In vitro effects of bisphenol F on antioxidant system indicators in the isolated hepatocytes of rainbow trout (Oncorhyncus mykiss)

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Abstract
Bisphenol F (BPF) has been used frequently in the plastics industry and the production of daily consumer products as an alternative to bisphenol A (BPA). It was aimed herein to determine the cytotoxic effects of BPF on hepatocytes isolated from the liver of rainbow trout (Oncorhyncus mykiss) using lactate dehydrogenase (LDH) assay and antioxidant defence system indicators. The cultured hepatocytes were exposed to seven concentrations (0, 15.63, 31.25, 62.50, 125, 250, and 500 µM) of BPF for 24 h. According to the LDH assay, the percentage of cytotoxicity was increased dose dependently in the cells. The malondialdehyde content, which is indicative of lipid peroxidation, was increased significantly at BPF concentrations between 15.63 and 250 µM, whereas it remained unchanged with a concentration of 500 µM. The activities of superoxide dismutase were increased, while those of catalase were decreased with all of the BPF concentrations. Elevated levels of reduced glutathione content were determined with BPF concentrations between 15.63 and 250 µM, but decreased significantly with a concentration of 500 µM. Significant increases in the activities of the glutathione peroxidase were found in hepatocytes treated with BPF at concentrations of 31.25 to 500 µM. GST activity was only significantly increased with a BPF concentration of 250 µM. The results showed that the toxic mechanism of BPF was mainly based on cell membrane damage and oxidative stress, which have an influence on antioxidant defences. Therefore, BPF should be reconsidered as a safe alternative instead of BPA in the manufacturing of industrial or daily products.

Keywords Bisphenol F · Oncorhyncus mykiss · Cultured fish hepatocytes · Cytotoxicity · Antioxidant system indicators

Introduction
Bisphenols are a chemical class known as diphenylmethanes that contain two hydroxyphenyl groups. Among this chemical group, bisphenol A (BPA) is the most commonly used chemical in the plastics industry and consumer product manufacturing. Nowadays, it is well-known from scientific reports over the last two decades that BPA is an endocrine-disrupting chemical that possesses suspected harmful effects on human and environmental health, such as carcinogenesis, obesity, diabetes, and behavioural, developmental, and reproductive abnormalities. Therefore, scientists and authorities have instructed manufacturers to restrict the utilization of BPA as they search for safer alternatives [1]. Eventually, a gradual replacement of BPA along with its analogues or derivatives, such as bisphenol AF (BPAF), bisphenol F (BPF), bisphenol S (BPS), tetrabromobisphenol A, and tetrachlorobisphenol A was found. Due to its more biodegradable features under anaerobic and aerobic conditions, and because it has lower environmental risks when compared to BPA, BPF has been frequently preferred in epoxy resin, plastic, coating, varnish, dental sealant, and food packaging production. On the other hand, BPF has been found in the paper, personal care, and food products used in daily life [2]. It has also been determined at an average concentration of 0.054 µg/g in household dust [3], and in surface waters, sediments, and effluents from sewage [4]. Recent studies have reported that BPA analogues, including BPF, have caused developmental abnormalities in the reproductive tissues of male rats, altering their hormonal balance and antioxidant defences [5]. Similar to BPA, the genotoxic, immunotoxic, and neurotoxic potentials of its
analogs have been reported in some experimental studies over last decade [6–8].

Fish constitute an important ring of the food chain for humans and other higher organisms, and they might be at risk of exposure to BPA analogues, which are distributed widely in aquatic environments. The detection of BPF was reported in the muscle and liver tissues of some species from the North East Atlantic Ocean [9]. Recent experimental studies have shown that BPF led to different types of harmful effects in fish. For example, it was reported that BPF altered gonad histology in zebrafish (*Danio rerio*) and impaired hormonal balance by changing the gene expression along the hypothalamic–pituitary–gonadal axis [10]. BPF has also led to the developmental defects, such as decreased heart rate, inhibited spontaneous movement, and spinal deformation, by downregulating the related genes in zebrafish at environmentally-relevant concentrations [11]. BPF has been reported to change the transcription of genes related to thyroid development, hormone transport and metabolism, and impaired thyroid hormone balance and function in developing zebrafish embryos/larvae [12]. Hence, BPF is a suspected alternative to BPA, although further studies are necessary to discern its hazardous impacts due to the limited data available in the literature.

Environmental pollutants are capable of increasing the levels of reactive oxygen species (ROS) in cells, which results in oxidative stress. As a status of the imbalance between antioxidant defences and the generation of ROS, oxidative stress causes deleterious effects that are damaging to cellular membrane lipids, proteins, or DNA, and consequently, a loss of function or death occurs in the cells. Lipid peroxidation is a significant indicator of oxidative damage that can be induced by environmental compounds [13]. Fish possess antioxidant defence systems, both enzymatic and nonenzymatic, that help to protect them against oxidative stress and respond to environmental pollutants via antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), and superoxide dismutase (SOD), as well as nonenzymatic enzymes, such as glutathione (GSH). These mentioned members of the antioxidant defence system have been frequently selected as oxidative stress biomarkers that are related to xenobiotics in fish [14]. Toxicity studies in in vitro have shown that BPF increased ROS levels, induced lipid peroxidation, and modified antioxidant enzyme activity in different cell types, such as human erythrocytes [15], and RWPE-1 cells [16].

Isolated fish hepatocytes have been used as a suitable model or screening tool to analyse the cytotoxic and oxidative stress-inducing potential of environmental chemicals [17]. Rainbow trout (*Oncorhyncus mykiss*) is a test animal that has been recommended by the Organisation for Economic Co-operation and Development in toxicological studies for testing chemicals [18]. As far as is known, to date, no studies have been performed to assess the cytotoxic and oxidative stress impacts of BPF, which is an analogue of BPA, using isolated rainbow trout hepatocytes. The aim herein was to evaluate the effects of BPF on selected antioxidant defence system indicators of oxidative stress, such as the GSH content and malondialdehyde (MDA) levels, as an indicator of lipid peroxidation, as well as antioxidant enzyme activities, such as SOD, CAT, GPx, and GST.

**Material and methods**

**Fish**

The fish used in this study comprised 3 female, juvenile rainbow trout (*Oncorhyncus mykiss*), weighing approximately 300 g, which were purchased from a rainbow trout farm in Van, Turkey. The fish were anesthetized with 2-phenoxethanol (0.32 mL/L) before hepatocyte isolation.

**Preparation of the isolated hepatocytes**

Isolation of the hepatocytes from the fish was performed according to the method of Mortensen et al. [19], with some modifications. All instruments and solutions were sterilized prior to the procedures. In brief, the livers of the fish were aseptically dissected and placed into a Petri dish that contained cold Ca²⁺-free solution-I, comprising 7.14 g/L of NaCl, 0.36 g/L of KCl, 0.15 g/L of MgSO₄, 1.6 g/L of Na₂HPO₄, 0.4 g/L of Na₂HPO₄, 0.31 g/L of NaHCO₃, and 20 ml/L of ethylene glycol tetraacetic acid (EGTA). While in this solution, the liver samples were cut into very small pieces, and cleared of blood and rude tissue components, such as vessels and connective tissue. Then, the liver was minced into pieces and mechanically dissociated into further small pieces using fine forceps and surgical blades. This process was repeated and continued, all while the samples were in the clear solution-I. Next, the whitened pieces of tissue were transferred into solution-II, which possessed the same compounds found in solution-I; however, rather than EGTA, included were 0.11 g/L of CaCl₂, and 0.025 mg/mL of Type IV collagenase, to break the Ca²⁺-dependent cell–cell connections and enzymatic dissociation of the cells. After softening of the tissue pieces in solution-II for 10 min, trituration was performed using different sized pipette tips. The crude particles were removed by means of a stainless-steel sieve and then the obtained cell suspension was passed carefully through a sterilized injector needle for further cell dissociation. The suspension was then transferred into Eppendorf tubes and centrifuged for 3 min at 60xg. The cell pellet was resuspended in Leibovitz 15 (L-15) medium that comprised 1% (v/v) antibiotic–antimycotic and 0.38 g/L of NaHCO₃. After the second wash with L-15, the cells were suspended in the medium. Next, a Thoma slide was
used to count the cells, and the Trypan blue exclusion test was used to assess cell viability. For the cell culture studies, observation of the cells was performed to possess more than 90% viability.

**Hepatocyte culture and BPF treatment**

The isolated hepatocytes were seeded into 24-well collagen-I coated culture plates (Gibco, Catalogue No: A11428-02, Thermo Fisher Scientific, MA, USA) within 1 mL of medium that contained a density of $5 \times 10^5$ cells in each well. A temperature of 14 °C was used to maintain the cells for 24 h in a sterile incubator (Binder, Tuttingen, Germany), under atmospheric air, with saturated humidity, before BPF treatment. A stock solution of 0.1 M of BPF (Sigma-Aldrich, MO, USA; CH$_2$(C$_6$H$_4$OH)$_2$, MW: 200.23, analytical standard) was prepared in absolute ethanol and exposure media that contained BPF concentrations of 15.63, 31.25, 62.50, 125, 250, and 500 µM were applied to the cells for 24 h. The final ethanol concentration did not exceed 0.5% in the media. Cells in the control group received L-15 medium that contained only 0.5% absolute ethanol. For the experimental groups, 5 replicate wells were constructed. The cell cultures were examined using a Leica DMI 6100B inverted microscope (Wetzlar, Germany).

**Lactate dehydrogenase cytotoxicity test**

Measurement of the lactate dehydrogenase (LDH) leakage into the culture media was used to determine the cytotoxic effects of the BPF after 24 h of treatment. After completion of the treatments, removal of the culture media samples from the wells was performed, which were then placed into Eppendorf tubes. Next, the samples (10 µL) were placed into a 96-well plate and an Abcam LDH-cytotoxicity assay kit (Cambridge, UK; Catalogue No: ab65393) was used to assay the LDH activity, following the manufacturer’s instructions. Finally, an ELISA DAS A3 plate reader (Rome, Italy) was used to measure the optic density (OD) values at 450 nm. For each experiment, five replicates were performed. All of the measurements were performed in duplicate, and the percentage of cytotoxicity was calculated for each sample using the formula provided in the booklet that came with the kit, as follows:

$$\text{Cytotoxicity (\%)} = \frac{(\text{OD of test sample} - \text{OD of low control well})}{(\text{OD of high control well} - \text{OD of low control well})} \times 100$$

**Measurement of the antioxidant defence indicators**

Following completion of the exposures, the culture media was gently pipetted out of the wells and 1 mL of ice-cold phosphate buffered saline, at a pH of 7.4, was used to rinse the hepatocytes. The cell suspensions obtained ($5 \times 10^5$/mL) were then placed into Eppendorf tubes and a Jencons glass-porcelain ultrasonic homogenizer (Herts, UK) was used to lyse them. Centrifugation of the homogenate was then performed for 15 min at 20,000×g. All of the procedures were performed on ice or at 4 °C. Next, the removal of the supernatant fractions was performed and they were then used for the determination of antioxidant defence indicators. For each experiment, five replicates were performed.

Measurement of the malondialdehyde (MDA) content, which is the result of lipid peroxidation, was performed at 532 nm, spectrophotometrically, using the method proposed by Jain et al., which was based on thiobarbituric acid reactivity [20]. The results were presented as nmol/5 × 10^5 cells.

The superoxide dismutase (SOD) activity was performed spectrophotometrically using a Ransel commercial kit (Randox Laboratories Ltd., UK), following the manufacturer’s instructions. The activity was presented as unit/5 × 10^5 cells. The catalase (CAT) activity was measured spectrophotometrically using the method reported by Aebi [21]. The results were expressed as µmole of consumed H$_2$O$_2$/min/5 × 10^5 cells. The reduced glutathione (GSH) content was assayed at 412 nm spectrophotometrically, according to the method of Beutler [22]. The results were presented as µmole/5 × 10^5 cells. The glutathione peroxidase (GPx) activity was performed using a Ransel commercial kit (Randox Laboratories Ltd.) at 37 °C and 340 nm, based on the manufacturer’s instructions. The results were presented as unit/5 × 10^5 cells.

Measurement of the glutathione S-transferase (GST) activity was performed using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate [23]. The activity of the GST was presented as nmol of CDNB-glutathione conjugate/min/5 × 10^5 cells.

**Statistical analysis**

SPSS Statistics for Windows 20.0 (IBM SPSS Statistics Inc., Chicago, IL, USA) was used to perform the statistical analyses. To analyse the differences among the groups, one-way ANOVA and the Duncan multiple comparison post-hoc test was used. The results were presented as the mean ± standard deviation (SD). Statistical significance was accepted as $P < 0.05$.

**Results**

The microscopic examination of the cells after completion of the trials showed that the control hepatocytes formed groups or cord-like arrangements possessed a healthy appearance (Fig. 1a). The treated hepatocytes displayed degenerative
changes and less healthy appearance when compared to the control cells (Fig. 1b).

All of the concentrations of BPF caused significantly increased leakage of the LDH into the media. The percentage of cellular cytotoxicity gradually increased with the BPF concentrations (P < 0.05). Cytotoxicity was observed with BPF concentrations of 7.27 ± 0.99%, 12.17 ± 0.87%, 16.58 ± 1.60%, 23.43 ± 3.39%, 29.39 ± 2.35%, and 32.55 ± 2.47% at 15.63, 31.25, 62.50, 125, 250, and 500 µM, respectively (Fig. 2).

The MDA content was significantly elevated with BPF concentrations of 15.63, 31.25, 62.50, 125, 250, and 500 µM; however, it did not change with 500 µM after 24 h-exposure to BPF (Fig. 3a). The hepatocyte SOD activity displayed significant increases (P < 0.05) (Fig. 3b), while the CAT activity was reduced significantly (P < 0.05) (Fig. 3c) with all of the concentrations of BPF. There were significant increases in the content of GSH with BPF concentrations of 15.63, 31.25, 62.50, 125, and 250 µM (P < 0.05); however, the GSH contents were significantly decreased with 500 µM of BPF (P < 0.05) (Fig. 3d). GPx activity was significantly increased with BPF concentration of 31.25, 62.50, 125, 250 and 500 µM (P < 0.05), while there was no significant change in the 15.63 µM concentration (Fig. 3e). GST activity was only significantly increased at the 125 µM concentrations of BPF (P < 0.05), while it did not change with the other concentrations (Fig. 3f).

**Fig. 1** Representative photomicrographs of the cultured hepatocytes of rainbow trout. a Healthy control cells forming groups. b Cells exposed to 500 µM of BPF for 24 h that displaying degenerative changes (arrows)

**Fig. 2** Cytotoxic effect of various concentrations of BPF on the cultured hepatocytes of rainbow trout after 24 h of exposure. Statistically significant differences between the columns are indicated by different letters

![Lactate Dehydrogenase (LDH) Activity](image)
Discussion

The toxic and endocrine potential of BPA have been well-studied; however, knowledge regarding its analogue, BPF, is limited, especially in aquatic organisms. BPF is frequently preferred in the manufacturing of industrial and daily products instead of BPA, and its presence has been widely detected in aquatic compartments. Fish, which are an important part of the food chain, are at risk of exposure to environmental chemicals via their surrounding environment, and thus, it is necessary to investigate and elucidate the toxic impacts of BPF and its underlying mechanisms in fish. With this aim, we first investigated herein the antioxidant indicators in primary cultured rainbow trout hepatocytes that had been treated with BPF for 24 h.

The LDH cytotoxicity test demonstrated that BPF exposure affected the cells in a dose-dependent manner and the percentage of cytotoxicity was observed to be 32.55 with the highest concentration of BPF. Several studies of different cell types have reported that BPF-induced cytotoxicity increased with increased concentrations and treatment times. For example, cytotoxicity was increased on a dose-dependent basis in human cell lines (HCLs), such as hepatoma HCL, HepG2; intestinal HCL, LS174T; and renal HCL, ACHN, treated with BPF concentrations ranging from 5 to 100 µM for 24 h [24]. Similarly, BPF-induced
cytotoxicity was reported with concentrations of 0–600 µM for 24 h in RWPE-1 cells [16]. Russo et al. reported that mouse embryo fibroblast cells and cancer cells exposed to BPF (0–300 µM for 48 h) resulted in increased cytotoxicity in a dose-dependent manner [25]. Jambor et al. [26] also determined escalated cytotoxic activity in mouse TM3 Leydig cells treated with BPF concentrations of 10–50 µg/mL for 24 h. On the other hand, BPF did not induce cytotoxicity, even at a concentration of 200 µM for 24 h, in the human adrenal carcinoma cell line (H295R), but was found to be cytotoxic at concentrations of 300 and 500 µM [27]. No significant changes in cell viability were observed in the human hepatoma cell line (HepG2) exposed to BPF concentrations of 12.5–100 µmol/L for 24 h [28]. Moreover, Hercog et al. [6] reported that no significant variations were observed in the viability of human hepatoma cells (HepG2) with BPF concentrations of 2.5 and 20 µg/mL for 24 h, while they determined significantly decreased cell viability at the highest BPF concentration for 72 h. Thus, the similar or different findings regarding the cytotoxic impact of BPF on cells from past studies to the current study might have resulted from the cell type used, experimental design, treatment time, or selected assays for the determination of cytotoxicity.

MDA is an end-product of lipid peroxidation and a commonly used assay for monitoring membrane damage or oxidative stress [14]. It has been reported that BPF enhances ROS levels and leads to increased levels of lipid peroxidation in human erythrocytes [15]. In another study, BPF resulted in a high level of MDA contents and finally led to apoptosis in the larvae of zebrafish after waterborne exposure to BPF [7]. Ullah et al. [5] also reported significant changes in cell viability were observed in the human hepatoma cell line (HepG2) exposed to BPF concentrations of 12.5–100 µmol/L for 24 h [28]. Moreover, Hercog et al. [6] reported that no significant variations were observed in the viability of human hepatoma cells (HepG2) with BPF concentrations of 2.5 and 20 µg/mL for 24 h, while they determined significantly decreased cell viability at the highest BPF concentration for 72 h. Thus, the similar or different findings regarding the cytotoxic impact of BPF on cells from past studies to the current study might have resulted from the cell type used, experimental design, treatment time, or selected assays for the determination of cytotoxicity.

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The current study results showed that 24-h exposure of the isolated hepatocytes to BPF affected enzymatic scavengers, including SOD, CAT, GPx, and GST, which were also utilized as biomarkers for a study of environmental toxicity in fish by van der Oost et al. [34]. CAT and SOD act as the first-line of defence against attacks by ROS in cells. Dismutation superoxide anion radicals are catalysed by SOD into hydrogen peroxide and molecular oxygen. In the present study, SOD activities were significantly increased with all of the BPF concentrations when compared to the control group, presumably in response to enhanced cellular superoxide anion radicals. In accordance with the present study, in vitro studies using fish hepatocytes have also reported increased SOD activities after exposure to different types of environmental chemicals, such as BPA [35], perfluorinated organic compounds [29], di(2-ethylhexyl) phthalate [36], and benzo[a]pyrene and nonylphenol [37]. On the other hand, no significant changes were seen in the SOD activity of juvenile common carp liver (Cyprinus carpio) samples after long-term (60 days) waterborne exposure to BPF [38]. Conversely, Gu et al. [7] stated decreased SOD activities in the larvae of zebrafish treated with BPF concentrations of 7–700 µg/L for 3 and 6 days. It can be said that the different findings regarding the SOD activities as a consequence of BPF exposure might have depended on the applied BPF concentration, and design and duration of the experiment. CAT degrades hydrogen peroxide, a hydroxyl radical precursor that results from SOD activity, into water and oxygen and protects unsaturated fatty acids on the cell membrane from peroxidation [14]. In the current study, exposure of hepatocytes to BPF resulted in significantly decreased CAT activity. Similar to these findings, Maćczak et al. found decreased levels of CAT in human erythrocytes treated with BPF for different periods of time (4 h and 24 h) [15]. Decreased CAT activity was also found in juvenile common carp [37], and the larvae of zebrafish after waterborne exposure to BPF [7]. Ullah et al., also reported that CAT activity was inhibited in the testicular tissues of rats exposed to BPF via drinking water [5]. Modesto and Martinez reported that many antioxidant enzymes might be inactivated by an excessive increase in oxidants or the substrate of the enzyme could be an oxidant [39]. Thus, the decreased CAT activity could be explained by accelerated SOD activity as a consequence of the excessive production of superoxide anion radicals,
which then lead to higher intracellular hydrogen peroxide generation [40, 41]. In accordance with the results herein, decreased CAT activity with increased SOD activity have been also found in the liver of goldfish (Carassius auratus) exposed to subacute concentrations of nickel [42], and liver samples of Nile tilapia (Oreochromis niloticus) collected from polluted waters [43].

GSH, a tripeptide that contains cysteine, g-glutamine, and glycine, is an antioxidant molecule that directly metabolizes and detoxifies xenobiotics that conjugate directly, in addition to protecting cells from oxidative damage. Both increases and decreases of the molecule are indicative of oxidative stress [44]. GSH levels in this study were observed to be increase in the hepatocytes with concentrations that ranged between 15.63 and 250 µM; however, a significant decrease in the GSH content was determined with 500 µM of BPF. Similar to the results herein, Kose et al. reported elevated GSH levels in RWPE-1 cells that were treated with BPA, as well as its analogues, BPF and BPS, for 24 h [16]. In another study, Maćczak et al. [15] found depleted GSH levels in human erythrocytes exposed to BPF. GSH levels might increase under slight oxidative stress, as an adaptive mechanism to counteract ROS attacks; however, severe oxidative stress could lead to the depletion of the GSH contents as a consequence of the disruption of adaptive mechanisms [45]. Thus, the decrease in the GSH level with 500 µM of BPF was probably due to the severity of the oxidative stress that occurred with such a high concentration.

GPx activity was found to increase remarkably with all of the BPF concentrations, with the exception of 15 µM. GPx is an antioxidant scavenging enzyme that catalyses the degradation of hydroperoxides into hydroxyl compounds, utilizing GSH as a cofactor. The activity of GPx is closely related with its cofactor, GSH, and a reduction of the molecule might cause a decrease in the activity of the enzyme in cells [46]. Thus, a probable increase in the levels of lipid hydroperoxides and hydroxyperoxides in the hepatocytes after BPF exposure could lead higher GPx activities, in an attempt to cope with the oxidative insult of the BPF. In parallel with the literature knowledge, synchronized increases in GPx activity with GSH levels were observed in the current study; however, an unchanged or decreased level of the enzyme did not occur, even though a significant drop was detected in the GSH level with the highest concentration of BPF. The unaffected levels of GPx activity at this concentration may have resulted because, even if a decrease in the GSH pool occurred with this concentration, the level of GSH was still adequate for the utilization of the enzyme. In support of this explanation, decreases in GSH levels were observed in human erythrocytes with minimal exposure to BPF (4 h), while decreased activities of GPx appeared after 24 h of exposure, depending on the BPF concentration applied [14].

GST is a phase-II detoxifying enzyme that has a critical role in cellular protection against ROS and toxic xenobiotics. GST catalyses GSH conjugation in reaction to endogenous and exogenous electrophiles [47]. In the study herein, significantly increased GST activity was observed with 125 µM of BPF, while it remained unchanged with the other concentrations. Previous studies performed in both the laboratory and field have displayed that exposure to environmental chemicals that possess endocrine-disrupting potential induced GST activity in the liver of fish; however, unchanged or reduced GST activities were also reported [34, 48, 49]. Due to the lack of research regarding the impact of BPF on liver GST activity, it was not possible to compare the results determined herein. On the other hand, similar to the findings of the current study, in a recent study, GST activity was induced in marine rotifer (Brachionus koreanus) after 24-h exposure to BPF [50].

Conclusion

Overall, it was observed that BPF could cause oxidative damage and lead to antioxidant responses in the isolated hepatocytes of rainbow trout. According to the results, BPF, which has been environmentally detected in aquatic environments and fish tissues, did not seem to be a safe alternative for BPA. Thus, authorities should take measures to prevent BPF contamination in aquatic environments and reconsider the status of BPF as a safe alternative. The present data will also offer new insight regarding the toxic impact mechanism of BPF, which are poorly understood in fish species.

Author contributions BK conceived the research. HA and BK contributed in isolation of the hepatocytes, hepatocyte culture and treatments. HA and BK performed the cytotoxicity test and measurement of antioxidant defense system indicators. BK analyzed the data and interpreted the results. BK drafted, edited and finalized the manuscript.

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Data availability The data and material used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The procedures conducted herein were in line with those of the National and Institutional Regulations for the Protection of Animal Welfare. This study received ethical approval by The Animal
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