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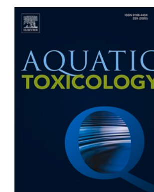

Acute exposure to realistic concentrations of Bisphenol-A trigger health damage in fish: Blood parameters, gene expression, oxidative stress

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A R T I C L E I N F O

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Gene expression altered

A B S T R A C T

Despite much information regarding BPA toxicity in fish and other aquatic organisms, data is still misleading as most studies have utilized concentrations several orders of magnitude higher than those typically found in the environment. As an illustration, eight of the ten studies investigating the impact of BPA on the biochemical and hematological parameters of fish have employed concentrations on the order of mg/L. Therefore, the results may not accurately represent the effects observed in the natural environment. Considering the information above, our study aimed to 1) determine whether or not realistic concentrations of BPA might alter the biochemical and blood parameters of *Danio rerio* and trigger an inflammatory response in the fish liver, brain, gills, and gut and 2) determine which organ could be more affected after exposure to this chemical. Findings pinpoint that realistic concentrations of BPA prompted a substantial increase in antioxidant and oxidant biomarkers in fish, triggering an oxidative stress response in all organs. Likewise, the expression of different genes related to inflammation and apoptosis response was significantly augmented in all organs. Our Pearson correlation shows gene expression was closely associated with the oxidative stress response. Regarding blood parameters, acute exposure to BPA generated biochemical and hematological parameters increased concentration-dependent. Thus, it can be concluded that BPA, at environmentally relevant concentrations, threatens aquatic species, as it prompts polychromasia and liver dysfunction in fish after acute exposure.

1. Introduction

The chemical compound known as Bisphenol A (BPA) was initially synthesized in 1891 by Aleksandr P. Dianin, who combined phenol with acetone under acidic conditions to bring it out (Xiao et al., 2020). During the 1950s, researchers observed that the reaction between BPA and carbonyl chloride yielded a transparent and rigid polymer known as polycarbonate. As a result of this discovery, BPA has been extensively employed in manufacturing plastic products and epoxy resins (Matuszczak, 2019; Bousoumah et al., 2021; Hahladakis et al., 2023). Nonetheless, this compound has also been utilized in manufacturing thermal papers, flame retardants, canned food containers, electronic products, medical equipment, dental sealants, and personal hygiene products (Ballesteros-Gómez et al., 2014; Testai et al., 2016; Björnsdotter et al., 2017; Lu et al., 2018). Therefore, BPA is among the most widely produced chemicals globally (Bousoumah et al., 2021). BPA's extensive production and widespread utilization have contributed to its continuous release and dissemination within the aquatic ecosystem. Moreover, given its ability to be detected in various sources of point and nonpoint pollution, it is considered an omnipresent contaminant. These sources include both industrial and domestic wastewater (Al-Saleh et al., 2017), treated and untreated sewage utilized for agricultural purposes (Lesser et al., 2018; Qian et al., 2021), sewage sludge or biosolids (Cavanagh et al., 2018), and landfill leachate (Qi et al., 2018). Thus far, the most significant concentrations of BPA have been identified in river water (3 μg/L) and sediments (10.5 μg/g)

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Table 1
Colorimetric methods employed.

Method	Procedure	Reference
HPC	To 100 mL of sample [previously deproteinized with 10% trichloroacetic acid (TCA)] were added 900 mL of reaction mixture [0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 4 mM butyl hydroxytoluene in 90% (v/v) methanol]. This mixture was incubated for 60 min at ambient temperature, and absorbance was determined at 560 nm against a blank containing reaction mixture only. HPC content was estimated using the molar extinction coefficient (MEC) of 4.3×10^4 M/ cm, and results were expressed as nM cumene hydroperoxide (CHP)/mg protein wet weight.	Jiang et al., 1992
LPO	To 500 mL of supernatant was added 1 mL Tris–HCl buffer solution (150 mM) pH 7.4. The resulting sample was incubated at 37 °C for 30 min, and then supplemented with 2 mL of 0.38% thiobarbituric acid (TBA) in 15% TCA. The sample was then centrifuged at 12,500 rpm at 4 °C for 15 min and absorbance was determined at 535 nm. Results were expressed as nM MDA, using the MEC of 1.56 $\times 10^5$ M/cm to calculate MDA content.	Buege and Aust, 1978
PCC	To 100 mL of supernatant were added 150 mL of 10 mM N-dimethylformamide in 2 M HCl, and the sample was then incubated at ambient temperature for 1 h in the dark. Next, 500 mL of 20% TCA were added and the sample was allowed to rest for 15 min at 4 °C, then centrifuged at 16,000 x g for 5 min. The bud was rinsed three times with ethanol:ethyl acetate (1:1), dissolved in 150 mL guanidine (6 M) at pH 2.3, and incubated at 37 °C for 30 min. Absorbance was read at 366 nm. Results were expressed as nM reactive carbonyls (C = O)/ mg protein wet tissue, using the MEC of 21,000 M^{-1} cm^{-1}	Levine et al., 1994
CAT	To 20 mL of supernatant was added 1 mL isolation buffer solution [0.3 M sucrose (Vetec, Sigma-Aldrich, St. Louis), 1 mM EDTA, 5 mM HEPES, 5 mM KH_2PO_4] plus 0.2 mL of 20 mM H_2O_2 solution. Absorbance was read at 240 nm, at 0 and 60 s. Values were calculated by substituting absorbance readings in the formula: CAT concentration = (A_0 - A_{60})/MEC, where the MEC of H_2O_2 is 0.043 mM/ cm. Results were expressed as units mg $protein^{-1}$. One unit is defined as the quantity of enzyme required to break down 1 μmol H_2O_2 min^{-1} at 25 °C and pH 7.0.	Radi et al., 1991
SOD	To 40 mL of supernatant in a 1-cm cuvette was added 260 mL carbonate buffer solution (50 mM sodium carbonate, 0.1 mM EDTA) pH 10.2 and 200 mL adrenaline (30 mM). Absorbance was read at 480 nm after 30 s and 5 min. Enzyme activity was determined by interpolating the data on a type curve.	Misra and Fridovich, 1972
TP	A total of 25 mL of supernatant were mixed with 75 mL deionized water and 2.5 mL Bradford reagent. The mixture was homogenized in a Vortex for 1 min and left in the dark for 5 min. Absorbance was read at 595 nm and results were interpolated on a bovine serum albumin type curve.	Bradford, 1976

located in Taiwan (Huang et al., 2012). However, its concentrations have also been reported to be high in the surface waters of China (3.3 μg/L) (Wang et al., 2017), Germany (0.56 μg/L) (Heemken et al., 2001, India (0.13 μg/L) (Selvaraj et al., 2014), and Spain (0.12 μg/L) (Esteban et al., 2014).

Empirical studies assessing BPA's biological and toxicological effects have validated that its presence can harm the environment and the organisms inhabiting it. For example, Tao et al., 2016 conducted an experiment in which *Gobiocypris rarus* larvae were subjected to high doses of BPA (1000 and 1225 μg/L) for seven days. Their outcomes demonstrated fish exposed showed a significant overproduction of H2O2 and concomitant immunosuppression. Similarly, Wang et al. (2019) investigated the impact of BPA at concentrations ranging from 1 to 500 μg/L on the development of the ovary and testis in *Carassius auratus* over 30 days. The results indicated that BPA could impede testis maturation by inducing apoptosis of both germ and Leydig cells and, consequently, reducing 11-KT levels. Moreover, Gu et al., 2021 assessed the detrimental effects of BPA (0.01 - 2 mg/L) on the liver of *Cyprinus carpio* after 30 d exposure. The data demonstrated that BPA exposure altered lipid metabolism and yielded oxidative damage, apoptosis, and inflammatory response in the liver tissues of fish. Even though there is plenty of information regarding BPA toxicity in fish and other hydrobionts, most studies have used concentrations several orders of magnitude above those in the environment. As an illustration, ten studies conducted since 2017 have examined the impact of BPA on the biochemical and hematological parameters of fish (George et al., 2017; Krishnapriya et al., 2017; Pal and Reddy, 2018; Faheem et al., 2019; Srivastava and Reddy et al., 2020; Gu et al., 2021; Sharma and Chadha, 2021; Minaz et al., 2022a,b; Afzal et al., 2022). However, eight of these studies employed concentrations on the order of mg/L, while the remaining two utilized concentrations exceeding 10.0 μg/L. Thus, outcomes can be misleading and not correctly reflect the effects observed in real life.

Considering the above, this study aimed to determine whether or not environmentally relevant concentrations of BPA might alter the biochemical and blood parameters of *Danio rerio* and trigger an inflammatory response in the fish liver, brain, gills, and gut. As this compound has been proven to induce autophagy in liver fish, we hypothesize BPA will damage the liver of *Danio rerio* and alter the blood levels of transaminases.

2. Method

2.1. Bisphenol-A and reagents

Bisphenol-A was acquired from Merck (Darmstadt, Germany), whereas the remaining reagents employed in the experiment were obtained from Sigma Aldrich (Saint Louis, Missouri).

2.2. Organisms maintenance and exposure

Five-month-old *Danio rerio* (AB strain) were maintained in 100 L water tanks (ratio 1fish:1 L) supplied with aerated, dechlorinated, charcoal-filtered, and UV-sterilized tap water. Tap water was supplemented with 0.3 g/L of Instant Ocean and renewed every other day. The experimental conditions, including temperature (27 °C ± 1 °C) and illumination length (14 h), were kept steady and under monitoring. To ensure all aquaria fulfilled the water quality parameters throughout the zebrafish maintenance and exposure, we measured the levels of oxygen dissolved (9.1 ± 0.3 mg/L), nitrate (0.027 ± 0.009 mg/L), nitrite (2.9 ± 0.3 mg/L), and un-ionized ammonia (0.011 ± 0.003 mg/L) in water every other day. Feeding was performed thrice daily with fresh brine shrimp (*Artemia* sp. *nauplii*). For each of the three individual experiments, 120 fish were employed. These fish were assigned to four tanks of 30 L, each containing 30 fish and one of the four test concentrations of BPA (0, 220, 1180, 1500 ng/L). Besides being realistic, the selected concentrations of BPA were chosen considering the LOAEL and LC50 of our previous research (Heredia-García et al., 2022). Acute exposure lasted 96 h, during which temperature and light length were constant as in fish upkeeping. Water renewal of each system was performed daily throughout the entire test period.

2.3. Tissue gathering

Euthanasia of fish from each concentration was carried out using immersion in eugenol 1%, which was chosen due to previous research demonstrating its ability to minimize stress during euthanasia of *Danio rerio* and enable the collection of significant volumes of postmortem

Table 2
qPCR genes evaluated.

Gen	Forward primer	Reverse primer	Source
nfkb	CAACGACACCACGAAAACG	CGTCAGGAATCTTGAATGGGT	Zheng et al., 2016
mapk14	AGCTACTGCGGGGGACTCGT	CTTTCCCTGCTCGTCCGCCC	Sanden et al., 2012
tnfa	ACAGACTGGGCACAGACACG	AACATTTTCCTCACTTTCGTTCA	Zheng et al., 2016
bax	GGC TAT TTC AAC CAG GGT TCC	TGC GAA TCA CCA ATG CTG T	Soares et al., 2018
casp3	CCG CTG CCC ATC ACT A	ATC CTT TCA CGA CCA TCT	Félix et al., 2018
p53	GCA GCG ATG AGG AGA TCT TT	GGG CTC AGA TGA TTC ACG AT	Lei et al., 2017
bcl2	TCACTCGTTCAGACCCTC	ACGCTTTCCACGCACAT	Kim et al., 2018

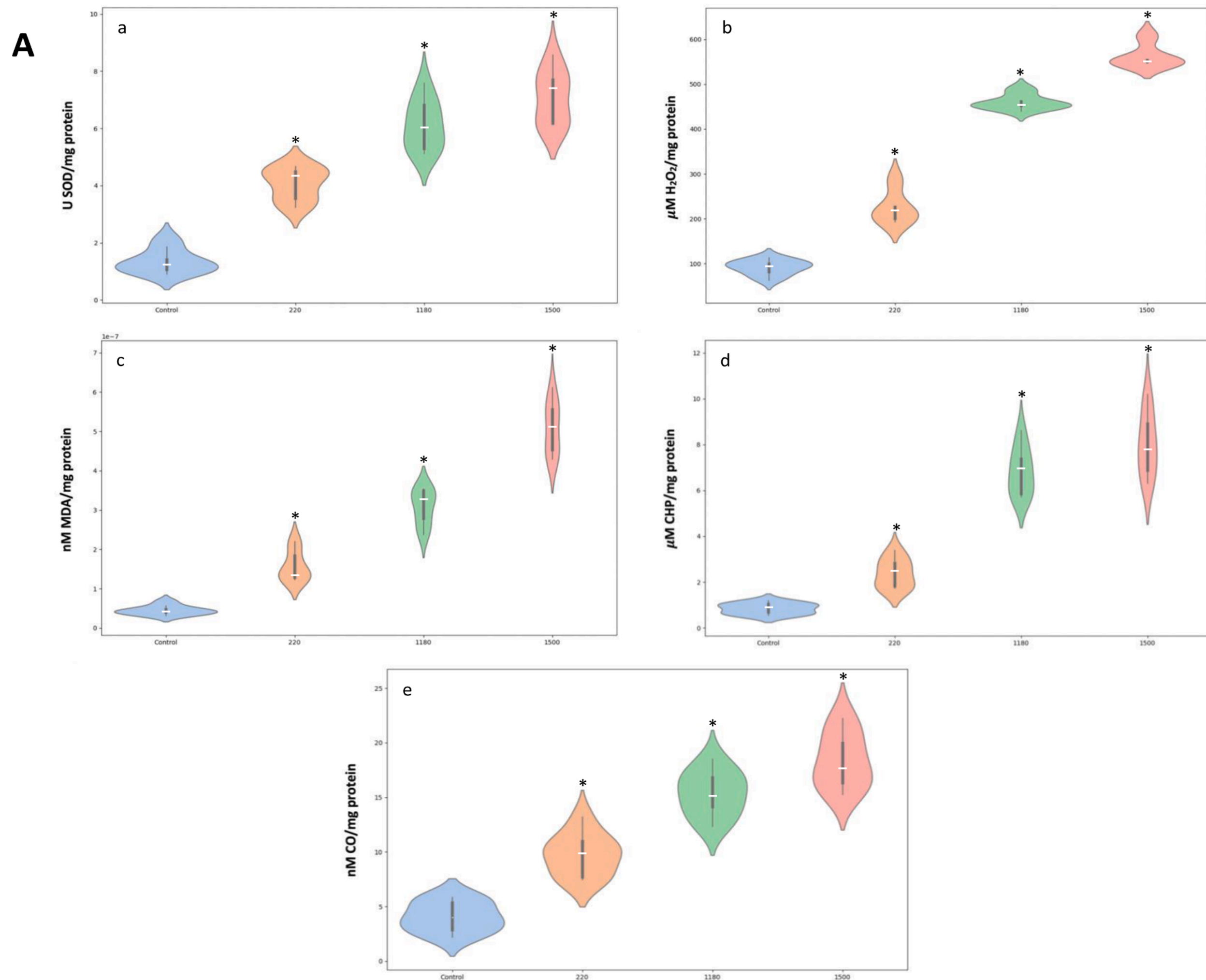

Fig. 1. Violin plots of oxidative stress biomarkers (a: SOD, b: CAT, c: LPX, d: HPX, e: PCC) from A: liver, B: brain, C: gills, and D: gut of *Danio rerio*. Data represent mean ± standard deviation of three independent experiments (n = 9). * denotes a significant change in comparison to the control group (one-way ANOVA test) ($p < 0.001$).

blood (Davis et al., 2015). Following euthanasia, a transverse incision of 0.1 to 0.3 cm was made in the caudal fin to collect the blood that flowed out using a micropipette. Around 40 to 50 μL of blood was collected from each incision and then transferred to BD micro containers. Once the blood was collected, the liver, brain, gills, and gut were removed and placed into microcentrifuge tubes. Microcentrifuge tubes employed for oxidative stress evaluation were filled with 1 mL of PBS (pH 7.4); meanwhile, those used for gene expression determination were free of RNases and contained RNALater (QIAGEN) 10 μL:1 mg of tissue. RNALater was used to avoid nucleic acid degradation.

2.4. Oxidative stress

The dissected organs were homogenized using a rotor-stator homogenizer (IKA, Germany) at 10,000 rpm for 20 min. The dissected organs were homogenized using a rotor-stator homogenizer (IKA, Germany) at 10,000 rpm for 20 min. In Tube 1, 300 μL of the homogenate was mixed with an equal volume of trichloroacetic acid (20%). Tube 2, on the other hand, consisted of 700 μL of the homogenate alone. Tube 2 was centrifuged at 12,500 rpm for 15 min at four-degree Celsius, and the resulting supernatant was used to measure the levels of superoxide dismutase (SOD) and catalase (CAT). Meanwhile, tube 1 was centrifuged at 11,495 rpm for 15 min at four degrees Celsius, and the resulting

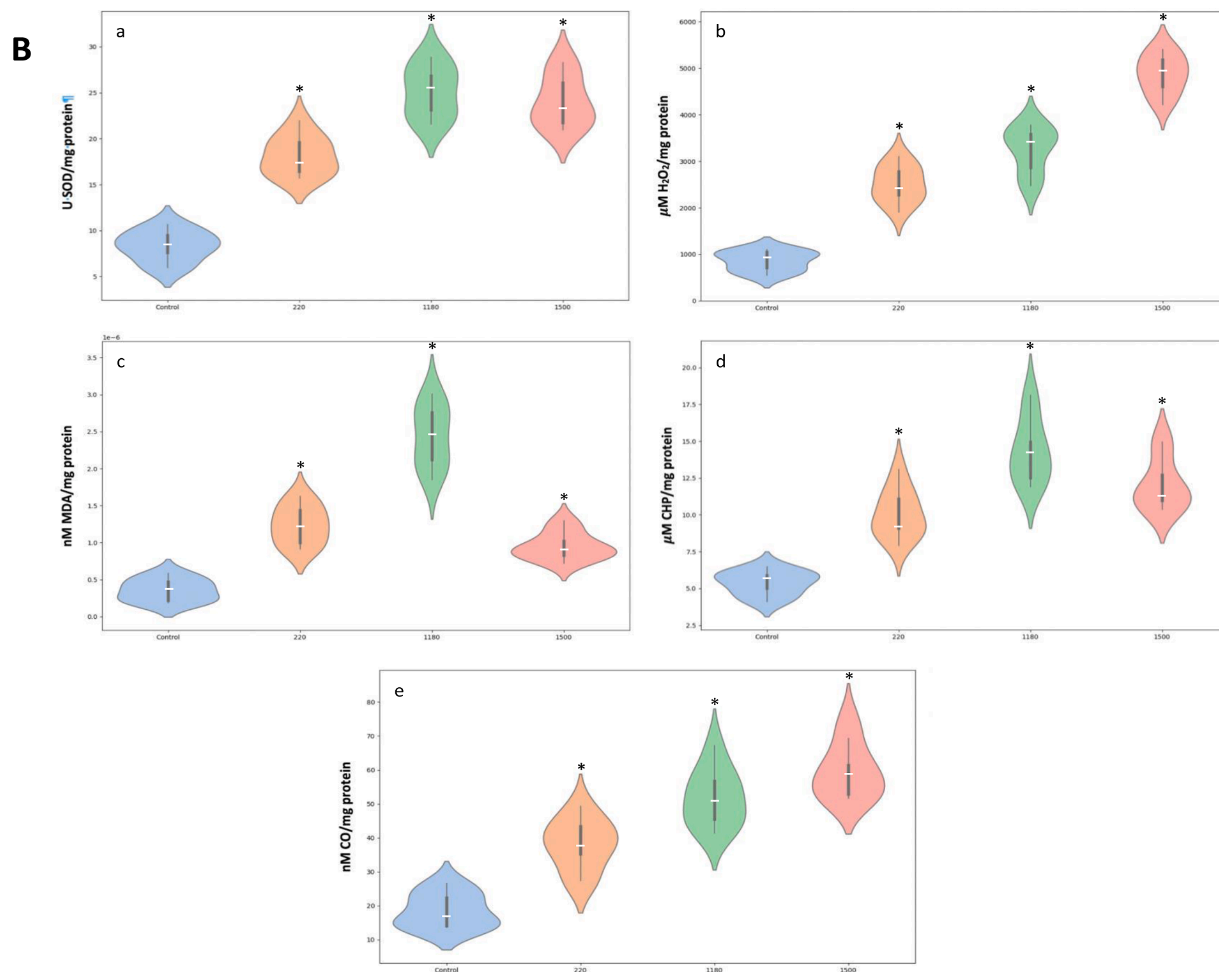

Fig. 1. (*continued*).

supernatant was used to ascertain lipid peroxidation (LPX) levels and hydroperoxides (HPC). Moreover, The precipitate obtained from this centrifugation was used to determine protein carbonyls (PCC). All methods employed in this experiment were colorimetric and followed the step-by-step protocols outlined in Table 1. Results were normalized against total proteins (TP). The organs from the three independent experiments were analyzed to evaluate oxidative stress. Additionally, samples from each of these experiments were evaluated three different times. Thus, nine results were obtained per biomarker and concentration ($n = 9$), which were used to calculate the mean and standard deviation.

2.5. qPCR

RNA was isolated using the RNeasy Mini Kit from QIAGEN, and its quality and purity were assessed by agarose gel electrophoresis (1%) and spectrophotometry (NanoDrop 2000/2000c Thermo Scientific), respectively. The resulting RNA was stored at −20 °C until use. The cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN), and its quality and purity were also assessed through agarose gel electrophoresis (1%) and spectrophotometry (NanoDrop 2000/2000c Thermo Scientific). qPCR reaction was performed by adding 25 uL of the 2x QuantiTect SYBR Green PCR (QIAGEN), 1.0 μL of each primer, 4.0 μL of cDNA, and 19 μL of RNase-free water. The genes evaluated are shown in Table 2. All reagents were mixed, and the qPCR was run using the following conditions: 94 °C for 15 s, followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. qPCR reaction was performed using Gene Q Rotor (QIAGEN) with the β-actin gene utilized as the housekeeping gene to normalize all samples. The $2^{-\Delta\Delta Cq}$ method (Pfaffl, 2001; Pfaffl et al., 2002; Schmittgen and Livak, 2008) was applied to calculate mRNA expression changes, and a dissociation curve was executed to distinguish between specific and non-specific products. qPCR reactions were carried out in triplicate, and data from all three experiments were gathered for statistical analysis. Samples lacking templates were used as a negative control, with RNase-free water used instead.

2.6. Blood analysis

Red-capped microtubes were centrifuged for 10 min at 2500 rpm. The serum was then used to quantify alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), creatinine (CRE), globulin (GLOB), glucose (GLU), phosphorus (Phos), total bilirubin (TBIL), total protein (TP), and blood urea nitrogen (BUN) using the VetScan VS2 system (Abaxis). Additionally, analysis of blood stored in purple-cap microtubes was conducted using HEMA-V6190 (Bioevopeak) to evaluate parameters such as red blood cell count (RBC), hemoglobin (Hb), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), percentage of lymphocytes (LYM), monocytes (MXD), neutrophils (NEU), eosinophils (EOS), and basophils (BAS). The above procedure was performed on blood samples from the three independent experiments. Moreover, samples from each experiment were analyzed in triplicate ($n = 9$ per concentration).

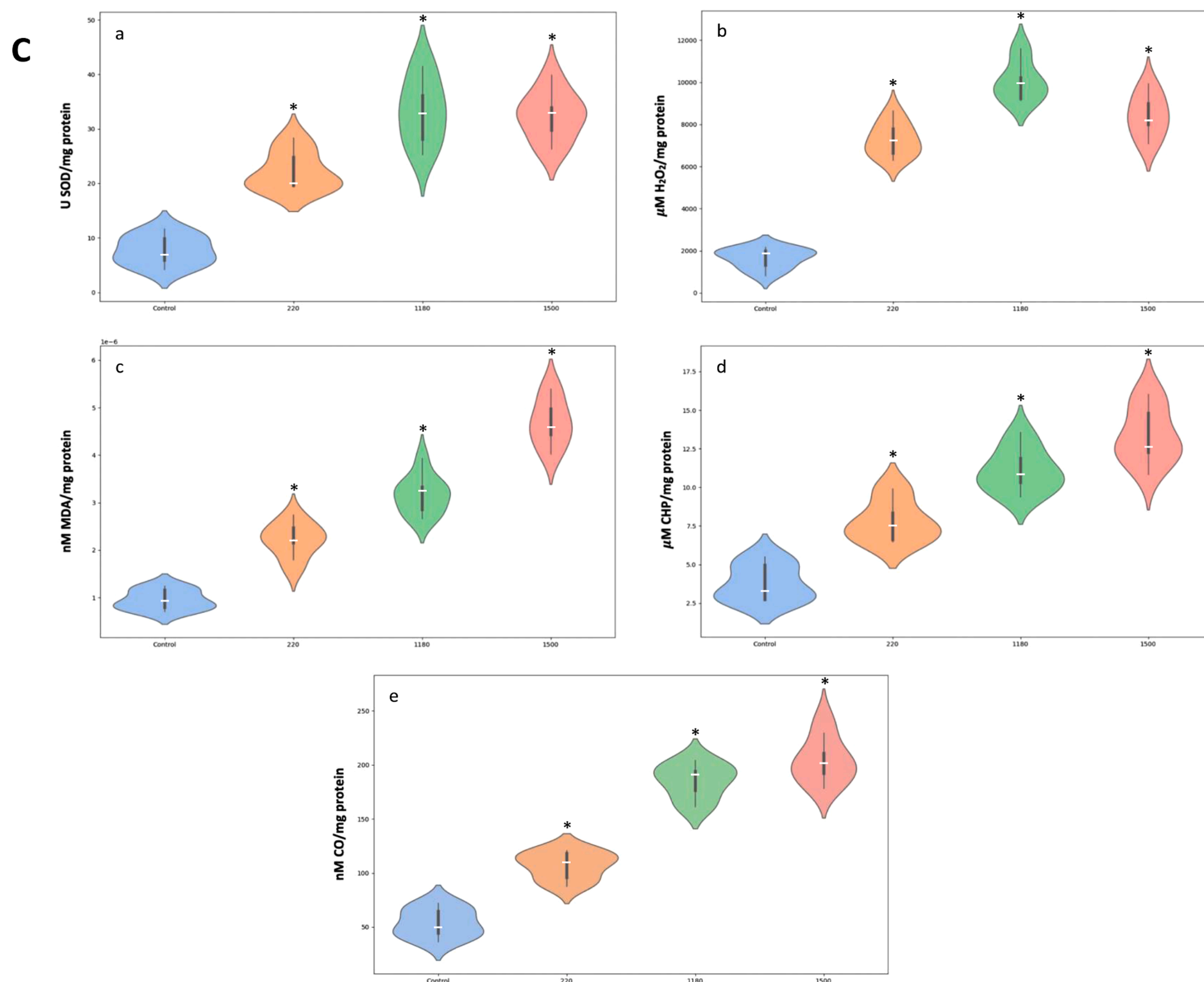

Fig. 1. (*continued*).

2.7. BPA quantification

Water samples of 10 mL were collected daily before the renewal of the systems. Chromatographic separations were conducted using a C18 column (2.6 μm, 4.6 × 100 mm) with an isocratic program employing a mobile phase consisting of 50% methanol (solvent B) and 50% water (solvent A). The flow rate was maintained at 0.5 mL/min, and a 10 μL injection volume was utilized. A G6460 triple quadrupole electrospray ionization mass spectrometer (Agilent Technologies) was used to quantify BPA. Organ homogenized were mixed using 200 μL of phosphate buffer (0.1 M, pH 7.4), 100 μL of C-BPA (200 μg/L), and 1 μL of dichloromethane. The mix was then centrifuged at 16,000 x g for 10 min, and 100 μL of the resulting supernatant was gathered in vials for analysis by G6460 triple quadrupole electrospray ionization mass spectrometer (Agilent Technologies). A C18 column holding a temperature of 40 °C and an outflow rate of 0.4 mL/min was employed to conduct the analysis. The mobile phase was initiated with 40% methanol and 60% water, but the methanol concentration was increased to 100% at seven minutes. The gas was dried at 350 °C, and the capillary voltage was set at 4.5 kV.

2.8. Statistics

Homoscedasticity and normality were verified by employing the Bartlett and Shapiro-Wilk tests, respectively. A Tukey test, with a significance level of $p < 0.05$, was used to pinpoint differences between means. Dissimilarities between treatment groups were ascertained using a one-way ANOVA test ($\alpha = 0.05$) (Sigma Plot 12.3). Two Pearson correlation analyses were executed to estimate the strength of the association between oxidative stress and gene expression and the extent of the connection between biochemical and hematological results. The significance level was set at $p < 0.05$, and the analyses were performed using the R software. Moreover, the resulting correlations were presented through various chord correlations and one correlogram.

3. Results

3.1. Oxidative stress

Levels of oxidative damage biomarkers were statistically different from the control group across all organs after acute exposure to BPA (Fig. 1). However, each organ had a different response. For example, in the fish brain and gills, some biomarkers (SOD, CAT, LPX, and HPX) decreased significantly at the highest concentration compared to the medium concentration. Moreover, in the gut, LPX, HPX, and PCC showed no significant difference between the middle and highest concentrations. The liver was the only organ with a concentration-dependent increase in all biomarkers. Therefore, it can be concluded that the liver exhibited the highest degree of oxidative stress following acute exposure to BPA.

3.2. qPCR

The heatmaps in Fig. 2 indicate that BPA exposure triggered a

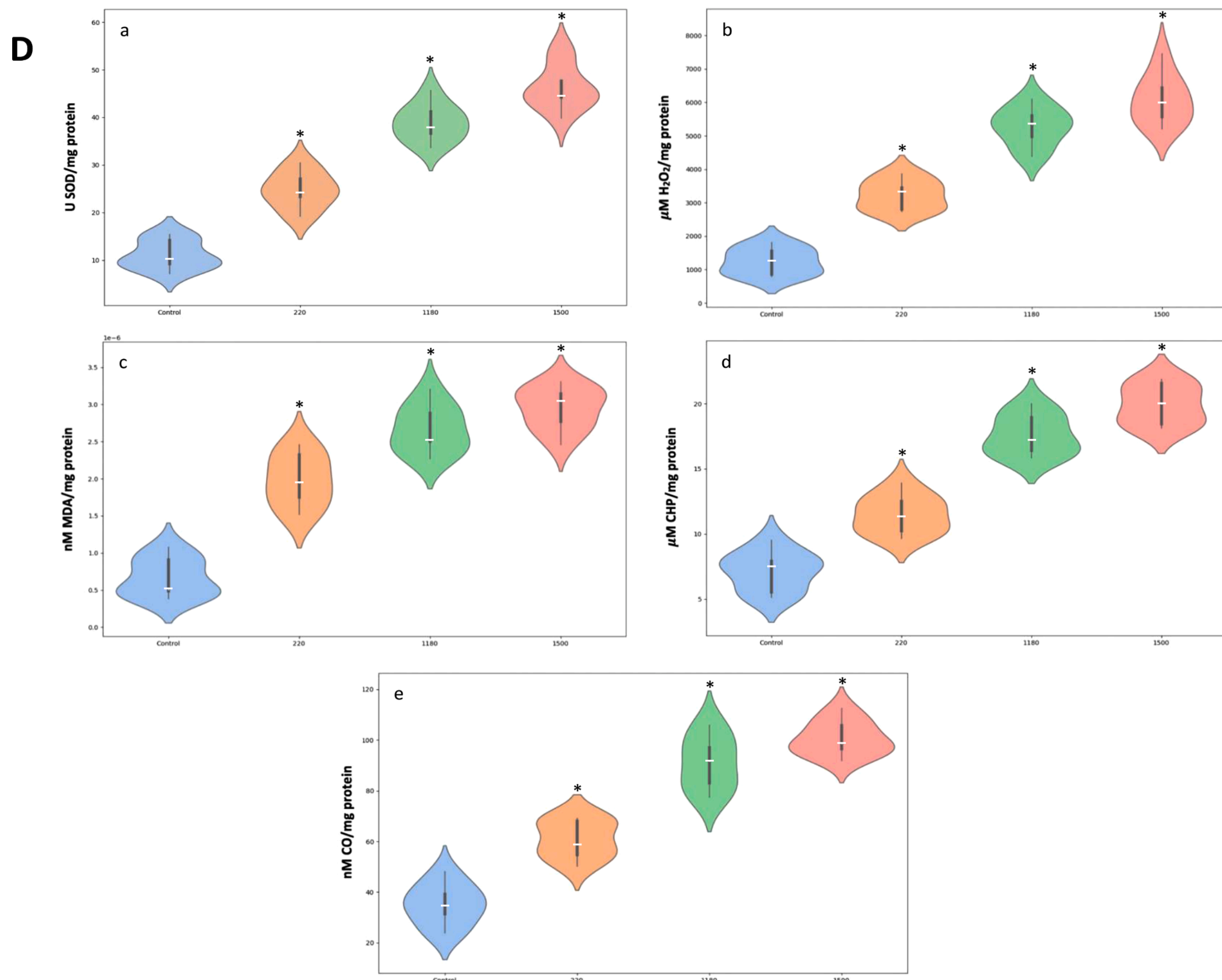

Fig. 1. (*continued*).

significant upregulation of apoptosis and inflammation genes in all examined organs. However, similar to the observed oxidative stress response, the fold changes in gene expression levels varied between organs. For example, the genes with the most significant increased expression in the liver were *nfkb, tnfa*, and *bax*. Even though the brain, gills, and gut followed the same trend, it can be seen that *blc2* in all of them was significantly more expressed than in the liver. Moreover, it can also be seen that as the *bcl2* expression augmented in those organs, the fold change of *p53* and *casp3* was lower. Based on the observations above, it can be inferred that the BPA led to a more significant inflammation and apoptosis response in the liver.

3.3. Blood parameters

All biochemical parameters, but amylase, increased concentration-dependent in fish exposed to BPA (Table 3). Amylase showed a significant reduction, also concentration-dependent, compared to the control group after acute exposure to the pollutant. Biochemical values from fish of the control group are closely related to reference ones reported in the literature. As in biochemical parameters, all hematological values, but HTC, of fish exposed to BPA increased concentration-dependent (Table 4). Interestingly, there was a minimum decrease in the HTC levels of fish exposed to the middle concentration of the chemical compared to the ones exposed to the lowest.

3.4. BPA quantification

BPA concentrations in water decreased in all systems but control, which remained below the limit of quantification. It is also noteworthy to pinpoint that BPA concentrations did not decrease beyond 84% in any of the systems. Given that the measured concentrations of BPA did not exhibit a decrease exceeding 20% compared to the nominal concentration, all results were analyzed solely based on the latter. The concentrations of BPA in all organs of the control group remained below the quantification limit. Conversely, organs of fish from treatment groups, excluding the control, demonstrated a significant escalation in the compound's concentration, as illustrated in Table 5. The gills displayed the highest BPA absorption, trailed by the gut, liver, and brain. BPA concentration in all organs demonstrated a concentration-dependent elevation.

3.5. Pearson correlations

Our Pearson correlation revealed all variables are positively correlated in all organs. However, it is paramount to note that the strength between variables varied from one organ to another. For example, the liver (0.85 - 0.99) showed the most potent relationship among all biomarkers evaluated, followed by the gills (0.77 – 0.99), gut (0.65 – 0.99), and brain (0.41 – 0.99). Moreover, it is noteworthy that the correlation of *casp3, blc2*, and *p53* with SOD, CAT, and PCC varied from 0.41 to 0.85 among all organs. The above can be observed in chord diagrams

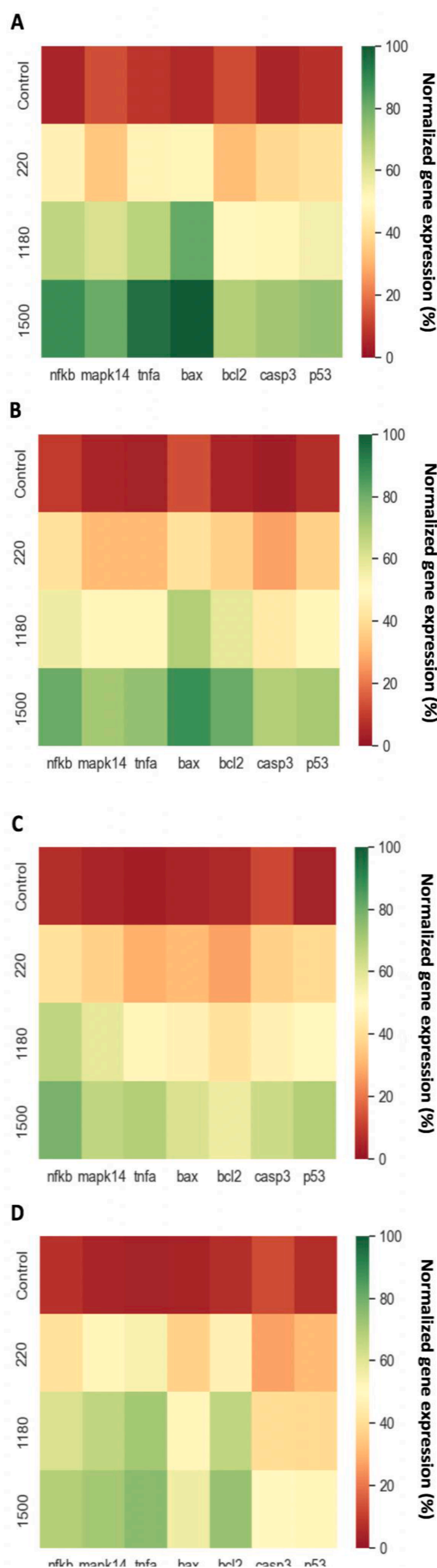

Fig. 2. BPA-induced gene expression change in **A:** liver, **B:** brain, **C:** gills, and **D:** gut of *Danio rerio*. Data represent mean ± standard deviation of three independent experiments ($n = 9$) ($p < 0.001$).

(Fig. 3A-D), as the size of the arcs among all nodes is high. Finally, concerning blood parameters, the Pearson correlation and heatmap (Fig. 4) showed all variables, but amylase and LYM, are strongly and positively correlated.

4. Discussion

Bisphenol A (BPA) is a chemical manufactured on a massive scale and extensively used across various industries (Bousoumah et al., 2021). These industries encompass the production of plastic products, epoxy resins, thermal papers, flame retardants, canned food containers, electronic products, medical equipment, dental sealants, and personal hygiene products (Björnsdotter et al., 2017; Lu et al., 2018; Matuszczak, 2019; Bousoumah et al., 2021; Hahladakis et al., 2023). Owing to its extensive production and broad application across various industries, BPA has emerged as a ubiquitous contaminant in water. Until now, BPA has reached concentrations of up to 3 μg/L in surface water (Huang et al., 2012); however, there is evidence it might bioaccumulate in fish and other aquatic species (Lv et al., 2019). Therefore, BPA poses a threat to hydrobionts and humans. Recently, BPA has attached the attention of the scientific community, and as a result, there is plenty of information concerning this chemical's toxic effects on aquatic organisms. Nonetheless, it is noteworthy that most of the findings have been from studies that employed concentrations of various orders of magnitude above the ones reported in surface water. Consequently, the results may not accurately reflect the actual impacts of BPA exposure. To address this issue, we exposed multiple *Danio rerio* adults to three environmentally relevant concentrations of BPA (220, 1180, and 1500 ng/L) and determined whether or not they might produce oxidative stress in organisms. Moreover, we also aimed to establish if these concentrations might also disrupt the expression of some genes related to inflammation and apoptosis, as well as the blood parameters of fish.

As to oxidative stress results, our findings demonstrated that BPA substantially augmented the production of reactive oxygen species in all organs compared to the control group. Moreover, in some organs, the activity of SOD and CAT of fish exposed to the highest concentration decreased compared to those exposed to the middle one. Concordantly with the above, two studies conducted on fish (*Aristichthys nobilis* and *Cyprinus carpio*) demonstrated BPA in concentrations ranging from 0.5 mg/L to 6 mg/L augmented the quantity of thiobarbituric acid reactive substance in the liver, gills, and brain of organisms (Akram et al., 2021; Afzal et al., 2022). Furthermore, it is remarkable that both studies also pinpointed that the antioxidant activity of SOD, CAT, and GPX was substantially decreased in all organs after 30 and 60 days of exposure. Interestingly, there is plenty of information regarding BPA-induced oxidative stress in the liver, gills, brain, and kidneys of fish. Nevertheless, to our knowledge, data regarding gut damage by this chemical is limited to one study. In that study, Chen et al., 2018 assessed the toxic effect of BPA and nano-TiO_2 prompted alone and in a binary mixture. According to their results, BPA alone (2 and 20 μg/L) generated oxidative stress in the gut's fish (*Danio rerio*) by altering the activity of SOD, CAT, GPX, and GSH. However, there were some differences in results between concentrations. For example, between concentrations, results indicated that ROS levels and the antioxidant activity of GPX and GSH were reduced at the highest concentration compared to the lowest. Even though our results did not show a reduction in ROS level between the middle and the highest concentration, we also did not observe any significant change between these. The above might be due to the low difference between the tested concentrations. BPA may induce oxidative damage through two main mechanisms: 1) inhibition of antioxidant defense mechanisms (Gassmann, 2017) and 2) mitochondrial function impairment (Nayak et al., 2022). However, it is paramount to note that regardless of the mechanism, different authors have indicated that BPA-induced oxidative damage is closely associated with triggering toxic responses in fish (Anet et al., 2019; Liu et al., 2021; Pradhan et al., 2021; Zhu et al., 2021).

Table 3
Biochemical biomarkers.

Biomarkers	Control	BPA 220 ng/L	1180 µg/L	1500 µg/L	Units	Reference values
Glucose	95.02±3.71	113.01±3.94*	128.32±4.11*	133.41±4.29*	mg/dL	62–91
BUN	3.84±0.54	5.16±0.94*	6.01±1.0 × 2	6.78±1.17*	mg/dL	3–4
CRE	0.97±0.04	1.45±0.21*	2.52±0.19*	3.07±0.30*	mg/dL	.5–0.9
TP	5.10±0.96	5.95±0.99	6.84±0.87*	7.11±1.01*	g/dL	4.4–5.8
ALT	395.02±5.83	413.19±6.01	452.71±5.95*	461.80±6.11*	U/L	343–410
ALP	9.51±1.12	11.34±1.77*	12.87±1.51*	13.37±1.68*	U/L	0–10
Amylase	1437.09±10.27	1320.48±10.03*	1108.91±9.83*	1092.76±9.99*	g/dL	20.3–24.3

Data represent mean ± standard deviation of three independent experiments ($n = 9$). * denote significant difference compared to the control group (one-way ANOVA test) ($p < 0.001$).

Table 4
Hematological biomarkers.

Biomarkers	Control	BPA 220 ng/L	1180 ng/L	1500 ng/L	Units	Reference values
RBC	3.12 ±0.43	4.09 ±0.50*	4.93 ±0.54*	5.80 ±0.63*	$X10^6$/mL	2.9–3.2
Hb	7.09 ±0.96	8.52 ±0.99*	9.34 ±1.22*	11.12 ±1.76*	g/dL	4.75–7.7
HTC	32.97 ±3.51	55.34 ±4.09*	54.67 ±4.11*	63.2 ± 5.03*	%	25–36
WBC	29.72 ±2.98	33.35 ±2.74*	36.99 ±2.91*	38.44 ±2.97*	mL	13.6–31.7
LYM	71.99 ±4.76	67.44 ±3.63*	62.34 ±3.91*	60.02 ±3.70*	%	71–92
MXD	13.25 ±0.99	15.72 ±1.03*	17.94 ±1.17*	18.76 ±1.34*	%	5–15
NEU	14.20 ±1.66	16.53 ±1.92*	19.90 ±2.01*	21.14 ±2.18*	%	2–18
EOS	0	0	0	0	%	0–2
BAS	0	0	0	0	%	0–2

Data represent mean ± standard deviation of three independent experiments ($n = 9$). * denote significant difference compared to the control group (one-way ANOVA test) ($p < 0.001$).

Table 5
BPA concentrations in water and organs.

	Control	BPA 220 ng/L	1180 ng/L	1500 ng/L
Exposure water	<LOQ	193.64±2.48 ng/L	1003.32±4.19 ng/L	1365.20±3.19 ng/L
Liver	<LOQ	3.98±0.07 ng/g	22.01±0.53 ng/g	26.78±0.81 ng/g
Gut	<LOQ	4.31±0.09 ng/g	25.69±0.87 ng/g	30.53±0.92 ng/g
Brain	<LOQ	3.47±0.06 ng/g	19.74±0.32 ng/g	23.12±0.71 ng/g
Gill	<LOQ	5.01±0.11 ng/g	23.42±0.64 ng/g	29.87±1.07 ng/g

Data represent mean ± standard deviation of three independent experiments. LOQ-liver: 0.97 ng/g; LOQ-gills: 1.46 ng/g; LOQ-brain: 1.19; LOQ-gut: 1.38 ng/g.

Cell inflammation and apoptosis are two of the most common toxic responses mediated by oxidative stress. Plenty of chemicals, including BPA, can induce inflammation and apoptosis by increasing reactive oxygen species (ROS) levels. For example, Biswas et al. (2020) demonstrated that chronic exposure to BPA (1–100 µg/L) caused oxidative and nitrosative stress responses, which mediated inflammatory processes in *Danio rerio* ovaries. Likewise, Gu et al., 2020 and Gu et al., 2021 noted that long-term exposure to BPA (0. 01 - 2.0 mg/L) caused a substantial redox imbalance in *Cyprinus carpio* that led to immune and inflammatory responses and apoptosis in the liver and gills of fish. The significant increase in the expression of *casp3, bax, tnf, il-1b*, and *il-6* supported the above. Additionally, Chen et al. (2022) demonstrated that exposure to BPA at a concentration of 200 nM increased reactive oxygen species (ROS) levels and activated different signaling pathways, leading to the upregulation of *bax* and *casp3* in *Ctenopharyngodon idella* hepatocytes. As can be seen, the common factor in all of the studies is oxidative stress, as a significant increase in reactive oxygen species (ROS) is necessary to initiate distinct signaling pathways that lead to apoptosis and inflammatory response. Based on the above data, the expression of apoptotic and inflammatory-related genes was evaluated. In agreement with prior findings, our results indicated BPA at realistic concentrations (220, 1180, and 1500 ng/L) produced a significant increase in the expression of *tnf, mapk14*, and *nfkb*. Tumor necrosis factor (tnf) is an essential signaling protein in the immune system, critical for tissue degeneration and repair processes. Its upregulation stimulates the production of inflammatory cytokines, programmed cell death, and necrosis under certain circumstances (Yang et al., 2018). The mitogen-activated protein kinases (MAPKs) family, including mapk14, is also well known for its involvement in inflammatory reactions, and BPA has been shown to activate them (Zhu et al., 2015). The upregulation of *mapk14* gene expression may trigger the secretion of cytokines or other signaling molecules, inducing an inflammatory response (Wang et al., 2015). Nuclear factor-κB (*nfkb*) is another hallmark inflammatory response gene regulating various genes involved in multiple aspects of immune and inflammatory responses, including the activation of inflammasomes (Liu et al., 2017). Recent studies have shown that its upregulation is closely related to chronic inflammatory diseases (Capece et al., 2022). The process of programmed cell death, known as apoptosis, is regulated by several genes acting as death switches (Maes et al., 2017). Among them, the *bcl2* gene family members play a crucial role by inhibiting various apoptotic signals (Radha and Raghavan, 2017). Experimental evidence confirms that bcl2 impedes the release of cytochrome C, a necessary factor for activating the caspases responsible for the apoptotic process (Gao and Wang, 2009). bax is recognized as the primary pro-apoptotic protein in the bcl2 family (Gavathiotis et al., 2010). The gene products from this family can create homo- and heterodimers with each other; for instance, bax can form dimers with bcl2 or itself. When excessively produced, bax homodimers trigger apoptosis. Conversely, when bcl2 is abundant, bcl2 homodimers prevail, protecting cells from death (Gao and Wang, 2009). Therefore, the relative levels of *bax* and *bcl2* expression play a vital role in regulating cellular survival, determining the cell's fate when exposed to an apoptotic trigger. This research reported higher levels of *bax* overexpression compared to *blc2* in the liver, brain, and gills, suggesting chronic exposure to realistic concentrations of BPA at 220, 1180, and 1500 ng/L may trigger cell death. Consistently, the expression of two genes frequently linked to programmed cell death (*p53* and *casp3*) was upregulated in all the organs mentioned above.

In addition to the upregulation of genes associated with apoptosis and inflammation, the findings indicate that BPA induced significant changes in the baseline levels of blood parameters. A clear example of the above phenomenon can be observed in the increase in glucose levels. Insulin enables the transportation of glucose molecules into cells,

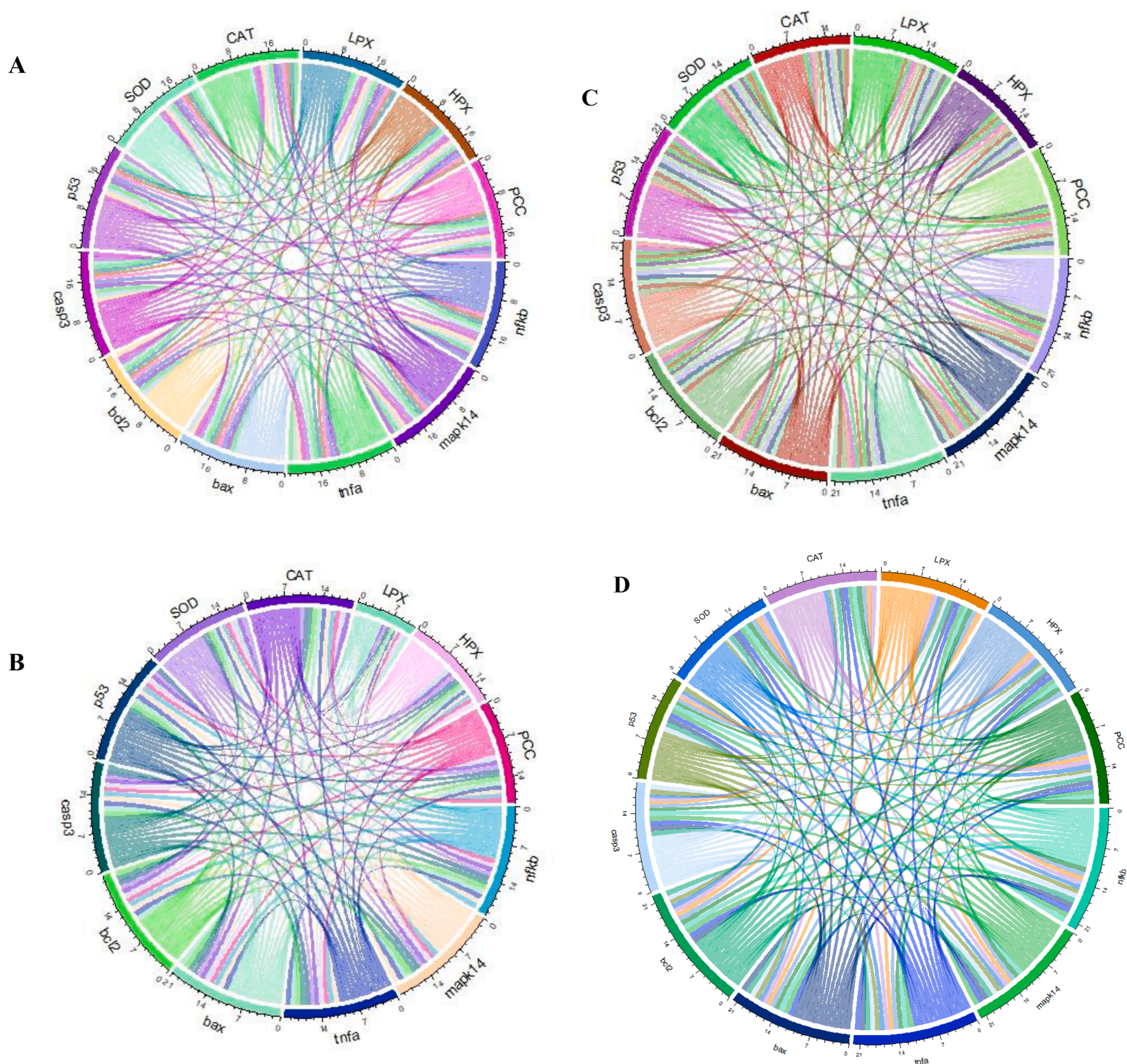

Fig. 3. Chord correlation of oxidative stress and gene expression outcomes in **A**: liver, **B**: brain **C**: gills, and **D**: gut.

facilitating their use for immediate energy generation or storage for later use (Lee et al., 2022). Nevertheless, oxidative stress has the potential to induce insulin resistance, thereby causing cells to exhibit reduced sensitivity toward this hormone-mediated actions (Luc et al., 2019). As a result, the transport of glucose molecules into cells is hindered, leading to an accumulation of glucose in the bloodstream, consequently leading to hyperglycemia. Based on the oxidative stress results, the increased glucose levels observed could be attributed to BPA-induced oxidative damage. However, further studies are warranted to determine whether insulin resistance, inflammation processes, or pancreatic disruption are involved in the underlying mechanisms of this phenomenon following BPA exposure. Concerning kidney function, it was observed that, as in glucose levels, there was a substantial boost in blood urea nitrogen (BUN) and creatinine (CRE) levels across all concentrations of BPA. BUN and CRE are standard diagnostic blood tests to assess kidney function. BUN measures the amount of nitrogen in the blood from urea, a byproduct of liver metabolism, during protein breakdown (Tomizawa et al., 2015). Conversely, creatinine is a byproduct generated during muscle metabolism and is eliminated from the body via renal excretion. In healthy individuals, the kidneys effectively eliminate both urea and creatinine from the body; however, elevated BUN and CRE levels may indicate renal dysfunction (Liu et al., 2021). In agreement with our results, two previous studies demonstrated fish (*Oreochromis niloticus* and *Catla catla*) suffered from kidney dysfunction after BPA exposure (1.64 - 100 μg/L) for two and six weeks, respectively (Abdel-Tawwab and Hamed, 2018; Faheem et al., 2019). The harmful impact of BPA on organs extends beyond the kidney, with previous studies highlighting its adverse effects on the liver. For example, different authors have indicated BPA in concentrations ranging from 10 μg/L to 2.14 mg/L can augment the levels of serum liver enzymes in *Heteropneustes fossilis* and *Catla catla*, respectively (Pal et al., 2018; Faheem et al., 2019; Srivastava and Reddy, 2020). Consistent with the observations mentioned above, the results of our study revealed a significant increase in the levels of alanine transaminase (ALT), alkaline phosphatase (ALP), and total protein (TP) in fish that were exposed to BPA. ALT and ALP are enzymes implicated in protein metabolism within the liver, while TP is a blood

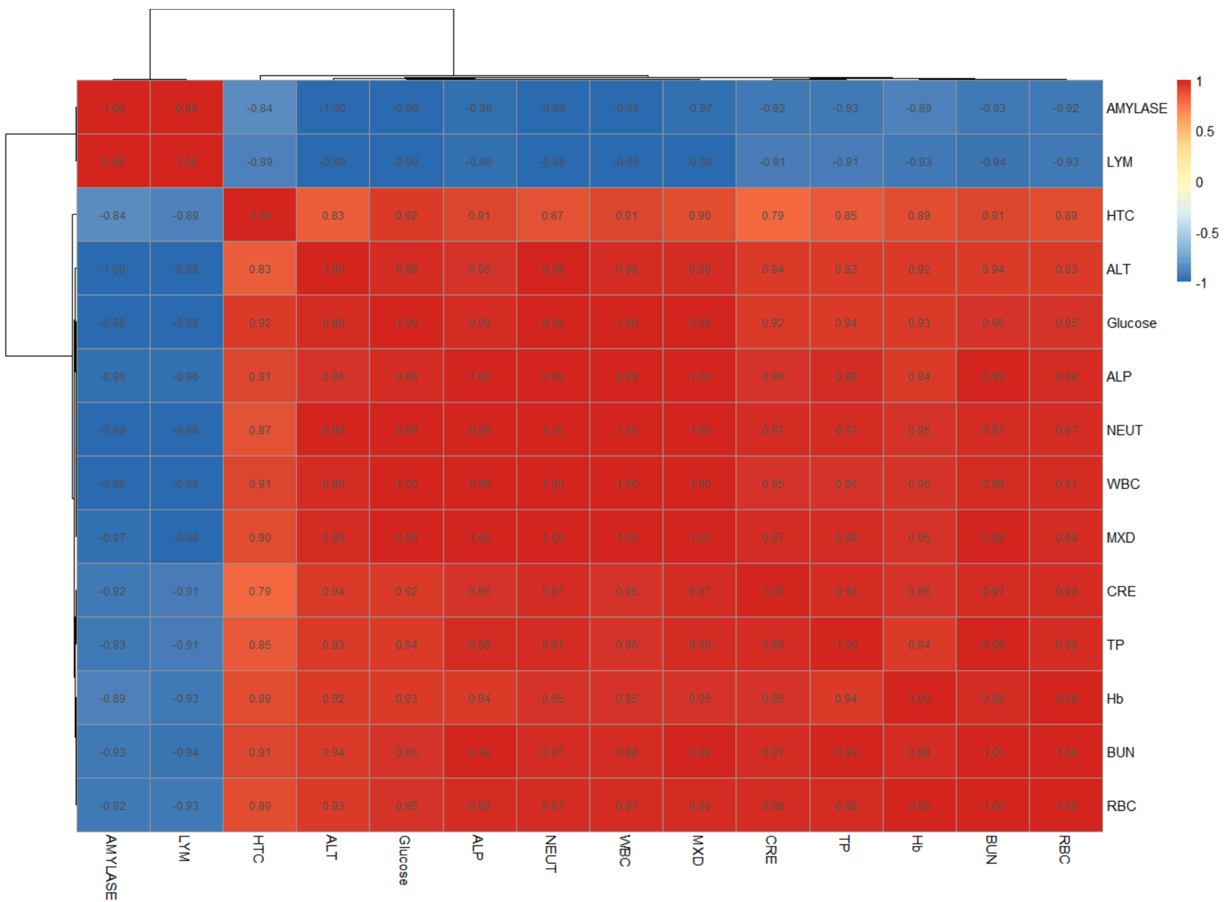

Fig. 4. Correlogram of biochemical and hematological results from fish exposed to BPA.

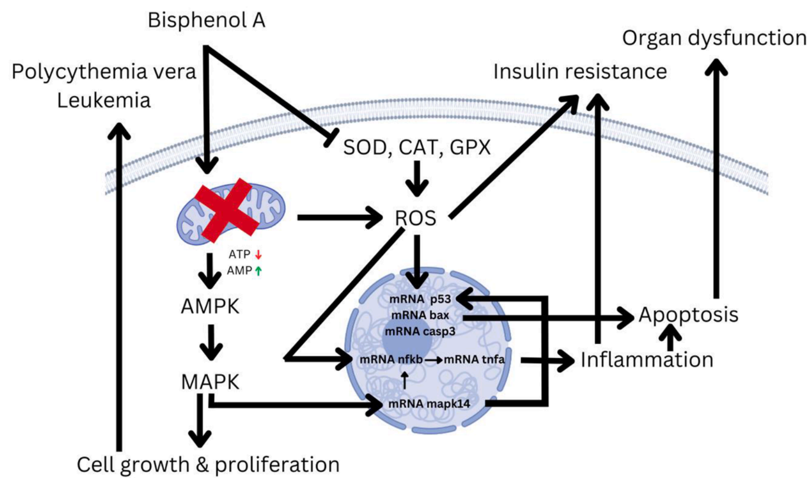

Fig. 5. Pathways through which BPA alters hematological parameters and prompts fish organ dysfunction.

marker often used to assess liver function and diagnose liver disease (Bruslé and Anadon, 2017). The concurrent increase in all of the markers mentioned above in BPA-exposed fish provides compelling evidence of liver dysfunction. This dysfunction is likely attributed to either the oxidative damage induced by BPA or the accompanying inflammatory and apoptotic responses.

Previously, it has been reported BPA (0.5 /L - 3.81 mg/L) could decrease the levels of RBCs, Hb, HTC, and MCV in fish exposed during 96 h and 21 days, respectively (Srivastava and Reddy, 2020; Sharma and Chadha, 2021; Minaz et al., 2022). However, a recent study indicated BPA alters hematopoiesis through EGFR/ERK signaling to induce cell proliferation and impaired differentiation (Sundarraj et al., 2021). Therefore, it enhances the myeloid repopulating activity and the accumulation of immature myeloblast cells. Herein, findings demonstrated that BPA (220 - 1500 ng/L) significantly increased RBCs, Hb, and HTC in *Danio rerio*, indicating BPA might induce polycythemia vera. Polycythemia vera is a disorder that results in the abnormal activation of hematopoiesis in the bone marrow, leading to the excessive production of morphologically normal blood cells (Spivak, 2018). Additionally, it is noteworthy that this condition can be preceded or accompanied by

long-term inflammation and could indicate an elevated susceptibility to other forms of cancer, such as leukemia (Hasselbalch and Bjørn, 2015). Leukemia is a type of blood cancer that occurs due to an abnormal increase in WBCs in the bone marrow of the human body (Ratley et al., 2020). Leukemia can be classified as acute and chronic leukemia, where acute leukemia proliferates, whereas chronic leukemia grows slowly. Further, both types have two subcategories lymphocytic and myeloid. Previous findings have demonstrated BPA can prompt acute myeloid leukemia by activating nfkb or epidermal growth factor receptor and extracellular signal-regulated kinase (EGFR/ERK) (Zhang et al., 2020; Sundarraj et al., 2021). Concordantly, this work demonstrated BPA (220 - 1500) acute exposure substantially increased WBCs, and levels of LYM, MXD, and NEU in *Danio rerio* adults. Even though an increase in the number of myeloid cells in the marrow characterizes acute myeloid leukemia (AML), prior studies have indicated lymphocytosis may occur in patients with AML (Röllig and Ehninger, 2015). The above may be an immunological response to a tumor.

In summary, Bisphenol A (BPA) has the potential to induce oxidative stress through mitochondrial dysfunction or inhibition of antioxidant enzymes (Gassmann, 2017; Nayak et al., 2022). Furthermore, the impairment of mitochondrial function can also result in the activation of the AMPK pathway due to ATP depletion (Elizalde-Velázquez et al., 2022a). AMPK can directly phosphorylate the RAF/KSR family kinases, pivotal components of the MAPK module, and consequently modify its activity under diverse conditions (Morrison, 2012). Activation of the MAPK pathway regulates cell growth and proliferation and is believed to be the pathway by which Bisphenol A (BPA) triggers the development of polycythemia vera and leukemia (Zhang et al., 2020; Sundarraj et al., 2021). Furthermore, MAPK activation can induce upregulation of *mapk14* gene expression, which may subsequently upregulate the expression of nfkb (Ozbek et al., 2009). *nfkb* can also be activated by ROS (Blaser et al., 2016). Once activated, nfkb can lead to cell inflammation by upregulating tnfa and interleukins, such as il-6 (Zhu et al., 2015). Cell inflammation may prompt apoptosis and insulin resistance. However, it is also known that the MAPK pathway and ROS overproduction can boost the fold change of several apoptotic-related genes, such as p53, bax, and casp3 (Stramucci et al., 2015). Finally, as observed herein, BPA-induced cell apoptosis can lead to fish organ dysfunction. Fig. 5 depicts the pathways described above.

5. Conclusions

Unlike previous findings, herein, results demonstrated BPA acute exposure to realistic concentrations (220, 1180, and 1500 ng/L) might lead to different toxic responses in *Danio rerio* adults. These responses included oxidative stress, inflammation, and apoptosis in the liver, gut, brain, and gills of organisms. Moreover, blood fish parameters also suffered substantial increases in almost all markers evaluated. Inflammatory and apoptotic responses in organ fish may result from BPA-induced oxidative damage. However, it is also suggested that increased biochemical and hematological parameters may lead fish to suffer two conditions, polycythemia vera and acute myeloid leukemia, that trigger these processes. According to our results, the liver was the most affected organ, followed by the brain, gills, and gut. Besides oxidative damage and inflammation, findings also show liver enzymes were significantly increased at all concentrations. Therefore, realistic concentrations of BPA can prompt liver dysfunction.

Credit author statement

Gustavo Axel Elizalde-Velázquez & Karina Elisa Rosales-Pérez performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez were involved in the conception

Leobardo Manuel Gómez-Oliván, Gustavo Axel Elizalde-Velázquez and Sandra García-Medina, were involved in the design and interpretation of the data and the writing of the manuscript with input from Selene Elizabeth Herrera-Vázquez, Nely SanJuan-Reyes Sandra & Marcela Galar-Martínez

Declaration of Competing Interest

The authors declare that they have no known competing financial interests- or personal relationships that could have appeared to influence the work reported in this paper.

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