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Effects of Bisphenol A on Male Gonadal Tissue of Viviparous Fish: Histopathological Evidence in *Goodea atripinnis*

Efectos del Bisfenol A en tejido Gonadal Masculino de Peces Vivíparos: Evidencia Histopatológica en *Goodea atripinnis*

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SUMMARY: BPA is a multifunctional endocrine disruptor with ubiquitous presence in aquatic ecosystems. The Mexican Central Plateau is an area severely impacted by pollution, inhabited by endemic viviparous fish. However, efforts to understand the effects of BPA on native species such as *Goodea atripinnis* are non-existent. This study focused on providing *in vivo* evidence of alterations in the testes of *G. atripinnis* males due to acute exposure to BPA at test concentrations of 1 mg/L, 10 mg/L, and 50 mg/L for 96 h. BPA exposition 1 mg/L and 10 mg/L showed degeneration and disorganization in germinal tissue. Furthermore, there was a notable decrease in sperm within the seminiferous tubules of males exposed to 10 mg/L of BPA. In all treatments, somatic cells had alterations by connective tissue thickening and an increase in collagen fibers. Additionally, inflammation and bleeding occurred in the testes of males exposed to 1 and 10 mg/L BPA. The alterations in the testes of *G. atripinnis* are related to BPA toxicity, which can lead to apoptosis in germ cells increasing connective tissue. Finally, even though the changes produced by BPA became evident in acute exposure (96 h), its effects are probably irreversible, compromising the reproduction of *G. atripinnis.*

KEY WORDS: Biomarker; Testes; Endocrine disruptor; Viviparous fish; Emerging pollutant.

INTRODUCTION

Since the late 90s, global efforts have been made to understand the adverse effects on wildlife and humans caused by short- and long-term exposure to endocrine disruptors (Vandenberg *et al.*, 2009). Bisphenol A (BPA) is a multifunctional endocrine disruptor (Canesi & Fabbri, 2015) with a ubiquitous presence in aquatic ecosystems (Corrales *et al*., 2015). BPA is a synthetic organic compound used in the industrial production of polycarbonate plastics and epoxy resins, both of which are in high demand worldwide (Kang *et al*., 2002; Vandenberg *et al*., 2009; Cruz-López *et al*., 2020). Its incorporation into aquatic ecosystems is indirectly associated with the diffuse entry of materials containing this pollutant, or discharged directly from wastewater treatment plants (Corrales *et al*., 2015). The BPA concentration considered environmentally relevant is ≤12 µg/L, as it affects the gonad functionality in aquatic organisms (Canesi & Fabbri, 2015).

Histology is useful for evaluating the effects of endocrine disruptors (EDCs) at the tissue level (Johnson *et al*., 2009) because it allows readily determining the modifications in gonadal tissue that are relevant at individual and even population levels (Bernet *et al*., 1999). For instance, changes in the ovarian tissue of *Danio rerio* have been reported after exposure to 100 and 1000 µg/L BPA (Molina *et al*., 2013). On the other hand, in *Carassius auratus*, a decrease in ovarian maturation and an increase in the number of primary follicles were observed in females exposed to BPA. Males showed a disruption in spermatogenesis, with only spermatogonia and somatic cells observed in the testicular tissue (Wang *et al*., 2019).

Several studies have focused on evaluating the disruptive effects of BPA in male fish due to their susceptible estrogenic activity. Furthermore, histopathological changes

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have been evidenced in the testes of teleost fish after chronic or acute exposure to sublethal and lethal concentrations of BPA (Kwak *et al*., 2001; Kang *et al*., 2002; Ribero *et al*., 2021). Although acute tests allow for determining the toxicity of this pollutant, they can also be valuable for physiological monitoring in fish (Minaz *et al*., 2022). This is especially relevant in natural environments where organisms are subject to temporal fluctuations in BPA concentration, as well as spatial variations, such as sites that receive leachates containing BPA concentrations from 5400 µg/L to 17.2 mg/L (Flint *et al*., 2012).

The presence of BPA in variable concentrations has been reported in freshwater bodies located in the central region of Mexico, particularly in densely populated areas (Vázquez-Tapia *et al*., 2022). In the Mezquital Valley, state of Hidalgo, BPA concentrations of 9340 ng/L have been recorded (Lesser *et al*., 2018). Water supplying Mexico City contained concentrations of 1 ng/L to 10 ng/L BPA (Félix-Cañedo *et al*., 2013). In the Chapultepec lacustrine system (Mexico City), BPA concentrations of 2.72 ± 2.99 µg/L were recorded in Lago Menor and 1.62 ± 3.25 µg/L in Lago Mayor (Olivares-Rubio *et al*., 2015). On the other hand, Xochimilco reported the highest BPA concentration in surface water in Mexico, with 140.33 ng/µL (Díaz-Torres *et al*., 2013; Vázquez-Tapia *et al*., 2022). Recently, BPA concentrations of 20 µg/L to 25 µg/L were detected in the Santa Catarina River in the city of Monterrey, north-western Mexico (Cruz-López *et al*., 2020).

The geographic regions most impacted by endocrine disruptors in Mexico include the Mexican Central Plateau and the Basin of Mexico (Olivares-Rubio *et al*., 2015). These regions are characterized by lacustrine systems that harbor viviparous fish of the Goodeinae subfamily (Domínguez-Domínguez *et al*., 2010). There are currently limited studies on the effects of EDCs in viviparous fish, especially in goodeids, although it has been reported that the presence of this type of substances in the natural habitat of these species puts their reproduction at risk, and consequently, their populations (Díaz-Torres *et al*., 2013; Olivares-Rubio *et al*., 2015). The reproductive strategies of viviparous fish are susceptible to EDCs, making them valuable non-conventional models (Edwards *et al*., 2010). *Goodea atripinnis* belongs to the subfamily Goodeinae and is widespread in the Mexican Central Plateau; it has been used as a sentinel (Reynoso Silva *et al*., 2014).

A previous study evaluated the expression of gene Foxl2 after subchronic exposure (14 and 28 days) to 1 mg/L BPA in *G. atripinnis*, showing its activity as an EDC by increasing the expression of this molecular marker in both female and male gonads (Cervantes-Camacho *et al*., 2020).

Histopathological damage to male gonads caused by acute and chronic exposure to BPA in fish, which are commonly used as conventional models in ecotoxicology, has been extensively studied over the past two decades. In Mexico, viviparous fish of the family Goodeidae are susceptible to BPA due to their distribution in lakes of the Mexican Central Plateau. Nevertheless, there is a notable gap in research as no studies have addressed the impact of this endocrine disruptor on testes of *G. atripinnis*. Given the effect of BPA on estrogenic activity, it is relevant to describe the effects of acute exposure to this compound in the testes of *G. atripinnis*, as a first approximation of the *in vivo* impact of BPA on the testicular tissue of male's viviparous fish.

MATERIAL AND METHOD

Chemicals. BPA [2,2-Bis (4-hydroxyphenyl) propane] (empirical formula: $C_{15}H_{16}O_2$; MW: 228.29; ≥99 %; Sigma-Aldrich, USA). The BPA stock solution was prepared in 1L MiliQ water and 0.0012 % dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), which was dissolved through ultrasonication at 60 ºC to 70 °C for four cycles (60 min sonication followed by a 10 min pause) and 42 kHz using an ultrasonic bath (Cole Parmer 8890). The stock solution was then used to prepare the dilutions for the test concentrations of 1 mg/L, 10 mg/L, and 50 mg/L BPA.

Test Organisms. Adult males of *G. atripinnis* (65 mm ± 0.7 mm total length [TL] and 3.3 g ± 1.0 g weight) were maintained in 50 L aquariums under controlled culture conditions of 20 ºC ± 2 °C and 12h/12h light/dark cycles. The fish were fed twice daily with commercial flakes (Wardley®, The Hartz Mountain Corporation). The physicochemical parameters were regularly monitored and maintained at pH = 7.5 ± 0.5, $CaCO_3$ = 100 mg/L ± 27 mg/L, and dissolved oxygen = 6 mg/L ± 1 mg/L. Two weeks before the commencement of the toxicological testing, fish were acclimatized in 20 L aquariums under standard conditions (American Public Health Association, 2005). They were fasted for 24 hours before the test. All organisms were treated ethically during the bioassay and sample collection processes, following the Technical Specification for the Production, Care, and Use of Laboratory Animals in the Official Mexican Standard NOM-062-ZOO-1999 (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 1999). This project received approval from the ethics committee of the Escuela Nacional de Ciencias Biológicas with number AUT-B-B-05219-052.

BPA Exposition and Sample Collection. Fishes underwent a 96-hour acute exposure to determine the BPA LC_{50} for *G. atripinnis*, following the protocol proposed by Cervantes-Camacho *et al*. (2020). Two control groups with and without

DMSO were used, and the test groups were exposed to BPA concentrations of 1 mg/L, 10 mg/L, and 50 mg/L. To maintain consistent BPA concentrations in each treatment, a static replacement was performed after the initial 48-hour testing period (Organization for Economic Cooperation and Development, 2019).

Histological Analysis. After completing the bioassay, organisms were promptly euthanized by decapitation following the protocol of physical methods of euthanasia (Stoskopf & Posner, 2008). Testis samples were collected and fixed in a 10 % neutral formalin solution for 48 hours. After fixation, samples were histologically processed with the routine technique, paraffin-embedded, and 5 µm thick sections were obtained. Sections were stained with the hematoxylin-eosin technique for light microscopy examination. Section examination and imaging were performed with a light microscope (Leica DM2700 M) using 4x, 10x, and 40x objectives. Histological changes were described following the recommendations of the guidance document on the diagnosis of endocrine-related histopathology in fish gonads (Johnson *et al.*, 2009).

RESULTS

In the control and test groups of *G. atripinnis*, testes were lobular, in pairs, and partially fused (Fig. 1 A). In *G. atripinnis*, the testicular structure is constituted by interstitial tissue (Figs. 1 A,B) that delimits the lobes. These show a progressive development (Fig. 1 A, B) of germ cells from spermatogonia to spermatozoa (Fig. 1 B), with the latter distributed in seminiferous tubules forming sperm clusters known as spermatozeugmata (Figs. 1B,C).

In control males (with and without DMSO), the gonad tissue exhibited no morphological variations, with the typical distribution of germ cell clusters and abundance sperm in the seminiferous tubules (Figs. 2A,B). Males exposed to 1 mg/L and 10 mg/L BPA displayed testicular tissue degeneration associated with vacuoles in germinal tissue, as well as cell disorganization in the testis lobes (Figs. 2C-F). The 10 mg/L BPA treatment produced a decrease in the number of spermatozoa.

All BPA treatments caused increased connective tissue with collagen fibers (Fig. 2C). Males exposed to 10 mg/L BPA also displayed the presence of fibroblasts within the germinal tissue (Fig. 2F). Organisms treated with 50 mg/L BPA exhibited an expanded interstitial space and slightly larger cells (Fig. 2H).

The 1 mg/L and 10 mg/L BPA treatments were associated with the presence of erythrocytes, indicating a mild hemorrhage (Fig. 2D). Polymorphonuclear cells were also found in individuals exposed to 1 mg/L BPA, suggesting an inflammatory process (Fig. 2D).

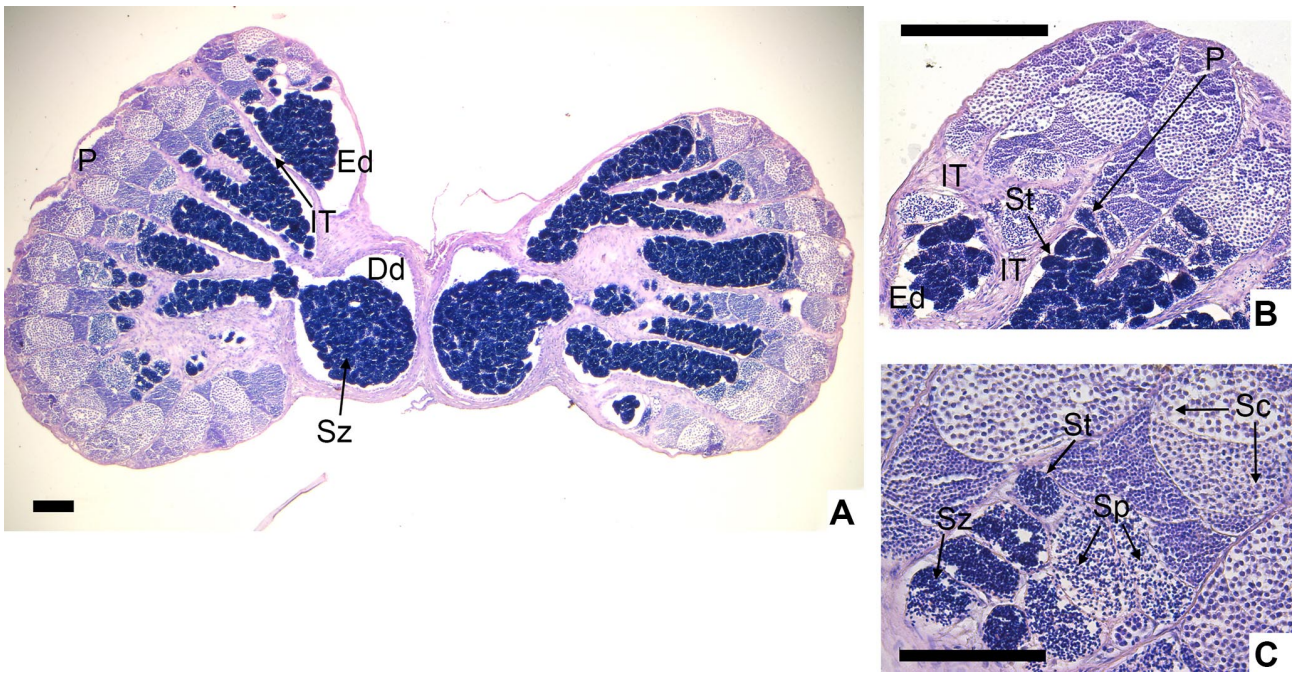

Fig. 1. Testes of *G. atripinnis* with no alterations (control). A. Cross-section of both testes. Spermatogenesis progresses from the periphery to the efferent ducts, with sperm contained in spermatozeugmata; lobes are separated by interstitial tissue. B. Lobe with progressive development of germ cells restricted in cysts. C. Cysts with germ cells in different spermatogenesis stages. P. Periphery; IT. Interstitial tissue; Ed. Efferent duct; Dd. Deferent duct; Sc. Spermatocyte; Sp. Spermatide; Sz. Spermatozoa; St. Spermatozeugmata. 100 µm scale bars.

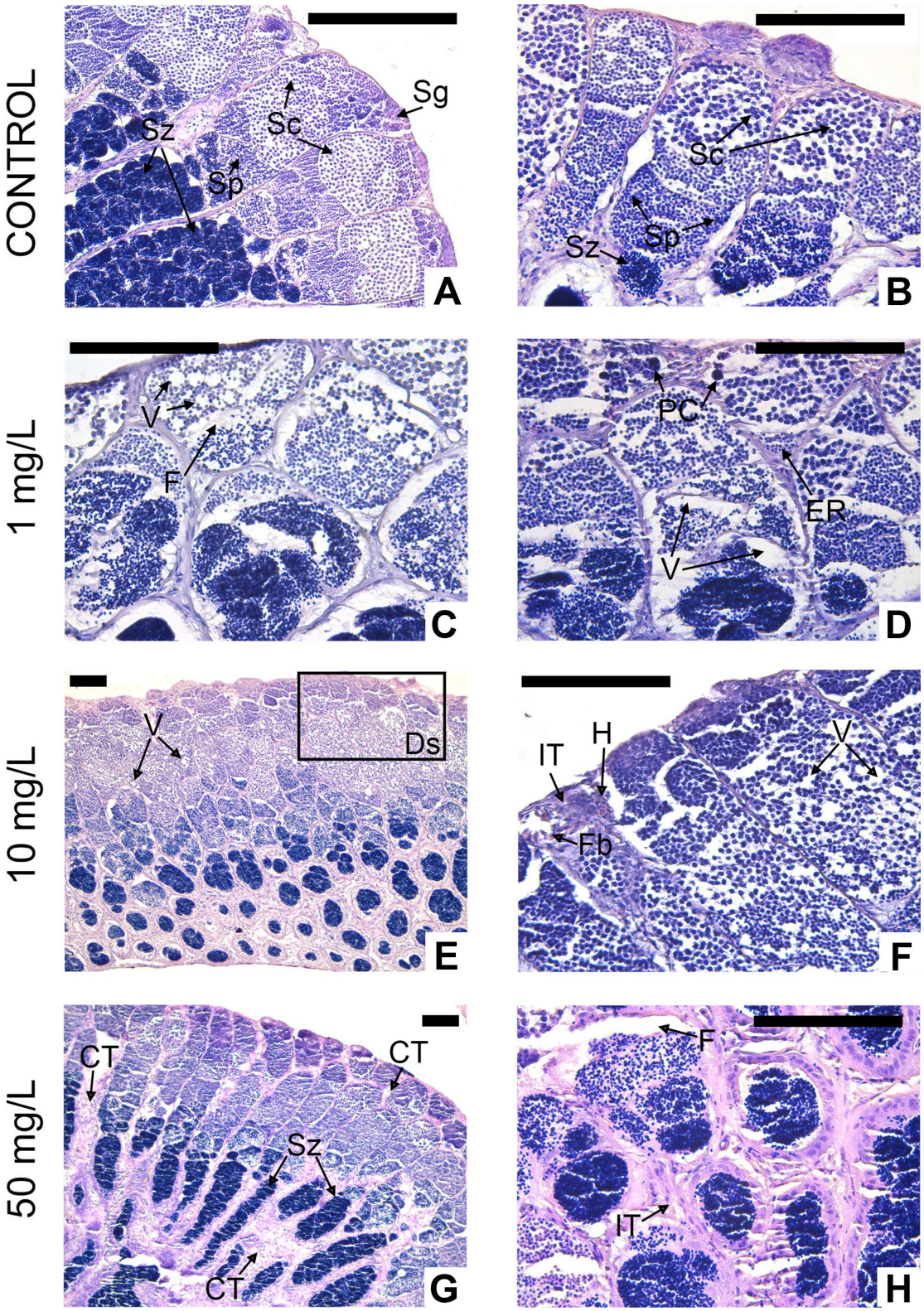

Fig. 2. Histological results of *G. atripinnis* testes after 96 h exposure in the control batch with DMSO (A, B) at 1 mg/L (C, D), 10 mg/L (E, F), and 50 mg/L (G, H). Sg. Spermatogonia; Sc. Spermatocyte; Sp. Spermatid; Sz. Spermatozoa; V. Vacuoles; F. Collagen fibers; Fb. Fibroblasts; PC. Polymorphonuclear cells; ER. Erythrocytes; CT. Connective tissue; IT. Interstitial tissue; H. Hemorrhage; Ds. Cell disorganization. 100 µm scale bars.

DISCUSSION

The organization of the testes in *G. atripinnis* depends on the progress and development of germ cells during spermatogenesis (Uribe *et al*., 2014). Spermatogenesis in teleost fish is regulated by steroid hormones including 17-b-ethinyl estradiol, which plays a transient role in the testes by stimulating spermatogonia proliferation and inducing mitosis. In contrast, 11-ketotestosterone (11-KT) controls the onset of meiosis, which increases progressively during spermatogenesis and decreases during spermiation (Schulz *et al*., 2010; Uribe *et al*., 2014).

Steroidogenesis is a process susceptible to endocrine disruption caused by BPA (Hatef *et al*., 2012). Negative regulation of 11-KT in teleost fish has been reported because of subchronic BPA exposure (Hatef *et al*., 2012; Forner-Piquer *et al*., 2019; Ribeiro *et al*., 2021). The BPA endocrine disruption has also been linked to changes in spermatogenesis, affecting the development of germ cells in testes (Ribeiro *et al*., 2021; Reis *et al*., 2022).

Acute exposure to BPA caused modifications in the structure of the testicular tissue of *G. atripinnis*. This is contrary to the findings reported for males of the viviparous fish *Xiphophorus hellerii* after acute exposure to BPA at 0.4 ppm, 2 ppm, and 10 ppm for 72 h, in which no lesions in the testes were observed, although the mixture of BPA with nonylphenol (NP) produced modifications in the testis architecture (Kwak *et al*., 2001). The effects of BPA on reproduction have been extensively studied in teleost fish. The variation of responses between fish models depends on the concentration/dose used, exposure time, the development stage of the organism, and the susceptibility of the species model (Forner-Piquer *et al*., 2019). The presence of vacuoles in the testis germ tissue is a sign of cell degeneration (Johnson *et al*., 2009). This process causes cellular disorganization, which was observed at the periphery of the testis lobes of *G. atripinnis* in males exposed to 1 mg/L and 10 mg/L BPA. Testis tissue degeneration has been reported previously in *Astyanax bimaculatus* after subchronic exposure to BPA at concentrations lower than those analyzed in the present study (Ribero *et al*., 2021). In addition, cell degeneration has been observed after subchronic exposure of *Danio rerio* females to BPA at concentrations of 10 µg/L to 1000 µg/L (Molina *et al*., 2013), and in males exposed for 21 days at BPA concentrations of 5 µg/L, 50 µg/L, and 500 µg/L (Reis *et al*., 2022).

G. atripinnis males exposed to 1 mg/L and 10 mg/L BPA showed erythrocytes and polymorphonuclear cells, suggesting bleeding and an inflammatory process in the testes. Reis *et al*. (2022), reported an aggravation of testicular alterations in *Danio rerio* males exposed to 500 µg/L BPA, with parenchyma hemorrhage among the most serious effects; these may be due not only to its estrogenic activity but also to its toxicity. In *G. atripinnis* and other goodeids, different functions of testis structures may be compromised by inflammatory processes and bleeding. This is because Leydig cells are close to the testis blood vessels, and in goodeids, these cells are abundant in the efferent ducts (Uribe *et al*., 2014). Therefore, a hemorrhage might alter Leydig cells, affecting the hormonal regulation of spermatogenesis and its fertilization capacity.

Changes were also observed in the interstitial tissue, mainly in the increase of connective tissue. The testis lobes are usually divided by a connective tissue compartment containing fibroblasts, collagen fibers, blood vessels, and immune cells (Uribe *et al*., 2014).

In all *G. atripinnis* males experimentally exposed to BPA, a thickening of the connective tissue was observed. Furthermore, BPA concentrations of 1 mg/L and 10 mg/L showed an increase in the number of collagen fibers. Testicular fibrosis has been reported in male fish exposed to endocrine-disrupting compounds (EDCs) with estrogenic activity (Kidd *et al*., 2007; Kaptaner & Ünal, 2011). Testicular fibrosis may be a response to estrogenic damage caused by EDCs, or a consequence of increased apoptotic activity induced by these pollutants (Kaptaner & Ünal, 2011).

EDCs with estrogenic activity capable of inducing apoptosis in testicular tissue were studied in the testes of *X. helleri*. Apoptosis was observed in germ cells of seminiferous tubules and interstitial tissue after exposure to 10 ppm BPA and 100 ppb nonylphenol (NP), with no evidence of fibrosis (Kwak *et al*., 2001). An advanced apoptotic process has also been observed in the goldfish *Carassius auratus* after subchronic exposure to BPA at 50 µg/L and 500 µg/L, along with the disappearance of germ cells in the testes and the decrease of 11-KT. Furthermore, permanent damage to the testes was apparent after replacing BPA-polluted with non-polluted water (Wang *et al*., 2019).

For its part, medaka fish *Oryzias latipes* individuals exposed to NP showed an increase of interlobular connective tissue with fibroblasts associated with testicular apoptosis (Weber *et al*., 2002). This may explain the increase in connective tissue with fibroblasts and the decrease in sperm in seminiferous tubules of *G. atripinnis* exposed to 10 mg/L BPA. Alterations in somatic and testis germinal tissue caused by EDCs exposure can affect spermatogenesis and spermiogenesis, disrupting the estrogenic activity of cells (Ribeiro *et al*., 2021).

According to the above, the changes in *G. atripinnis* testes may also be related to BPA toxicity. A previous study in *G. atripinnis* where DNA damage was observed in somatic and testicular germ cells after a 14-day exposure to 1 mg/L BPA. A higher percentage of comets (cells with DNA fragmentation) and an aggravation of DNA damage evidenced through the tail moment of comets were reported (Cervantes-Camacho *et al*., 2020).

Although the concentrations used in this study are deemed high (≥1000 µg/L of BPA in water) (vom Saal & Welshons, 2006), the prevalence of this pollutant in Mexico remains poorly understood. It has been detected in concentrations above 1000 µg/L in water bodies inhabited by *G. atripinnis* and other goodeids (Díaz-Torres *et al*., 2013; Vázquez-Tapia *et al*., 2022). Additionally, the present study suggests that there are serious alterations in the testes after short exposure to BPA, consistent with reports for fish under subchronic exposure to BPA or other EDCs. Although high BPA concentrations can cause mortality in exposed organisms, some organisms survive and even reproduce despite the toxicity (Akram *et al*., 2021). The damage caused by BPA exposure to germ cells is usually irreversible (Wang *et al*., 2019) and indirectly affects the progeny. Embryos of adult fish exposed to BPA have been reported to experience high mortality and cardiac and musculoskeletal malformations (Kang *et al*., 2002; Reis *et al*., 2022).

CONCLUSION

Acute exposure to *G. atripinnis* males to BPA caused alterations in testes germ and somatic cells, which may compromise their reproductive function early and irreversibly. This is relevant given the endemic nature of this subfamily, particularly in those species that inhabit polluted water bodies and may be exposed to BPA chronically, over short periods, or to varying concentrations of this endocrine disruptor. Additionally, *G. atripinnis* remains an unconventional model that may be used as a sentinel organism in the evaluation of EDCs in freshwater bodies of Mexico.

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CERVANTES-CAMACHO, I.; RUIZ-PICOS, R.A. & LÓPEZ-LÓPEZ, E. Efectos del bisfenol A en tejido gonadal masculino de peces vivíparos: Evidencia histopatológica en *Goodea atripinnis*. *Int. J. Morphol., 42(4)*:977-983, 2024.

RESUMEN: El BPA es un disruptor endocrino multifuncional con presencia ubicua en los ecosistemas acuáticos. La Meseta Central mexicana habitada por peces vivíparos endémicos, es una zona severamente impactada por la contaminación. Sin embargo, los esfuerzos por comprender los efectos del BPA en especies nativas como *Goodea atripinnis* son inexistentes. Este estudio se centró en proporcionar evidencia *in vivo* de alteraciones en los testículos de machos de *G. atripinnis* debido a la exposición aguda al BPA en concentraciones de prueba de 1 mg/L, 10 mg/L y 50 mg/L durante 96 h. La exposición a BPA 1 mg/L y 10 mg/L mostró degeneración y desorganización en el tejido germinal. Además, hubo una disminución notable de los espermatozoides dentro de los túbulos seminíferos de machos expuestos a 10 mg/L de BPA. En todos los tratamientos las células somáticas presentaron alteraciones por engrosamiento del tejido conectivo y aumento de las fibras de colágeno. Además, se produjo inflamación y sangrado en los testículos de machos expuestos a 1 y 10 mg/L de BPA. Las alteraciones en los testículos de *G. atripinnis* están relacionadas con la toxicidad del BPA, lo que puede provocar apoptosis en las células germinales aumentando el tejido conectivo. Finalmente, si bien los cambios producidos por el BPA se hicieron evidentes en la exposición aguda (96 h), sus efectos probablemente sean irreversibles, comprometiendo la reproducción de *G. atripinnis*.

PALABRAS CLAVE: Biomarcador; Testículos; Disruptor endocrino; Peces vivíparos; Contaminante emergente.

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