been used as models to study innate immunity[27]. Some studies performed in the last decade have suggested that the use of *Eisenia* would be relevant to the understanding of invertebrate innate immunity[13].

BPA is considered to be one of the most widely used synthetic compounds in the world, resistant to degradation, and able to bioaccumulate in the environment[28]. In general, the results showed that BPA affected the analyzed markers, but this effect did not follow a dose-response pattern and was limited to the lowest concentrations (0.1–1 µM). The more intense effect of low BPA doses has been previously described in other studies, suggesting that BPA interacts with and disrupts several signaling pathways[29]. Considering regulatory resolutions regarding exposure to BPA, the four concentrations tested in this study were lower than the maximum dose recommended by the Brazilian health regulatory agency (Anvisa, Health Ministry)[30]: 0.1, 0.3, 1.0, and 3.0 µM, which is approximately equal to 0.02, 0.06, 0.3, and 0.6 mg/kg food/day, respectively. Only 10 µM (6 mg/kg food/day) was above the maximum recommended limit. However, this maximum limit is relatively high compared to other regulatory agencies, such as European Food Safety Authority (EFSA) [31], which lowered the maximum limit to 0.05 mg/Kg food/day in January 2018. Even though the concentrations tested were below the exposure limits recommended by the Ministry of Health in Brazil and other regulatory agencies, such as the Food and Drug Administration (FDA), the effect of BPA on earthworms is worrying. This is because earthworms are highly relevant in the maintenance of soil quality, and the effect of exposure to hormonal switching agents could have unpredictable consequences.

BPA is considered an important risk factor for endocrine, immune, and CNT diseases, including neurological, cardiovascular, reproductive, and oncological conditions[32]. A large proportion of these illnesses associated with BPA exposure are related to the establishment of low-grade chronic inflammatory states, which ultimately represent an immunocompetence impairment in the resolution of inflammatory responses[33]. The data described herein corroborate this assumption. A synthesis of the potential mechanisms underlying the alterations that lead to impaired immunocompetence triggered by BPA in *E. fetida* is presented in Figure 8.

The interpretation of the results obtained is complex and requires speculations based on previous evidence related to oxidative metabolism in the inflammatory response, mainly involving the superoxide anion and NO. After 24h of exposure to BPA, earthworms showed divergent results with respect to the evaluated oxidative markers. Regarding the results involving the superoxide anion and NO, we calculated a ratio index of the superoxide levels divided by the NO levels for the control and BPA-treated earthworms. The results showed a significant increase in these indices in earthworms exposed to all BPA concentrations investigated, indicating that this molecule induced an S-NO imbalance via an increase in the NO levels.

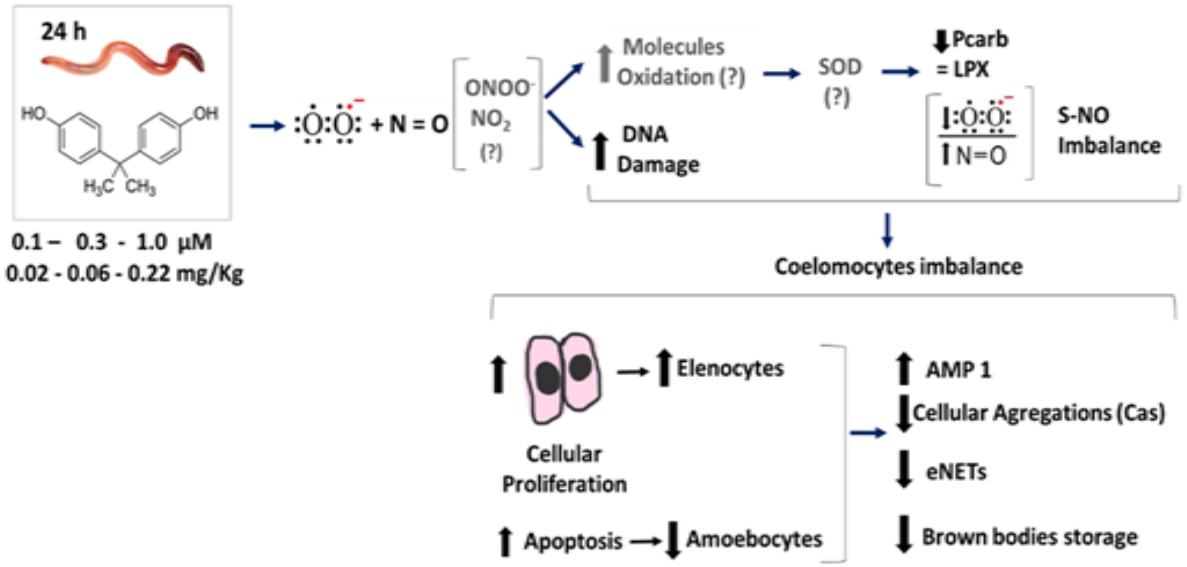

Figure 8 – Putative model of BPA effects on innate metabolism involving oxidate based in data described here using *E. fetida* as experimental model

However, the oxidation of macromolecules, such as lipids and proteins, was not pronounced in earthworms exposed to BPA. In contrast, all the concentrations of this molecule induced a significant decrease in protein carbonylation. These data seem inconsistent when considering the increase in the rate of DNA damage demonstrated by the presence of micronuclei and other chromosomal alterations, as well as the frequency of apoptosis. A previous report on the effect of BPA on earthworms also reported similar toxic effects in this organism [11-34].

Similar to leukocytes, coelomocytes are heterogeneous cells that are generically classified into two groups: amoebocytes and eleocytes, also known as chloragocytes[35]. Previous studies have shown that amoebocyte immune responses involve phagocytosis, ROS production, and cytotoxic effects against microorganisms. Therefore, ROS molecules are relevant components of earthworm defense against pathogens, including an increase in some oxidant molecules, such as superoxide anions and NO[35-36]. In particular, superoxide plays a strategic role in phagocytic cells, which use this ROS to kill

and digest microorganisms. Therefore, in phagocytic cells, such as coelomocytes, an elevation in the superoxide levels can originate from spontaneous processes or by the mediation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, which is strategically immunocompetent[37].

NO also has a relevant role in the inflammatory response, mainly related to phagocytic cells, and has been shown to suppress neutrophil phagocytic activity. In this context, the evidence suggests that superoxide and NO interplay fine-tunes mechanisms regulating the life and death of neutrophils[38].

In this context, we postulated that BPA could increase superoxide levels in coelomocytes, inducing spontaneous reactions with NO molecules, which generate reactive nitrogen species (RNS), especially peroxynitrite ($ONOO^-$). Peroxynitrite, in turn, forms NO_2 and hydroxyl radicals (OH^-), which have a high affinity for DNA, causing extensive damage to this molecule[38]. This could be the main route that explains the impact of BPA in increasing DNA damage, as assessed through micronucleus analysis. Increased genotoxicity is also closely related to the induction of apoptotic events, as observed in the results described here. However, because the ROS levels need to be finely regulated to avoid undesirable effects, it has been well established that coelomocytes have antioxidant defense strategies, including enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT)[39]. For this reason, it is possible that after 24 h, the oxidation rates of lipids and proteins may have decreased, while the DNA damage rate was assessed through MN and apoptosis rates indicating relevant BPA genotoxic effect.

Genotoxicity triggered by BPA exposure has been previously described in rodents, causing an increase in the frequency of MN in polychromatic erythrocytes (PCEs), structural chromosome aberrations in bone marrow cells, and DNA damage in blood lymphocytes[40]. BPA also induces micronucleus formation in cultured lymphocytes. As micronucleus tests provide important information about a chemical's ability to interfere with chromosome structure and function, this assay confirms that BPA has genotoxic effects in several species, including earthworms. It is also important to note that MN tests must be performed on actively dividing cells. In our study, we found that BPA induced an increase in the rate of cell proliferation, thus making it possible to perform the test. However, performing these analyses was challenging because the collected coelomocytes present in the coelomic fluid tended to migrate, cluster, and form large polynucleated cytoplasmic masses (Figure 1B). Nevertheless, it was possible to observe the formation of micronuclei in different types of coelomocytes.

The induction of cell proliferation, increasing the amoebocyte concentration, confirmed the action of BPA on the *E. fetida* immune system. Coelomocyte functions are associated with the expression of genes, such as eaTLR[20], as well as AMP-like proteins, also known as lumbricin 1[41].

However, exposure to BPA induced an antagonistic response in the two genes studied, as the AMP1 gene was activated and eaTLR did not modify its expression. This result is quite exciting, considering that these genes are highly sensitive to biotic and abiotic environmental factors that pose a danger to earthworm homeostasis. Generally, these genes are overexpressed in the presence of pathogens[20-42-43]. Antimicrobial peptides, such as AMP1, are important contributors to non-specific host defense in both vertebrates and invertebrates, and are structurally conserved during phylogenesis[44]. Therefore, it was not surprising that BPA induced an increase in AMP-1 expression.

Immune cells express pattern recognition receptors (PRRs), which are able to detect danger by recognizing specific pathogen-associated molecular patterns (PAMPs)[45]. However, the non-responsiveness of the eaTLR gene to BPA is an unexpected result, considering that exposure to this pollutant induces DNA damage, apoptosis, and increases the concentration of earthworm amoebocytes. This type of action has been previously observed when earthworms are exposed to other toxic agents, such as rotenone, and previous studies have shown that some pollutants, such as rotenone[15] and titanium dioxide nanoparticles[46], can induce the downregulation of the eaTLR gene in earthworms.

This antagonistic effect between the expression of AMP1 and eaTLR genes may be related to the decrease in the immunocompetence of earthworms caused by BPA in response to exposure to yeasts. In this way, molecules that decrease or do not induce the upregulation of TLR genes, such as BPA, could impair the earthworm's immune system, with unpredictable consequences for their survival.

However, we must be careful when translating these results to humans. This is because our species has hepatic mechanisms that are highly efficient for BPA clearance[47]. However, there is evidence that BPA is associated with an increased risk of certain types of cancers[32]. The extent to which this association could be linked to the BPA induction of DNA damage and the kind of "blinding of the immune system" triggers less efficiency in recognizing and killing carcinogenic cells. Although this assumption is speculative, it requires further investigation.

CONCLUSION

The results described here support the hypothesis that BPA induces oxidative stress, causing an imbalance in superoxide and NO levels and DNA damage, which may induce alterations in the earthworm immune response. Despite the inflammatory activation triggered by BPA, the immunocompetence of earthworms in capturing and destroying external agents, such as yeast, is impaired. Therefore, the association between DNA damage and changes in innate immune metabolism could be a relevant part of the action of BPA associated with the risk of developing NCDs, especially cancer.

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