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Original Article

Degenerative changes in testis, epididymis, and sperm quality in ICR mice treated with methoxychlor and bisphenol A

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ABSTRACT Endocrine-disrupting chemicals found in many commercial products may interfere with the normal functioning of the endocrine system and are unsafe because of their cumulative effect on the human body. However, little is known about the effects of combinations of endocrine-disrupting chemicals in humans. Methoxychlor and bisphenol A are toxic to male reproductive organs. Therefore, we studied the effects of methoxychlor and bisphenol A on male reproductive function. Male mice were divided into four treatment groups: control, 400 mg methoxychlor, 1 mg bisphenol A, and 400 mg methoxychlor + 1 mg bisphenol A/kg/day. Methoxychlor and bisphenol A were dissolved in sesame oil and acetone and administered orally for 4 weeks. After administration, the weight and histological changes in the testicles and epididymis, sperm count and health were observed biochemical tests and whole blood counts were performed. The results showed that the mice in the bisphenol A and methoxychlor + bisphenol A groups gained more weight than those in the control and methoxychlor group. The weights of the testes and epididymis were higher in the experimental groups than in the control. Sperm motility and progression were significantly reduced in the bisphenol A and methoxychlor + bisphenol A groups. Histological observation showed a reduced number of sperm, smaller seminiferous tubules, and destroyed lumen in the methoxychlor + bisphenol A group compared to the other groups. In conclusion, our study showed that methoxychlor and bisphenol A destroy male reproductive tissues and decrease sperm quality.

Keywords: bisphenol A, endocrine disruptor, male reproductivesystem, methoxychlor

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are defined by the U.S. Environmental Protection Agency as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body". EDCs are thought to primarily function through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors. However, current research has shown that they have multiple mechanisms of action such as working with non-nucleic steroid hormone recipients, non-steroid recipients, and orphan receivers. EDCs are found in chemicals and plastics used in our daily lives including synthetic chemicals such as industrial solvents or lubricants and by-products such as polychlorinated biphenyls (PCBs), polybrominated

biphenyls (PBBs), dioxins, plastics [bisphenol A (BPA)], pesticides [methoxychlor (MXC)], chlorpyrifos, dichlorodiphenyltrichloroethane (DDT), and pharmaceutical agents [diethylstilbestrol (DES)]. Some EDCs were banned decades ago, whereas others have only been banned recently. However, a major problem with EDCs is that they continue to be secreted by the already-manufactured products. EDCs are pollutants because they are industrial chemicals that can leach into the soil and groundwater. Animals and humans can be exposed to EDCs through the contaminated water, air, food, or soil. As some EDCs have long half-life, they might be beneficial for industrial use. However, they are harmful to wildlife and humans. These compounds are not easily degradable, accumulate in the environment and become toxic. Even EDCs banned decades ago remain abundant in the environment and can still be detected in animals and humans (Diamanti-Kandarakis et al., 2009).

MXC is an organochlorine-based insecticide developed as an alternative to DDT and 2,2-dichlorovinyl dimethyl phosphate (DDVP). Since DDT was banned in 1972, MXC has been used owing to its low toxicity and rapid metabolism in the body. However, MXC causes side effects, such as dysplasia and infertility, because it acts like estrogen in living organisms. Studies have shown that MXC reduces serum testosterone levels and inhibits the development of the male reproductive tract. In addition, sperm motility in the epididymis is reduced in rats treated with MXC (Cooke and Eroschenko, 1990; Latchoumycandane et al., 2002). BPA, a common EDC present in plastics, also exhibits an estrogenic activity similar to that of MXC. Studies have shown that BPA decreases sperm motility and concentration, and serum testosterone levels in the epidural space (Jang et al., 2011; Yi et al., 2021).

As infertility rates increase globally, research on EDCs and their effects on fertility is needed. Most of the previous studies related to EDC have examined the effect of only one EDC substance, and lack adequate information on the combined effect of multiple EDCs on male fertility. In particular, studies using a combination of MXC and BPA are rare, and each EDC has a different mechanism of action, resulting in different pathological tissue changes in the testis. In previous studies, MXC was found to disrupt seminiferous and luminal structures in the testis, and BPA caused a decrease in sperm count (Burks et al., 2011; Chen et al., 2014). In addition, studies in which two or more types of EDC are administered at high concentrations for a short time are rare. In this study, the experiment was conducted by increasing MXC and BPA concentrations beyond the level that affect fertility. EDC accumulates faster in the body at high-dose short-term administration than at low-dose long-term administration, and induces convulsions and heart failure due to damage to the nervous system (Konieczna et al., 2015). However, previous studies have not reported the effects of high-concentration EDC exposure on reproductive organs. Therefore, we investigated the effects of the short-term administration of high concentrations of MXC and BPA on the fertility of male mice.

MATERIALS AND METHODS

Animals and groupings

Male ICR mice were obtained from DOOYEOLBIOTECH (Seoul, South Korea). The animals were maintained under a 12 h light/dark cycle at 20 ± 2℃ room temperature. Food and water were provided ad libitum. The experimental group consisted of pre-pubescent mice (PND 21). Twenty mice were divided into four groups, as follows: A, Control group; B, MXC administration group; C, BPA administration group; D, MXC and BPA combination administration group. After acclimatizing the mice for one week, BPA and MXC were orally administered for four weeks.

Ethical statement

All procedures were performed in accordance with the protocol approved by the Gachon University Animal Experimental Ethics Committee (approval number: GU1-2021-IA0014-00). At the end of the 4-week experiment, the animals were fasted for 24 h, anesthetized through inhalation of isoflurane in a closed chamber, and euthanized. Blood samples (1 mL) were collected by inserting a 25G needle into the inferior vena cava through an abdominal incision. The blood was divided into two parts. One portion (0.6 mL) was stored in an EDTA tube for biochemical analysis, and the remaining blood (0.4 mL) was placed in an SST tube for hormonal analysis. All the blood samples were gently shaken and stored at 4℃.

Tissue processing after animal slaughter

Tissue pretreatment with hematoxylin and eosin (H&E)

was performed. Reproductive organ tissues fixed in Bouin's solution were embedded in paraffin, according to standard procedures. The tissues were fixed in Bouin's solution for 12 h and then washed under running water for 10 h. Tissue dehydration and fixation were performed using an Automatic Tissue Processor TP1020 (Leica, South Korea). The automatic tissue processor was programmed to process tissue samples at 1 h per reagent. After preprocessing, tissue paraffin blocks were formed using the KD-BM II & BL Tissue Embedding & Cooling System (Wincom, Hunan, China). Tissue samples were serially sectioned (5 nm thick), mounted on glass slides, and stained with Harris hematoxylin and eosin after bleaching with 1% HCl (Cardiff et al., 2014; Bilinska et al., 2018).

Chemicals

BPA and MXC were purchased from Sigma-Aldrich (CAS #80-05-7, purity ≥99%; CAS #:72-43-5, purity ≥98%) and animals treated with sesame oil, 400 mg MXC, 1 mg BPA, and 400 mg MXC + 1 mg BPA/kg/day in 0.2 mL vehicle for 4 weeks through oral gavage on alternate days. Both BPA and MXC were dissolved in acetone and sesame oil (1:19) (Latchoumycandane et al., 2002) and the combined 0.2 mL dose 0.2 mL was orally administered simultaneously.

The LD_{50} concentration of MXC, indicating acute toxicity was 1,850 mg/kg, and higher than the concentration of 400 mg/kg used in this experiment (Magos, 1992). However, in a previous study, 125 mg/kg of MXC acted as an estrogen competitor, affecting reproduction and reducing mating numbers, resulting in a decrease in the number of offspring in pregnant mice (Hartley and Kidd, 1987; Magos, 1992). The LD_{50} concentration of BPA was 3,250 g/kg, which is also higher than the BPA concentration used in this experiment (Chapin et al., 2008). Nonetheless, the acceptable daily dose of BPA is 50 μg/kg, which is lower than the dose used in this experiment (Rubin et al., 2001). Therefore, in this experiment, we used a high concentration of EDC, which does not affect the survival but change only the reproductive system of mice.

Biochemical analysis

Biochemical and white blood cell (WBC) tests were performed to determine MXC toxicity. Blood samples were collected in EDTA tubes during the autopsy at the end of the experiment and tests were performed using an AU480 (Beckman Coulter, Inc., United States). A WBC was performed using an automated hematology analyzer BC-5000vet (Mindray, China). WBCs were counted to measure the number of monocytes, neutrophils, leukocytes, eosinophils, and basophils.

Histological analysis

Histological analysis was performed to evaluate the tissue endocrine-disrupting effects of MXC. The testes and epididymis were stained with H&E and observed at 100× and 400× magnifications. Lumens and seminiferous tubules were observed in stained testes and epididymis.

Computer-assisted sperm analysis (CASA)

Sperms were extracted from the epididymis stored in PBS at 37℃ and analyzed using CASA and Hamilton-Thorne IVOS 12.3 (Hamilton Thorne, USA). Sperm motility, sperm progression, average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL) were analyzed in 20 mouse sperm samples (n = 5 per group) (Amann and Waberski, 2014).

Statistical analysis

All results are expressed as mean ± standard error (SE). Tukey's HSD tests were used as post-hoc in the one-way ANOVA of SPSS (software version 26.0.0.0, IBM SPSS Statistics for Windows, IBM Corp., USA). Mean comparisons between the groups were conducted at a significance level of $p < 0.05$.

RESULTS

Weight measurement

Body weight was measured to analyze the effects of endocrine disruptors on ICR mice (Fig. 1). The BPA and MXC + BPA groups gained more body weight than the control and MXC groups (Fig. 1A). The relative weight gains showed a change of 32.58%, 34.3%, 61.93% and 54.60% in the control, MXC, BPA, and MXC + BPA groups respectively (Fig. 1B). The relative masses of the epididymis and testis in the MXC and BPA groups were not significantly different from those of the control group. However, the testicular and epididymal masses in the MXC + BPA group were significantly higher than the control (Fig. 2A and 2B, respectively).

Biochemical analysis

The mean corpuscular volume (MCV) was significantly lower in the BPA and MXC + BPA groups than in the control. There was a significant decrease in the mean corpuscular hemoglobin concentration (MCHC) in the MXC and MXC + BPA groups compared to the control (Fig. 3). The experimental groups did not show significant differences in WBC counts compared to the control (Table 1). MXC and BPA did not induce an inflammatory response.

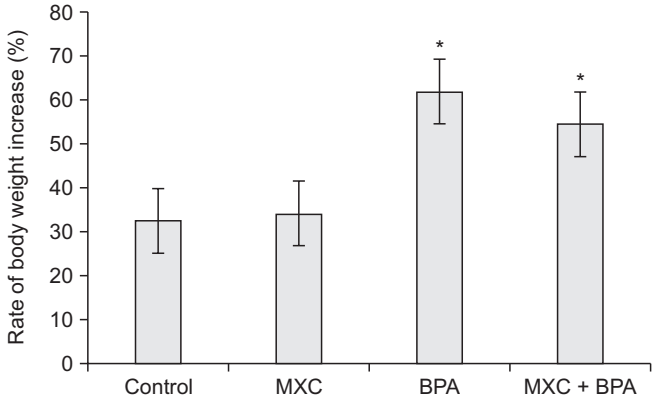

Fig. 1. Body weight and rate of weight increase in ICR mice. Relative weight gain of mice after four weeks. Values are represented as mean ± SE (n = 5), *indicates significant differences compared to variable values in all groups at $p < 0.05$.

Computer-assisted sperm analysis (CASA)

Sperm motility and progression significantly decreased in the BPA and MXC + BPA groups compared to the control (Fig. 4A and 4B). No significant differences existed in all the treatments for the VAP, VSL, and VCL, but these decreased the most in the MXC + BPA group (Fig. 4C).

Histological analysis

The effects of BPA and MXC on the male genitalia were investigated using H&E staining. Compared with the control, we observed inconsistent thickness of seminiferous

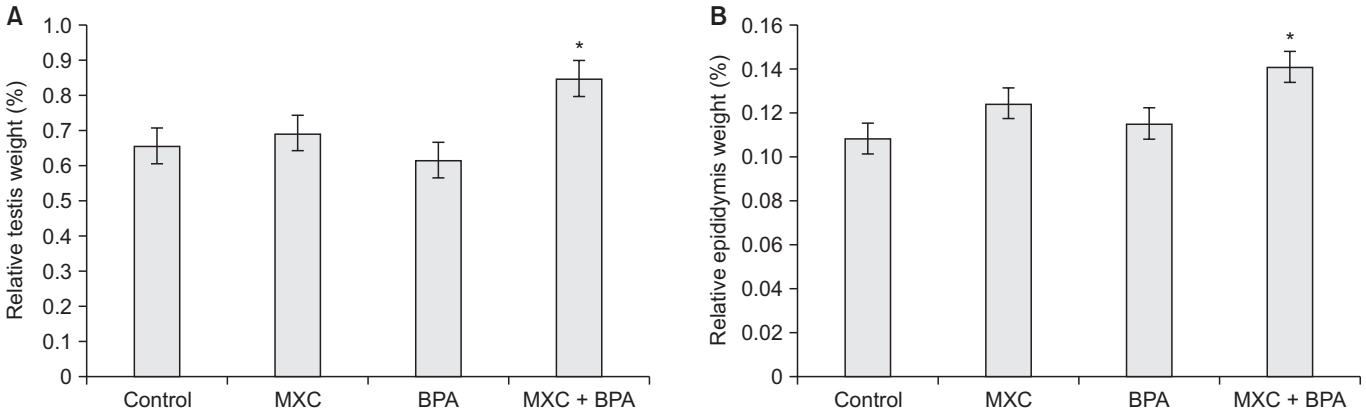

Fig. 2. Relative weights of testis and epididymis in 8-week-old ICR mice. (A) Relative testis weight in each group. (B) Relative epididymal weights in each group. Values are represented as mean ± SE (n = 5), *indicates significant differences compared to variable values in all groups at $p < 0.05$.

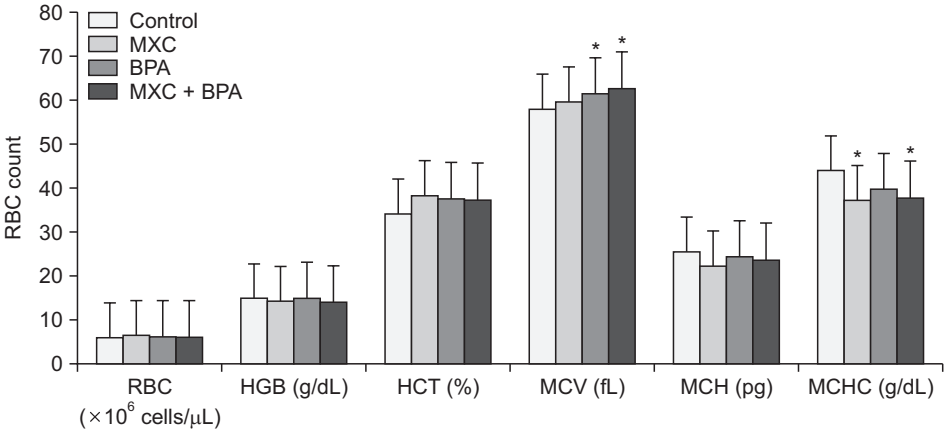

Fig. 3. CBC count for 8-week-old ICR mice. The measured amounts of RBC, HGB, HCT, MCV, MCH, and MCHC in the CBC test are shown. Values are represented as mean ± SE (n = 5), *indicates significant differences compared to variable values in all groups at $p < 0.05$. RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

tubules in all experimental groups and destruction of lumen (Fig. 5). In addition, normal sperms were observed at the center of the sperm tube in the control, whereas abnormal sperm were observed in the MXC group. The number of sperms in the MXC was lower than the control And sperm was not observed in the BPA and MXC + BPA groups. Epididymal H&E staining showed that all experimental groups had a lower sperm count than the control group (Fig. 6). In the MXC + BPA group, the epidural tissue lumen was damaged and the sperm count was the lowest among the experimental groups (Fig. 6D).

Table 1. PLT and WBC from each group

Group	PLT × 10^3cells/uL	WBC × 10^3cells/uL
Control	1410.25	3.05
MXC	1609.75	2.57
BPA	1628.6	2.34
MCX + BPA	1394.8	2.84

Group	WBC differential counting (%)			
	Neut	Lymph	Mono	Eos
Control	13.0	77.4	1.2	4.9
MXC	10.8	80.4	2.0	3.8
BPA	9.2	78.1	1.7	8.4
MCX + BPA	9.4	78.2	1.4	8.7

Group	WBC differential counting (× 10^3cells/uL)			
	Neut	Lymph	Mono	Eos
Control	0.41	2.36	0.04	0.14
MXC	0.30	2.06	0.04	0.09
BPA	0.21	1.83	0.03	0.21
MCX + BPA	0.26	2.25	0.04	0.24

Values are represented as the mean of four mouse groups (n = 5), $p < 0.05$. There were no significant differences between the groups. PLT, platelet; WBC, white blood cell; MXC, methoxychlor; BPA, bisphenol A.

DISCUSSION

Humans are exposed to many endocrine disruptors through plastic products and industrial chemicals. These cause air, soil, and water pollution, resulting in significant harm to humans. Endocrine disruptors increase the risk of reproductive function, growth disorders, and cancer in living organisms besides accumulating in the body, and are not easily decomposed (An et al., 2021). BPA and MXC have been studied as EDCs that show reproductive toxicity in previous studies. However, studies on male fertility using high concentrations of BPA and MXC combination are rare. With increasing infertility rates, research on endocrine disruptors that leads to reproductive dysfunction is essential.

Several studies have shown that oral exposure to MXC lead to weight loss in rats, rabbits, and dogs (Hartley and Kidd, 1987; Miller et al., 2002). However, in our study, MXC did not affect weight in the group administered with 400 mg/kg MXC. Other studies have reported a correlation between BPA exposure and obesity. *In vitro* and rodent studies have shown that BPA exposure induces fat accumulation and promotes weight gain (Wu et al., 2020). Consistent with these results, our results also showed significant weight gain in the group administered with 1 mg/kg BPA and significant weight gain in the group administered with both MXC and BPA. BPA affects weight gain via

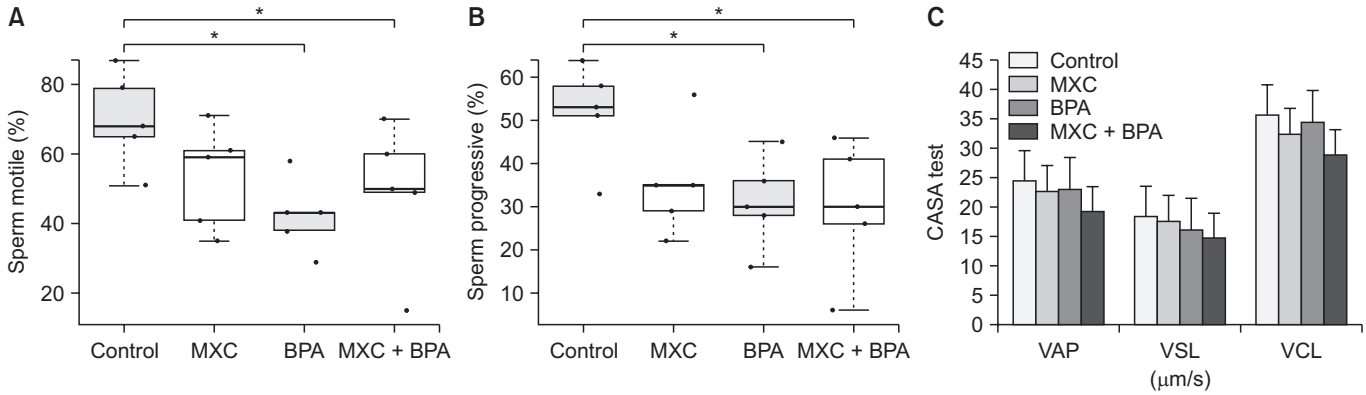

Fig. 4. CASA test results of 8-week-old ICR mice. (A) Motile sperms (%) in each group. (B) Sperm progression (%) in each group. (C) Average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL) of each group. Values are represented as mean ± SE (N = 5), *$p < 0.05$.

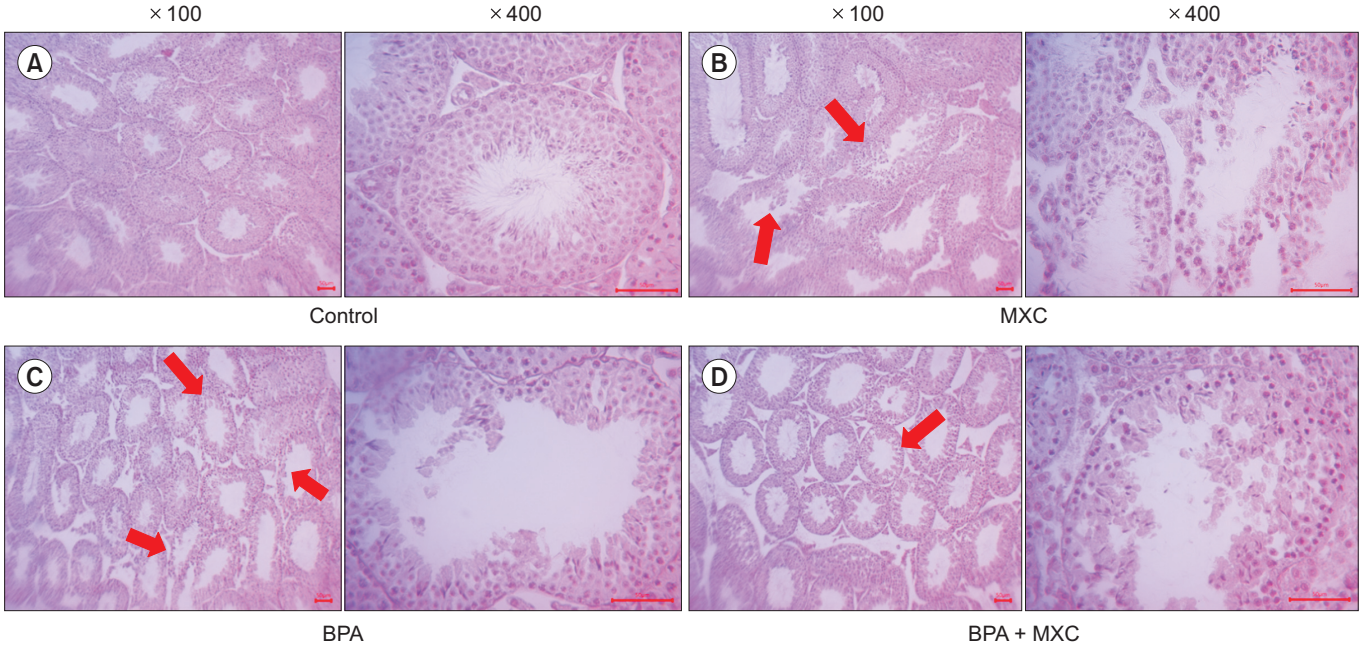

Fig. 5. Testis H&E staining results of 8-week-old ICR mice. Testes were stained with hematoxylin and eosin (H&E) and imaged. (A) Control, (B) methoxychlor (MXC), (C) bisphenol A (BPA), and (D) MXC + BPA groups. The destroyed lumina of the seminiferous tubules are indicated by the arrows. Scale bar = 50 μm, original magnification: 100× and 400×.

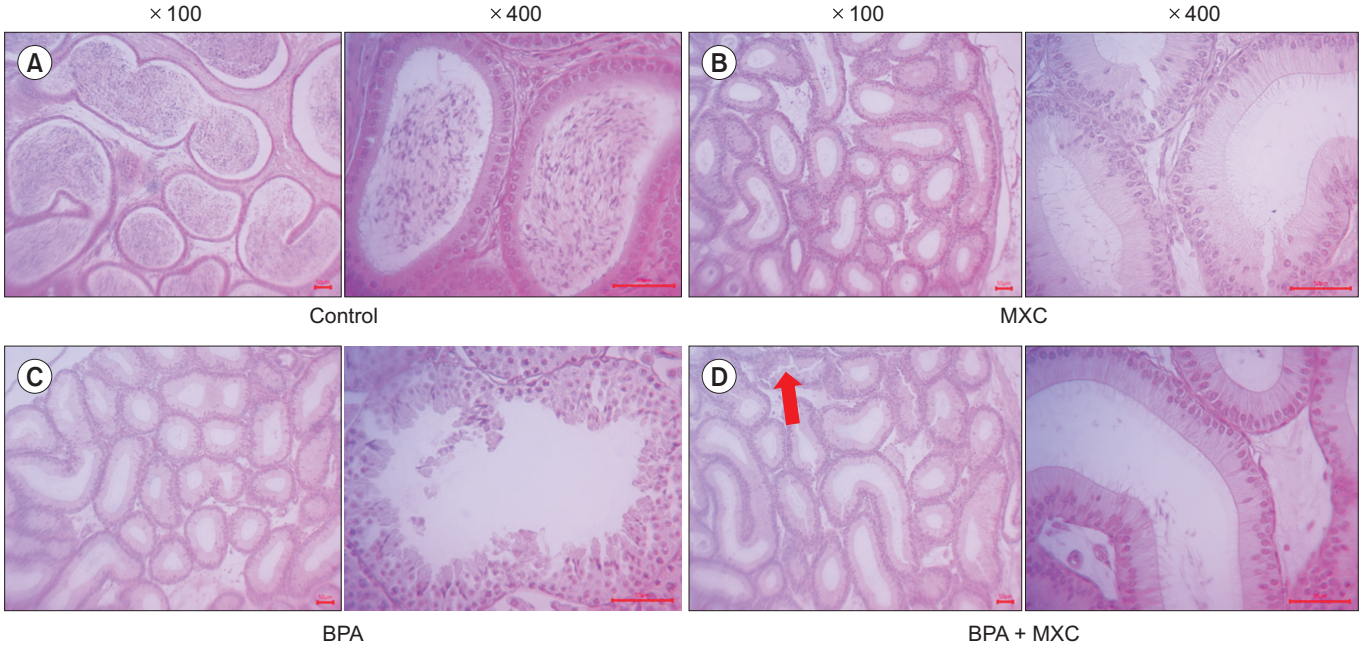

Fig. 6. Epididymis H&E staining results of 8-week-old ICR mice. All epididymis were stained with hematoxylin and eosin (H&E). (A) Control, (B) methoxychlor (MXC), (C) bisphenol A (BPA), and (D) MXC + BPA groups. Arrows indicate damaged epidural lumen tissue. Scale bar = 50 μm, original magnification: 100× and 400×.

various mechanisms and enhances adipocyte differentiation and lipid accumulation (Masuno et al., 2002; Masuno et al., 2005). BPA also improves glucose uptake in 3T3-F443A adipocytes in mature mice by increasing GLUT4 protein (Sakurai et al., 2004; Rubin and Soto, 2009). In addition, BPA binds to thyroid hormone receptors and

acts as an antagonist to inhibit the transcriptional activity stimulated by the thyroid hormone triiodothyronine (T3) (Moriyama et al., 2002; Zoeller, 2005; Rubin and Soto, 2009). Halogenated derivatives of BPA inhibit the binding of T3 to thyroid hormone receptors (Kitamura et al., 2002; Rubin and Soto, 2009). Given the important role of thyroid hormones in energy homeostasis, BPA exposure is likely to affect weight gain.

Varied results are obtained in studies on changes in weight of reproductive organ due to MXC and BPA exposure. Some studies reported no weight change, whereas others reported weight loss after MXC or BPA administration. This is because the concentration and duration of drug administration differed between the studies. In our study, no significant difference was observed in the weight of the testis and epididymis between the MXC 400 mg/kg and BPA 1 mg/kg groups. In contrast, the reproductive organs in the MXC + BPA group weighed more than those of the other groups. However, because environmentally toxic endocrine disruptors are exposed to low concentrations for a long period, long-term research is required to accurately determine their toxicity (Kim et al., 2002).

Biochemical tests showed no significant differences in WBC counts and therefore, no specific inflammatory reaction was observed. Serum hepatic biomarkers (alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased in experimental animals administered with high concentrations of MXC and BPA, indicating mild hepatotoxicity (Miller et al., 2002; Thoene et al., 2017). Therefore, MXC and BPA are likely to cause hepatotoxicity in the male ICR mice in our study.

MXC and BPA can reduce sperm quality by reducing testosterone and increasing estradiol levels through antiandrogen activity. BPA interferes with sperm formation by activating apoptotic pathways in reproductive cells by reducing serum reproductive hormone levels (follicle-stimulating hormone [FSH], luteinizing hormone [LH], and GnRH) in rats and gonadotropin-releasing hormone (Jin et al., 2013). Consistent with previous studies, the sperm count in the epididymis of rats exposed to MXC 50-400 mg/kg per day decreased compared with the control (Gray et al., 1999). The BPA and MXC + BPA groups showed significantly decreased sperm motility and slightly decreased VAP, VSL, and VCL levels compared with those in the control. This indicates that MXC and BPA affect sperm quality (Du et al., 2014; Olukole et al., 2020; Virant-Klun et al., 2022).

Our results show that MXC and BPA are toxic to the testes and epididymis of male ICR mice. In the drug administration group, destruction of the testicular lumen and a reduction in the number of sperm in the seminiferous tubules and epidural space were observed. In the MXC + BPA group, we observed a low sperm count in the seminiferous tubules and damaged lumen cells, making the lumen indistinguishable from the other structures. In addition, the thickness and density of the seminiferous tubules in the testicles decreased. These results are consistent with those of previous studies in which testicular tissue damage was observed after MXC and BPA administration (Gray et al., 1989; Kazemi et al., 2016).

Sesame oil and acetone were used to dissolve MXC and BPA. I reason why we used acetone and sesame oil as solvents instead of DMSO is because DMSO is toxic to the body and unsafe. In addition, the solubility of EDC was increased by adding a low dose of acetone because it is difficult to completely dissolve BPA and MXC with only sesame oil (Yoon et al., 2006). Sesame oil prevented the side effects of acetone in mice while addition of small amount of acetone minimized its toxicity.

In conclusion, the effects of EDC on the reproductive system have been studied, but lack molecular analysis. Therefore, in a follow-up study, hormone analysis and reproductive system oxidative stress will be measured using the administration of concomitant concentration and EDC conditions. Nevertheless, our experimental results are mostly consistent with those of previous studies which suggest that representative EDCs, MXC, and BPA can lead to reproductive degradation. In addition to MXC and BPA, several environmentally toxic substances can cause reproductive dysfunction. This calls for additional studies on these chemicals.

CONCLUSION

Twenty male ICR mice were divided into four groups, and were orally administered sesame oil, MXC 400 mg/kg, BPA 1 mg/kg, and MXC 400 mg/kg + BPA 1 mg/kg, respectively, every alternate day for 28 days. Significant weight gain and a reduction in sperm motility and progression rates were observed in the BPA and MXC + BPA groups. In addition, testicular and epididymal tis-

sue damage and sperm count reduction in the epididymis were observed in all experimental groups except for the control. This study suggests that MXC and BPA degrade reproductive function. As EDCs can cause male infertility, studies on fertility-enhancing substances that can replace endocrine disruptors should be conducted.

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Ethical Approval: All procedures were performed in accordance with the protocol approved by the Gachon University Animal Experimental Ethics Committee (approval number: GU1-2021-IA0014-00).

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

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Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

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