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Gene Expression Analysis, Biochemical and Histological Alterations in the Nile Tilapia (*Oreochromis niloticus*) Exposed to Bisphenol A: The Protective Role of Proanthocyanidin

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ABSTRACT

One of the major contaminants in aquatic ecosystems is bisphenol A (BPA), a synthetic industrial chemical widely used in the production of epoxy resins and plastics. BPA has negative effects on fish health. Proanthocyanidin, a natural immune stimulant extracted from grape seeds (GR), possesses various biological and antioxidant properties. This study aimed to mitigate the harmful effects of BPA by supplementing the fish diet with GR. Healthy Nile tilapia (120 fish, 14.95±0.10g) were divided into four groups: Group 1 served as the control; group 2 was fed 400mg GR per kg of feed; group 3 was exposed to 3mg of BPA per liter of water without GR supplementation, and group 4 was exposed to 3mg of BPA per liter of water and fed a GR-supplemented diet (400mg GR per kg). After 28 days, blood and tissue samples were collected to assess serum biochemical parameters, cytokines, catalase (CAT) activity, malondialdehyde (MDA) levels, and the histological features of the liver, spleen, and kidney. Co-treatment with GR improved serum protein levels as well as urea and creatinine levels that were previously altered by BPA exposure. BPA exposure elevated serum interleukin (IL)-1β and hepatic MDA levels, which were reduced by GR administration. Additionally, CAT activity decreased in the BPA group but increased in the GR+BPA group. BPA also significantly upregulated the mRNA transcripts of IL-6 and tumor necrosis factor (TNF-α), which may trigger inflammation, while GR significantly downregulated these genes in the BPA-treated group. Histological analysis showed that GR alleviated tissue damage caused by BPA. In conclusion, GR supplementation improved fish biochemical and histological parameters and mitigated the toxic effects of BPA.

INTRODUCTION

Bisphenol A (BPA) is an organic compound that is vastly consumed in industry, including epoxy resins, polycarbonate, and plastic manufacturing. It is considered an efficient plasticizer due to its cross-linking characteristics **(Faheem & Bhandari, 2021)**.

Moreover, its production has expanded constantly due to the intense production of plastic **(Onay *et al.*, 2023)**. It is highly detected in aquatic surroundings due to the high frequency of production and consumption. An estimated 1000000 of BPA pounds are leaked into the environment each year **(McCracken *et al.*, 2017)**. Additionally, it has gathered attention due to its detrimental effects on aquatic ecosystems, especially fish **(Bhandari *et al.*, 2015)**. Mainly, fish are the most affected aquatic vertebrates with chemical substances and toxins **(El-Garawani *et al.*, 2021)**.

Fish are the most influenced species by pollution because they absorb toxins via their skin and gills **(Alm-Eldeen *et al.*, 2018)** leading to disruption in the biochemical and physiological processes **(Afzal *et al.*, 2022)**. Prolonged exposure to water contaminants can alter the histology, biochemistry, and morphology of their tissues, even at small concentrations **(Haredi *et al.*, 2020)**. It is soundly established that aquatic creatures including fish subjected to various contaminants might generate reactive oxygen species (ROS) that damage cell macromolecules. On the other hand, fish have a tangled antioxidant system like catalase (CAT) that competes against the oxidative damage of the ROS **(Sayed & Soliman, 2018)**. Fish exposed to various contaminants can exhibit bioindicators of oxidative stress, such as the rise of lipid peroxidation and the generation of antioxidant enzymes that are further depleted **(Karami *et al.*, 2016)**. Additionally, fish play a remarkable part in the trophic chain and can accumulate toxic substances like BPA, even in small quantities; therefore they are implemented for monitoring contamination in the aquatic ecosystem **(Milla *et al.*, 2011)**. The Nile tilapia, *Oreochromis niloticus*, is ultimately considered the uppermost commercially cultured species in countless areas of the world, including Egypt, and it is a well-thought-out appropriate aquatic class to perform toxicological research **(Abdel-Tawwab & Hamed, 2018; Al-Awadhi *et al.*, 2024)**.

BPA is deemed as an endocrine-disrupting toxicant for vertebrates and invertebrates by disturbing the hormonal gesturing lane **(Qin *et al.*, 2021, Minaz *et al.*, 2023)**. It is a very hazardous compound as it affects both the larval stage as well as the adult stage **(Brown *et al.*, 2018)**. In addition, it increases the mortality rate and decreases the growth rate of fish **(Hanson *et al.*, 2014)**. Moreover, it disrupts the sexual differentiation in a broad spectrum of aquatic species **(Bhandari *et al.*, 2015)**. It also induces transgenerational effects via sperm epigenome **(Drobná *et al.*, 2018)**. Consequently, it has deleterious effects on fish reproduction. It negatively affects gamete quality and gonad development. Furthermore, it diminishes sperm quality and density, which are the main detrimental reproductive consequences in males, even at low BPA concentrations **(Faheem & Bhandari, 2021)**. Additionally, fish exposure to BPA is typically associated with alterations in DNA methylation throughout the entire genome. These alterations were more prevalent in pathways related to metabolism and the neurological system **(Qin *et al.*, 2021)**. Moreover, BPA alters the expression of many

genes during the developmental stages of fish organs, which is highly impacted via environmental and genetic insults **(Afzal *et al.*, 2022)**. Furthermore, BPA can significantly affect fish growth, morphology, metabolic parameters, genotoxicity, behavior, and histological structure **(Yaghoobi *et al.*, 2017; Faheem & Bhandari, 2021)**. Continuous acquaintance with BPA could also cause harm to fish's liver and kidneys, as indicated by a rise in creatinine levels **(Pal & Reddy, 2018)**. Fish exposed to BPA exhibited an H_2O_2 overproduction and accompanied immunosuppression. Additionally, BPA disrupts lipid metabolism, induces apoptosis and inflammatory response, and enhances oxidative damage in fish liver tissues. It was proved that liver tissues are the most affected organ by BPA **(Elizalde-Velázquez *et al.*, 2023)**.

Fish exposed to BPA suffered from oxidative stress in response to ROS **(Faheem & Lone, 2018)**. A change in the balance between antioxidants and oxidants results in an increment in ROS, leading to oxidative stress **(Kurutas, 2015)**. Fish antioxidant defense system includes CAT, and the lipid oxidation system includes malondialdehyde (MDA). They both serve as biomarkers for indicating the existence of contaminants. CAT breaks down hydrogen peroxide into water and molecular oxygen **(Hossain *et al.*, 2021)**. On the other hand, MDA is a marker of lipid oxidation that diminishes lipid peroxides **(Lykkesfeldt, 2007)**. Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) hold a decisive interplay in the pathogenesis of disease in fish by regulating their immune response. They are labial to undesirable stress circumstances and trigger inflammatory responses in the Nile tilapia **(Nájera-Martínez *et al.*, 2020)**. Consequently, TNF-α and IL-6 levels are significant bioindicators for assessing the pollution level in the aquatic environment **(Hossain *et al.*, 2021)**.

Food additives derived from plant extracts like grape seeds (GR) have gained significant attention as dietary supplements that enhance the fish's health status and growth **(Yang *et al.*, 2023)**. GR constitutes the highest concentration of bioactive substances. They retain about 30% of the total proanthocyanidins. They are known for their role in prohibiting lipid peroxidation, formation of H_2O_2, and protein oxidation ability **(Unusan, 2020)**. Moreover, they have potent antioxidant activity due to hydrogen donation's high affinity **(Zhai *et al.*, 2014)** and reducing ROS in the Nile tilapia **(Yang *et al.*, 2023)**. Consequently, the current research intends to explore the toxic influence of BPA and the protective role of dietary GR supplementation on the Nile tilapia by studying different biochemical, histopathological mutations, and gene expression analyses of cytokines such as TNF-α and IL-6.

MATERIALS AND METHODS

1. Experimental setup

The Nile tilapia (*Oreochromis niloticus*, n=120) were obtained from the Fish Research Center at Suez Canal University in Ismailia Governorate, Egypt, and transferred

to the laboratory. Fish were stocked for one week of acclimation before the commencement of the experiment. Fish weighing 14.95±0.10g were housed in 60L tanks, with 10 fish in each of the four groups (triplicates/group), as shown in Table (1). The water in the tanks was thoroughly replaced every day. The fish were given food 3 times per day. Every day, the tanks were cleaned by siphoning off feces and uneaten food. Each tank was aerated continuously by an air compressor that was centrally located and attached to an air stone. During the experimental duration, the temperature of water in tanks was 26.8–28.4ºC, and pH was 7.3–7.7.

Table 1. Experimental design lasted for 28 days

Experimental group (10 fish/group)	**Standard diet (0 mg GR Kg $^{-1}$)**	**400 mg GR Kg $^{-1}$**	**3 mg BPA L $^{-1}$**
Group 1: Control	+	–	–
Group 2: GR	–	+	–
Group 3: BPA	+	–	+
Group 4: GR+BPA	–	+	+

GR: Grape seeds extract, **BPA:** Bisphenol A.

2. Diet preparation and BPA dose

GR, which contains 95% pure proanthocyanidin (Hangzhou Jaymore Technology Company, China), was mixed with the food items to generate experimental meals at levels of 0 (normal diet) and 400mg/ kg (supplemented diet), which were previously determined by **Zhai *et al.* (2014)**. The dietary composition of the fish diet was formulated according to **El-Fahla *et al.* (2022)** method.

BPA (>99% purity) was obtained from (St. Louis, MO, USA) Sigma-Aldrich company. To create an effective dose of BPA (3mg/ L), a stock solution of 10mg BPA is dissolved in ethanol, as BPA is less soluble in water **(Abdel-Tawwab & Hamed, 2018)**.

3. Samples

At the experimental termination (day 28), all fish were starved for 12h. Fish were exposed to clove oil/ethanol (1:10) for anesthesia, and 0.5ml cardiac blood/fish were obtained from six fish/replication **(López-Cánovas *et al.*, 2020)**. Pooling was done to six samples into 3 samples to afford sufficient volume for different analyses. Blood clotting was allowed, and then centrifugation for 10min at 1000 ×*g* was performed. The separated sera were stored at –80°C to determine biochemical parameters (total protein, albumin, globulin, creatinine, urea, and IL-1β). Following that, the fish viscera were removed on

ice, and the liver was stored in –80°C freezer to analyze CAT and lipid peroxidation (MDA) **(Tuck *et al.*, 2009)**. Liver samples (6 tissues per group) were stored frozen in RNA later and then tested for mRNA expression IL-6 and TNF-α. The hepatopancreas, spleen, and kidney were taken for histological study.

4. Determination of serum total protein, albumin and globulin levels

Serum samples were implemented to gauge the levels of total protein, albumin, and globulin using commercial kits **(Henok *et al.*, 2020)**. Total protein concentrations were calculated using a specific Biuret kit (Cat. No. 41951, CliniChem Co., USA; detection range: up to 12.0g/ dL, protein concentration at 546nm, sensitivity: 0.018g/ dL). The albumin concentrations were calculated using a specific Biuret kit (Cat. No. 41253, CliniChem Co., USA; detection range: up to 69g/ l (6.90 g/dl), sensitivity: 0.06g/ L (0.006g/ dL)). Calculation of globulin level was done via the subtraction of serum albumin from the total protein.

5. Determination of serum urea and creatinine levels

Urea concentrations were evaluated by a specific fish urea kit (Cat. No. 46661, CliniChem Co., USA; detection range: 66.7mmol/ l (400mg/ dl), sensitivity: 0.009mmol/ L (0.05mg/ dl)) **(Mikkelsen, 1990)**. The creatinine concentration was measured using a fish kit (Cat. No. 41751, CliniChem Co., USA; detection range: 1326µmol/ L (15mg/ dL), sensitivity: 3.82µmol/ L (0.04mg/ dL)).

6. Determination of fish IL-1β concentration

Fish IL-1β in sera of different treatments was determined by a specific ELISA kit (Cat. No. CSB-E13259Fh, AFG Scientific Co., USA, sensitivity: less than 0.78pg/ mL, detection range: 3.12-200pg/ mL) pursuing the described method by **Liang *et al.* (2016)**.

7. Determination of MDA level and CAT activity

Hepatic tissue samples were subjected to homogenization in (20mM, pH 7.4, 4°C) pre-chilled phosphate buffer saline. The samples were then centrifuged (2000 ×*g*, 15min, 4°C), and then placement of the supernatant into micro tubes was done. MDA and CAT were assessed in the supernatant using commercial-specific fish kits.

MDA was measured using a specific kit (Cat. No. EK750261, AFG Scientific Co., USA, sensitivity: 0.312-20nmol/ mL, detection range: 0.312-20nmol/ mL) **(Nisimoto *et al.*, 2010)**. CAT activity was measured with a specific kit (Cat. No. SL0028FI, SunLong Co., USA, sensitivity: 2.5pg/ mL, detection range: 10–800pg/ mL).

8. Quantitative real-time PCR

Liver tissues were collected for RNA extraction. Extraction of the total RNA was done using the ABT Total RNA kit (Cat. No. ABT002, Applied Biotechnology, Egypt) following the manufacturer's recommendations. The isolated mRNA was subjected to

reverse transcription into cDNA using the ABT 2x RT Mix Oligo kit (Cat. No. AMP11, Applied Biotechnology, Egypt) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to estimate IL-6 and TNF-α expression levels using the Applied Biosystems, StepOne™ Real-Time PCR System. β-actin was cast off as a housekeeping gene. Primer sets with their accession number are demonstrated in Table (2). The qRT-PCR was conducted using the ABT 2x qPCR Mix (SYBR) high ROX kit (Cat. No. AMP04, Applied Biotechnology, Egypt). The thermal cycling conditions for the target primers were 45 cycles for 10min at 95°C, 95°C for 10s followed by 40s at 60°C, and finally 72°C for 40s. Melting curves were inspected to ensure the specificity of the amplification process. The fold expression was performed in triplicates for the target primer sequences. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method **(Livak & Schmittgen, 2001)**.

Table 2. Nile tilapia *Oreochromis niloticus* primer sequences for qRT-PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)	Accession number	References
IL-6	**GTCGCCTCCAGTGGTTACAA**	**GAAGTCCAGCACCTCTTGCT**	**XM_019360734.2**	**NCBI**
TNF-α	**AAGCCAAGGCAGCCATCCAT**	**TTGACCATTCCTCCACTCCAGA**	**NM_001279533.1**	**(Limbu *et al.*, 2018)**
β-actin	**AAACCCAAACAACTGGCTCG**	**CAGTTCTGATGGGCAGTGAGC**	**KJ126772**	**(Abu Almaaty *et al.*, 2019, El-kady et al., 2022)**

NCBI: National Center for Biotechnology Information

9. Histological study

The hepatopancreatic, spleen, and kidney tissues were removed from each fish, then immediately fixed in buffered formalin (10%) and preserved in 70% ethanol. Paraffin wax was applied to the preserved tissues, sliced at 4-5μm in thickness, and then subjected to hematoxylin and eosin staining **(Bancroft & Gamble, 2008)**. Finally, the stained sections were photographed using an Olympus microscope supplemented with a Digital Camera (14 MP USB) to examine the histological changes.

10. Statistical analysis

Statistical analysis was made using the SPSS 20 program for Windows. All data exist as mean ± SE. One-way ANOVA followed by post hoc Duncan's test was applied to evaluate the significance between control and treatment groups. *P* values equal to or lesser than 0.05 were considered statistically significant.

RESULTS

1. Effect of GR on serum protein levels in BPA-treated fish

Total protein and albumin levels exhibited a significant ($P< 0.05$) increase in a supplemented group with GR than the control group. In contrast, the BPA group showed decreased levels ($P< 0.05$) in all proteins. The BPA group supplemented with GR revealed levels higher than the levels of the BPA group (Table 3).

2. Effect of GR on serum creatinine and urea levels in BPA-treated fish

The concentrations of creatinine and urea were statistically high ($P< 0.05$) in the BPA group as matched to the remaining groups (Table 3). The supplement of GR to the diet of the BPA group significantly ($P< 0.05$) decreased these concentrations more than that of the BPA group. No differences in urea and creatinine levels between the control and GR groups.

3. Effect of GR on serum IL-1β level in BPA-treated fish

A concentration of 3mg BPA L^{-1} raised significantly ($P<0.05$) the concentrations of IL-1β in BPA tilapia sera. In the BPA+GR group, the concentrations were statistically ($P< 0.05$) diminished as compared to the BPA group. As shown in Table (3), the control and GR groups displayed no significant difference in the IL-1β levels.

Table 3. Effect of BPA exposure and GR as a treatment on serum biochemical parameters and IL-1β levels in the Nile tilapia after 28 days of the experiment

Group / Parameter	Control (0 mg BPA L^{-1} + 0 mg GR Kg^{-1})	400 mg GR Kg^{-1}	3 mg BPA L^{-1}	3 mg BPA L^{-1} 400 mg GR Kg^{-1}
Total protein (g/dL)	**4.11±0.03^{b}**	**4.27±0.02^{a}**	**2.95±0.03^{d}**	**3.64±0.02^{c}**
Albumin (g/dL)	**2.45±0.02^{b}**	**2.55±0.01^{a}**	**2.01±0.03^{d}**	**2.27±0.02^{c}**
Globulin (g/dL)	**1.66±0.01^{a}**	**1.57±0.01^{a}**	**0.94±0.02^{c}**	**1.53±0.01^{b}**
Creatinine (mg/dL)	**0.33±0.01^{c}**	**0.32±0.02^{c}**	**0.89±0.02^{a}**	**0.58±0.02^{b}**
Urea (mg/dL)	**11.35±0.16^{c}**	**11.34±0.12^{c}**	**14.62±0.11^{a}**	**12.94±0.09^{b}**
IL-1β (pg/mL)	**4.58±0.047^{c}**	**4.74±0.021^{c}**	**7.55±0.059^{a}**	**6.02±0.042^{b}**

Values expressed as mean ± SE (n = 6 per group). a, b, c and d letters denote that the corresponding means differ significantly at $P < 0.05$, according to the one-way ANOVA followed by Duncan's multiple tests. **GR:** Grape seeds extract, **BPA:** Bisphenol A, **IL-1β:** interleukin-1β.

4. Effect of GR on serum MDA level and CAT activity in BPA-treated fish

Table (4) concludes that the hepatic MDA contents were elevated statistically ($P< 0.05$) in the BPA group; on the contrary, adding GR to the diet of the BPA group lowered statistically ($P< 0.05$) the MDA levels. Dietary GR positively influenced the antioxidant status, as it increased statistically ($P< 0.05$) CAT activity in the BPA+GR group compared with the BPA group. There was no statistical variation between the control and GR groups in the content of MDA and CAT activities.

Table 4. Effect of BPA exposure and GR on MDA level and CAT activity in hepatic tissues of the Nile tilapia after 28 days of experiment

Group / **Parameter**	**Control (0 mg BPA L^{-1} + 0 mg GR Kg^{-1})**	**400 mg GR Kg^{-1}**	**3 mg BPA L^{-1}**	**3 mg BPA L^{-1} 400 mg GR Kg^{-1}**
CAT (pg/mg wet liver tissue)	**904.76±5.94^{a}**	**909.68±3.5^{a}**	**683.92±3.49^{c}**	**788.82±3.61^{b}**
MDA (pg/mg wet liver tissue)	**3.39±0.02^{c}**	**3.39±0.01^{c}**	**8.12±0.03^{a}**	**5.5±0.07^{b}**

Values expressed as mean ± SE (n = 6 per group). a, b and c letters denote that the corresponding means differ significantly at $P< 0.05$, according to the one-way ANOVA followed by Duncan's multiple tests.
GR: Grape seeds extract, **BPA:** Bisphenol A, **MDA**: Malondialdehyde, **CAT**: Catalase.

5. Effect of GR on hepatic IL-6 and TNF-α expression levels in BPA-treated fish

Our findings disclosed that the transcript levels of the proinflammatory cytokines varied among different treated groups. The results of the gene expression analysis were represented by log fold change of the target TNF-α and IL-6 (Fig. 1). Our results showed that the BPA demonstrated a statistical ($P< 0.05$) upregulation in the expression level of TNF-α compared with the control ones. Moreover, it significantly ($P < 0.0001$) reinforced the upregulation of the IL-6 transcript level compared with the control ones. In addition, the supplemented fish with GR revealed a statistical ($P< 0.05$) downregulation in the mRNA fold change expression level of TNF-α. In contrast, it revealed a non-significant change in the IL-6 when compared with the control ones. Notably, the supplementation of GR to the diet of the BPA group significantly ($P< 0.0001$) enhanced the downregulation of both transcript levels when matched with the BPA-intoxicated fish. Interestingly, the group supplemented with GR showed a significant ($P < 0.0001$) downregulation in the transcript levels of TNF-α and IL-6 compared to the BPA group.

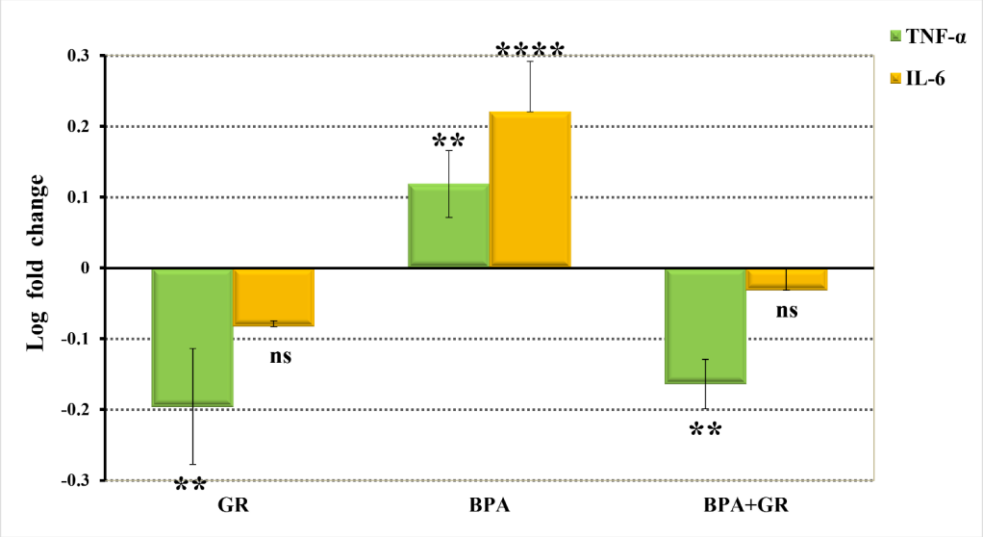

Fig. 1. Gene expression analysis of TNF-α and IL-6 in the hepatic tissue of the Nile tilapia (*Oreochromis niloticus*) represented by log fold change compared to the control group. It shows significant upregulation of the expressed genes in the group supplemented with BPA compared to the control ones. On the contrary, it shows significant downregulation in the transcript levels of TNF-α in the GR group and the BPA group supplemented with GR compared to the control ones. *, **, ***, and **** denote significant differences between groups at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$, respectively. **ns:** Non-significant

6. Effect of GR on histological alterations in BPA-treated fish

The hepatic tissues from both the control and GR-supplemented groups showed normal polygonal hepatocytes and glycogen stores cells arranged around vascular sinusoids with no signs of alterations (Fig. 2a, b). As depicted in Fig. (2c), 28 days of BPA toxicity caused hepatocyte abnormalities such as vacuolization, degenerative parenchyma, and karyolysis and karyopyknotic in hepatocyte nuclei. In addition, large-scale necrotic areas within the hepatic parenchyma were associated with hemorrhage, which was the most frequent lesion within this group. Adding GR extract to the diet of the BPA group enhanced the hepatic appearance to be histologically comparable to those of normal groups (Fig. 2d).

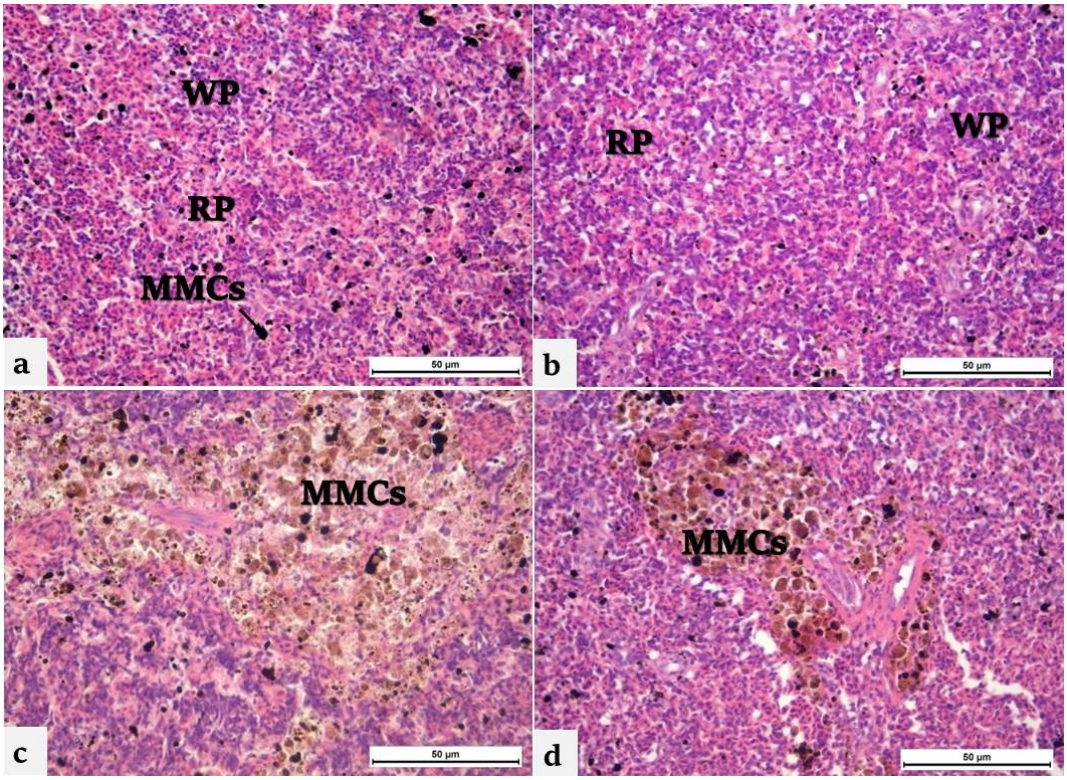

Fig. 2a–d. Histological sections of spleen stained with hematoxylin and eosin (x 400). **(a)** Control **(b)** GR-supplemented groups showed normal splenic parenchyma with white pulp (WP) and red pulp (RP) without distinct boundaries. **(c)** BPA-administered spleen revealed an increased area of melanomacrophage centers (MMCs) with deformities of splenic parenchyma. **(d)** Decreased area occluded with MMCs in BPA-administered spleen fed on GR extract

Splenic sections of both the control and GR-supplemented groups exhibited normal architecture, in which the parenchyma was observed to be arranged to a red and white pulp without a borderline between the two regions, as illustrated in **Fig. (3a, b)**. However, the splenic sections subjected to 3mg BPA L^{-1} revealed a larger melanomacrophage centers (MMCs) area than the other examined groups. Additionally, it was noted that the MMCs of the BPA group were spread in the whole splenic parenchyma and disorganized (Fig. 3c). A relatively smaller sized area of MMCs was noted in the group that was intoxicated with BPA and nourished on GR extract (Fig. 3d).

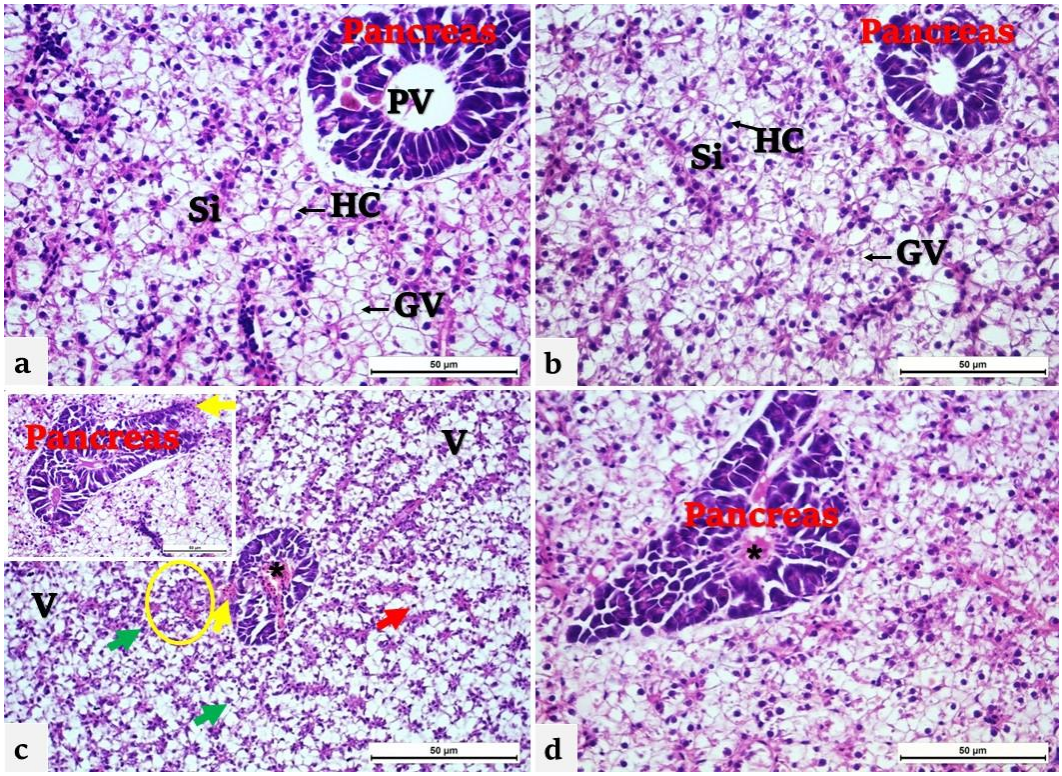

Fig. 3a–d. Histological sections of hepatopancreas (left lobe) stained with hematoxylin and eosin (x 400). **(a)** Control **(b)** GR-supplemented groups showed normal hepatic and pancreatic parenchyma with normal hepatocytes (HC), glycogen vacuoles (GV), and sinusoids (Si). **(c)** BPA-administered hepatic tissue revealed hepatocyte degeneration (vacuolization, V) with signs of karyopyknotic (red arrow) and karyolysis (green arrow) nuclei, necrosis (yellow circle) and hemorrhage (yellow arrow). Moreover, congestion (*) of the pancreatic vein can be seen (PV). **(d)** Supplementation of the BPA group with GR extract led to an improved appearance of the hepatic parenchyma, indicating retrogressive changes

No lesions were noticed in the kidneys of the control and GR-supplemented fish, in which the renal tissues exhibited normal hematopoietic tissues with a normal appearance of renal tubules and bowmen's capsule with well-defined glomerulus (Fig. 4a, b). After 28 days of exposure, kidney sections of the BPA-treated group displayed degenerated renal tubules, severely chief to the existence of necrotic tubules. Moreover, renal tubule atrophy was also noticed. Glomerular degeneration was distinguished, and renal blood vessel dilatation with congestion can also be seen in Fig. (4c). In contrast, a supplement of GR to the diet of the BPA-treated group improved the appearance of renal tissues, with some lesions still present (Fig. 4d).

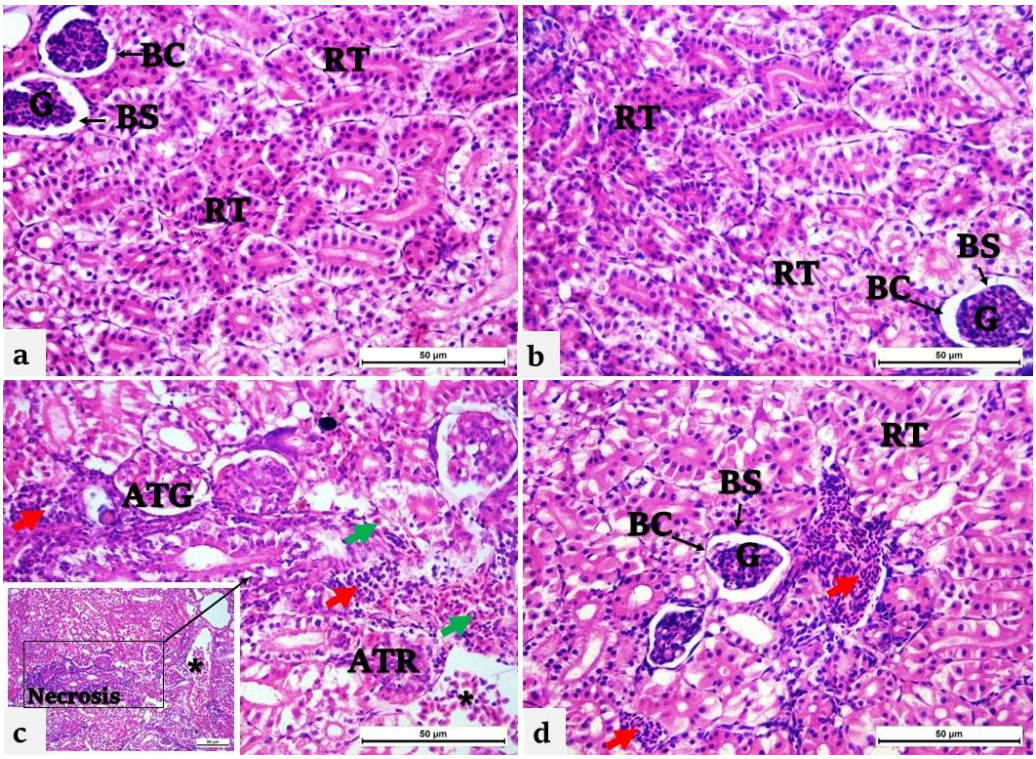

Fig. 4a–d. Histological sections of renal tissues stained with hematoxylin and eosin (x 400). **(a)** Control **(b)** GR-supplemented groups showed normal renal hematopoietic tissues, including Bowman's capsule (BC) with glomerulus (G) and Bowman space (BS), along with disorganized renal tubules (RT). **(c)** BPA-administered renal tissue, the magnified window revealed area included necrotic tubules, atrophy in the glomerulus (ATG) and renal tubules (ATR) with hemorrhage (green arrows), and aggregation of inflammatory cells (red arrows), necrosis (yellow circle) and hemorrhage (yellow arrow). Besides, congestion and dilatation (*) of the renal vein. **(d)** Supplementation of the BPA group with GR extract led to an improved appearance of the renal tissues; however, the detectable lesion of inflammatory cell aggregation was still seen

DISCUSSION

BPA is introduced into the water environment through sewage treatment wastewater, landfill leachate, or natural degradation and is hazardous to fish **(Wang *et al.*, 2019)**. Even though lethal dosages are rare in aqueous environments, it is well-established that low amounts of BPA significantly impact the growth, physiological state, morphology, and behavior of eggs, larvae, and fries **(Pastva *et al.*, 2001)**. On the other hand, GR contains some polyphenols (such as gallic acid, catechin, procyanidin, and epicatechin) that are considered influential antioxidants and promote fish immunity and upgrade beneficial intestinal bacteria populations **(Yilmaz & Toledo, 2004)**. The herein research assessed the possible ameliorative influence of GR extract against BPA toxicity.

Biochemical analysis is commonly used to assess fish's nutritional status, health, and adaptability to their environment **(Abdel-Tawwab *et al.*, 2018)**. In this research, fish subjected to BPA had lower serum albumin, total protein, and globulin levels in comparison with the control fish; these results were documented previously in the Nile tilapia after BPA exposure **(Abdel-Tawwab *et al.*, 2017)**. BPA-intoxicated fish may have depleted blood total protein, albumin, and globulin levels due to liver and renal failure **(Pal & Reddy, 2018)**. In the group that was supplemented with GR, the level of total protein and albumin increased significantly than the control; this means that it has a good effect against toxins, as reported in other studies **(Ali Rajput *et al.*, 2017, Mehrinakhi *et al.*, 2020)**. In the same vein, prior research by **Mehrinakhi *et al*. (2020)** detected that the supplement of 300mg GR Kg^{-1} in the common carp diet amplified all the protein profile levels.

Exposure to BPA resulted in elevated levels of creatinine and urea in comparison with the BPA-free groups, indicating that BPA poisoning caused significant kidney damage and degenerative processes. This is parallel to a previous research, which verified that changes in kidney and liver tissue due to exposure to BPA commanded increased levels of urea and creatinine **(Koriem, 2022)**. Urea and creatinine are commonly implemented to screen renal function and assess renal integrity structure **(Solomon, 2014)**. The addition of GR extract to the Nile tilapia diet reserved the urea and creatinine values at the same levels as the control group. Furthermore, GR supplementation to the BPA group lowered urea and creatinine values as compared with the BPA-intoxicated fish. This might be attributed to the antioxidant potential of GR to reserve the renal tissues from BPA-generated ROS, resulting in keeping the creatinine and urea within normal range. This suggestion is in accordance with the study of **Ulusoy *et al.* (2012)**, which confirmed that the administration of GR extract protected the renal tissues of rats from amikacin antibiotic toxicity and kept urea levels in normal values via enhancing antioxidants.

The IL-6 and IL-1β are pro-inflammatory cytokines that legalize inflammation, homeostasis, and cell growth. They are also linked to autologous immune metabolism **(Jiang & Li, 2022)**. In the current study, fish IL-1β has been raised after 28 days of BPA exposure; this result is harmonized with **Soliman *et al.* (2023)** study that confirmed microplastic exposure triggered IL-1β in the sera of the African catfish. Exposure to microplastics disrupted homeostasis and caused damage by pro-inflammatory cytokines in the catfish, as seen by elevated cytokine levels, a characteristic immunological retort in cellular damage and inflammation **(Wang *et al.*, 2022; Soliman *et al.*, 2023)**. In addition, they identified an elevation of IL-1β expression in the snake fish subjected to nano-microplastics. Once cellular homeostasis is disordered by stressors (i.e., tissue injuries), interleukins initiate instantaneous immunological responses counter to the stressor.

Extreme production of interleukin leads to pathological sequelae like severe immune disorders and systemic inflammatory response **(Jiang *et al.*, 2021)**.

Feeding the BPA group with GR lowered the level of IL-1β; this is inconsistent with research which found that procyanidin reduced the level of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α while upregulating expression of IL-10 as an anti-inflammatory cytokine **(Lu *et al.*, 2020)**. Thus, it is suggested that procyanidin may regulate fish immunity by inhibiting pro-inflammatory cytokine production, thereby balancing the fish's physiological status and health. These results are aligned with a previous study on the zebrafish: pre-incubation with GR extract reduced inflammatory responses and mortality in the infected zebrafish **(Kao *et al.*, 2010)**.

The study observed that BPA toxicity caused oxidative load by decreasing the echelons of CAT and increasing the levels of MDA. The same findings were described in *Cyprinus carpio* fish (common carp) after prolonged acquaintance with BPA **(Gu *et al.*, 2020)**. Enriching the diet of the BPA group within GR increased CAT levels and decreased MDA levels. Similar data were obtained in the zebrafish **(Hoseinifar *et al.*, 2023)**.

The current study was intended to inspect the toxic influence of the BPA and the protective role of the GR on the Nile tilapia by studying the expression of the TNF-α and IL-6 genes. These cytokines represent reliable bioindicators for assessing pollutants and chemical substances in the aquatic ecosystem. The current study indicated an upregulation in the transcript levels of the studied genes; these outcomes are in harmony with those of **Abd El Tawab *et al.* (2020)**, who confirmed a promotion in the level of the mRNA expression for TNF-α in the Nile tilapia upon exposure to pollutants **(Abd El Tawab *et al.*, 2020)**. Fish immunological system is triggered by declining water quality parameters and increasing environmental stress, consequently affecting the mRNA expression of certain biomarkers **(Ismail *et al.*, 2017, El-Mezayen *et al.*, 2018)**. Moreover, others stated that plastic materials increase oxidative stress, trigger ROS accumulation **(Hayati *et al.*, 2023)**, and enhance the proclamation of proinflammatory cytokines, among which TNF-α, resulting in activation of the immune system, causing inflammatory effects **(Weber *et al.*, 2022, Hayati *et al.*, 2023)**. Other previous studies also described the upregulation in the expression levels of interleukins upon exposing the Nile tilapia to environmental stress factors that lead to stimulating their immune system **(Abd El Tawab *et al.*, 2020)**. Moreover, a recent study agrees with our findings, and they explained that plastic materials increase neutrophils count and lead to liver inflammation in the zebrafish with increasing plastic particle concentration **(Cheng *et al.*, 2022)**. In line with our data, **Huang *et al.* (2020)** observed an elevation in the IL-6 mRNA level in the intestine of the juvenile guppy once exposed to microplastics **(Huang *et al.*, 2020)**.

On the opposite line, dietary supplementation with GR results in a downregulation in the mRNA IL-6 and TNF-α levels expression. **Hayati *et al.* (2023)** agree with our findings that the use of antioxidants leads to a reduction in the stress levels and the transcript level of the proinflammatory cytokine TNF-α **(Hayati *et al.*, 2023)**. Additionally, GR reduced the mRNA expression levels of TNF-α and IL-6 in *Ctenopharyngodon idella* (the grass carp) **(Quagliardi *et al.*, 2024)**. Indeed, GR was declared to have significant anti-inflammatory activity in the hepatic tissue, interposed by genes responsible for lipogenesis that affects the regulation of lipid metabolism and improves immunity **(Lu *et al.*, 2020**, **Quagliardi *et al.*, 2024)**. Consistent with the research of **Lu *et al.* (2020)**, it was elucidated that GR dietary supplementation diminishes the mRNA of the proinflammatory cytokines IL-6 and TNF-α when utilizing the GR in the diet of *Ctenopharyngodon idella* **(Lu *et al.*, 2020)**. Our findings can be explained by the fact that antioxidants like GR can conquer the oxidative stress caused by BPA.

The liver acts as the central regulator of fish metabolism and is highly sensitive to external chemical substances **(Roy & Bhattacharya, 2006)**. Acquaintance to pollutants that exist in the environment can lead to changes in the physiological characteristics and structure of fish liver **(Rashidian *et al.*, 2020)**. BPA induction of hepatic toxicity has been documented in numerous organisms, such as the zebrafish **(Marqueño *et al.*, 2021)**, mice **(Al-Griw *et al.*, 2023)**, and medaka **(Czarny-Krzymińska *et al.*, 2023)**. In the current study, the liver tissue showed deformities in the hepatocytes, such as vacuolation, karyopyknosis, and karyolysis, indicating that exposure to BPA results in liver injuries. These alterations were in line with those of a previous study that established the capability of BPA to tempt hepatic injury in marine medaka by inducing liver cell vacuolation and edema **(Li *et al.*, 2024)**. On the other side, the interruption of the hepatic antioxidant CAT balance may be connected to this spectacle, as confirmed by **Li *et al.* (2024)**. The exposure of the Nile tilapia to 3mg BPA L^{-1} in this study caused liver alterations that resulted in a reduction of hepatic CAT activity, leading to decreased levels of total protein, albumin, and globulin. This disturbance outline may specify that the antioxidative system of the tilapia loses its equilibrium after 4 weeks of exposure to BPA.

The fish spleen in various vertebrate species, including fish, has imperative immunological functions and is also subtle to pollutants **(David & Kartheek, 2015)**. One important physiological feature of the fish spleen is the existence of MMCs, which are the functional equivalents of the spleen's germinal centers **(Agius & Roberts, 2003)**. In the existing work, it was detected that BPA exposure could harshly restrict MMCs organization and area. MMCs serve as important biomarkers in toxicological assessments **(Steinel & Bolnick, 2017)**. Fish suffering from prolonged stressful conditions often display larger MMCs **(Ribeiro *et al.*, 2011)**. Stressful environmental conditions

frequently cause an animal's splenic MMC count to rise **(David & Kartheek, 2015)**, which is consistent with the current finding that numerous MMCs are found in the splenic sections of fish subjected to 3mg BPA L^{-1}. This increment in the MMCs area is linked to their part in immune response and detoxification; however, it might be associated with the damage caused by oxidant load **(Da Silva *et al.*, 2012, Mela *et al.*, 2013, Oliveira *et al.*, 2018)**. These are consistent with the present findings concerning the increased levels of IL-1β and MDA. On the contrary, an earlier study by **Pronina *et al.* (2014)** documented that the intensification in the MMC area could be related to its protecting function in dispensing the detrimental products of metabolic squalor.

Fish kidneys are crucial organs for excretion, osmoregulation, and hematopoiesis **(Cengiz, 2006)**. Toxicants enter the kidney through the bloodstream **(Sanz *et al.*, 2008)**, several research confirmed that kidneys are the primary sites affected by phenolic toxicants **(Faheem *et al.*, 2016, Smorodinskaya *et al.*, 2023, Waris *et al.*, 2023)**. This is in accordance with the current findings; compared to the other studied tissues, the most deleterious abrasions were noticed in the kidneys and resulted in renal failure. In the present study, the kidney of the BPA group displayed degeneration and necrosis of renal tubules, glomerulus expansion or atrophies, aggregation of lymphocytes, and hemorrhage within the hematopoietic tissues. In addition, severe dilatation of renal vessels with hemolysis of endothelium was also observed. These findings led to kidney failure and, thus, were supported by the current elevation of urea and creatinine levels in sera of BPA-intoxicated tilapias. Similar alterations were shown in the kidneys of different fish species when exposed to different levels of BPA **(Faheem *et al.*, 2016, Pal & Reddy, 2018)**.

Environmental stress leads to the accumulation of ROS and oxidative stress in fish **(Gu *et al.*, 2020)**. Promoting the antioxidative capacity is a viable method to advance fish health and maintain a balanced physiological status. GR extract, a natural antioxidant, has been reported to enhance fish growth and immunity by enhancing antioxidative status **(Xu *et al.*, 2022, Yang *et al.*, 2023)**. Similar to later studies, dietary GR statistically increased the antioxidative CAT in the current work, thereby dropping the levels of ROS (i.e., MDA) in the hepatic tissue of the Nile tilapia. These supported the improved appearance of hepatic, splenic, and renal tissues in the BPA group feed diet enriched with GR, owing to the antioxidant effect of GR.

CONCLUSION

Subjecting BPA at 3mg L^{-1} concentration caused toxicity in the physiologic status of the tilapias and histologically altered the normal features of the liver, spleen, and kidneys. BPA exposure validates the alterations in biochemical parameters of total protein, albumin, globulin, and IL-1β. Additionally, it caused a reduction in CAT activities and an increment in MDA levels, ultimately impacting the overall health of the

fish. These impacts of BPA promoted necrosis in the hepatic tissues and had an interference influence on the spleen, as observed by perturbation in the MMCs area. In addition, it triggers kidney failure. Furthermore, BPA causes upregulation in the mRNA transcript echelons of the proinflammatory cytokines TNF-α and IL-6, which may augment inflammation, while GR supplementation downregulates them. Consequently, dietary GR ameliorates the negative impacts of BPA exposure via the activation of the antioxidant pathway. Overall, GR in the diet is an auspicious natural feed additive that could ameliorate the toxic effects of toxicants that are frequently exposed to via the applied GR in an optimum concentration within the diet.

ETHICAL APPROVAL

The study design was approved by the Scientific Research Ethics Committee (SCU-VET 2024033) at the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

DECLARATION OF COMPETING INTEREST

The authors claim that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

Conceptualization, R.M.A., H.M.A.A; Methodology, R.M.A., H.M.A.A, A.S.F, H.A.A.M., N.M.T., N.A.E.; Formal analysis, R.M.A., H.M.A.A., A.S.F, N.M.T., N.A.E; Investigation, R.M.A., H.M.A.A., N.M.T., N.A.E.; Resources, R.M.A., H.M.A.A., A.S.F, H.A.A.M., N.M.T., N.A.E.; Writing- original draft preparation, N.M.T., N.A.E.; Writing-review and Editing, R.M.A., H.M.A.A., H.A.A.M., N.M.T., N.A.E., all authors have read and agreed to the published version of the manuscript.

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تحليل التعبير الجيني والتغيرات البيوكيميائية والنسيجية المرضية في البلطي النيلى المعرض لثنائي الفينول أ:

الدور الوقائي للبروانثوسيانيدين

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أحد الملوثات الرئيسية في النظم الايكولوجية المائية هو ثنائي الفينول أ، وهو مادة كيميائية مصنعة تستخدم على نطاق واسع في إنتاج بلاستيك البولي كربونات وراتنجات الايبوكسي ولها آثار سلبية على صحة الأسماك. البروانثوسيانيدين هو مستخلص طبيعي ومنشط مناعي يتم الحصول عليه من بذور العنب وله مجموعة متنوعة من الخصائص البيولوجية ومضادات الأكسدة. تهدف الدراسة إلى التقليل من التأثيرات الضارة لثنائي الفينول أ عن طريق إثراء النظام الغذائي للأسماك بالبروانثوسيانيدين. تم تقسيم 120 سمكة بلطي نيلي صحية بمتوسط وزن قدره 0.10±14.95 جم إلى أربع مجموعات متساوية. كانت المجموعة الأولى هي المجموعة الضابطة، والمجموعة الثانية تم تغذيتها على 400 مجم من بذور العنب، وتعرضت المجموعة الثالثة لـ 3 مجم من ثنائي الفينول أ مع نظام غذائي خال من البروانثوسيانيدين بينما تعرضت المجموعة الرابعة لـ 3 مجم من ثنائي الفينول وتغذت على نظام غذائي مكمل 400 مجم من بذور العنب. بعد 28 يوما من التجربة، تم الحصول على عينات الدم والأنسجة لتحليل الدلالات الكيميائية الحيوية في الدم والسيتوكينات، ونشاط الكتاليز، والمالونديالدهيد، والسمات النسيجية للكبد والطحال والكلى. أدى العلاج بالبروانثوسيانيدين إلى تحسين مستويات البروتين في المصل وكذلك مستويات اليوريا، ومستويات الكرياتينين الذين تأثروا بالتعرض لثنائي الفينول أ. وفي الوقت نفسه، ارتفعت مستويات إنترلوكين-1β في المصل والمالونديالدهيد بسبب التعرض لثنائي الفينول أ وانخفضت مستوياتهم بسبب تناول البروانثوسيانيدين. بالإضافة إلى ذلك، انخفضت أنشطة الكتاليز للمجموعة الثالثة من الأسماك، بينما زادت للمجموعة الرابعة. علاوة على ذلك، فقد زادت بشكل كبير مستويات نسخ الحمض النووي الريبوزى الرسول لكلا من عامل نخر الورم الفا وإنترلوكين-6 نتيجة التعرض لثنائي الفينول أ، مما يشير ربما إلى تفاعلات التهابية، بينما انخفضت مستوياتهم عند إضافة البروانثوسيانيدين. ولوحظ أيضا أن تناول بذور العنب أدى الى تحسين البنية النسيجية للأنسجة التي تم فحصها. علاوة على ذلك، أدى تناول البروانثوسيانيدين إلى تحسين السمات الكيميائية الحيوية والنسيجية للأسماك والتخفيف من سمية البروانثوسيانيدين.

الكلمات الدالة: بروانثوسيانيدين، إنترلوكين-1β، إنترلوكين-6، عامل نخر الورم الفا، تفاعل البوليميراز المتسلسل الكمي، الكبد، الطحال، الكلى