

Immunohistochemical investigation of the effects of different doses of some endocrine-disrupting chemicals on receptor-interacting serine/threonine-protein kinase 1 (RIPK1) levels in the testis

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Received: 22/08/2023

Accepted: 11/09/2023

Published: 22/09/2023

ABSTRACT

Aims: The reproductive toxicity of endocrine-disrupting chemicals (EDCs) is well known, but the underlying mechanism remains unclear. Receptor-interacting serine/threonine protein kinase 1 (RIPK1) mainly mediates cell death and inflammation. It is crucial to both necroptosis and apoptosis. Therefore, in this study, we aimed to investigate the changes in RIPK1 expression levels immunohistochemically in the adverse mechanism of EDCs on spermatogenic cells.

Methods: Forty-two wistar albino male rats (10 weeks old, 250±50 g) were assigned into seven groups. Bisphenol A (BPA), 4-nonylphenol (NP), and their mixtures at low 25 mg/kg doses and high 100 mg/kg doses were used. The control group received corn oil via gastric tube. At the end of the 21 days of the experimental protocol, the testis tissues were extracted and immersed in Bouin's solution. Five µm sections were taken from the tissue and stained immunohistochemically with an anti-RIPK1 antibody. Histo (H)-score was calculated by determining the intensity of immunoreactivity in the sections.

Results: The results showed that the mixed dose group and all high-dose group round spermatids had excessive RIPK1 immunoreactivities compared to the control group and individually low-dose group of both ($p<0.01$). This result might be responsible for poor sperm quality, motility disruptions, or other reproductive consequences.

Conclusion: In conclusion, RIPK1 overactivation may be essential in pathophysiological conditions caused by EDC exposure at high doses and their mixture. Further studies are now needed to evaluate RIPK1 overactivation in testis tissues.

Keywords: Apoptosis, endocrine-disrupting chemicals, necroptosis, RIPK1.

INTRODUCTION

Endocrine-disrupting chemicals (EDCs), one of the most critical toxic substances in industrialized countries, are used in many areas of daily life and constitute an essential risk factor for human health. Epoxy resins and polycarbonates are present in many daily materials (food packaging, carboys, etc.). Therefore, it is essential to know these chemicals' short- and long-term biological consequences for health.¹ Bisphenol A (BPA) is a monomer in the structure of food packaging and carboys. 4-Nonylphenol (NP) is a chemical compound found in various detergents, plastics, paints, resins, cosmetics, emulsifiers, solvents, and other daily products.^{1,2}

Most of these chemicals or their degradation products, which are xenoestrogens, can be mutagenic, estrogenic, toxic, or carcinogenic.¹ These substances can influence the metabolic

functions of hormones involved in reproduction and development by blocking or altering the natural hormone's ability to bind to a specific receptor site or by acting as a hormone. These chemicals may have anti-hormonal effects, seriously harming the body's tissues and organs, including the immunological, neuroendocrine, and reproductive systems.³ For this reason, it is vital to examine the possible indirect or direct effects of BPA and NP at the molecular level, and it is predicted that this subject is among the significant research areas.¹

These substances are exposed daily through direct absorption, inhalation, contact with contaminated water, packaging of vegetables, fish, milk, fruit, and grains, and ingestion.⁴ NP and its metabolites are lipophilic and accumulate in the body due to their long half-life and can be found in human milk, blood, urine, and body tissues.⁵ According to studies, NP has several

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Cite this article as: Tüfekci KK. Immunohistochemical investigation of the effects of different doses of some endocrine-disrupting chemicals on receptor-interacting serine/threonine-protein kinase 1 (RIPK1) levels in the testis. *Kastamonu Med J.* 2023;3(3):165-169.

harmful effects on the body, including toxicity to the immune system, the nervous system, the embryo, and the reproductive system. The primary mechanism of toxicity of BPA and NP is to increase lipid peroxidation and ROS by disrupting the oxidant-antioxidant balance.^{6,7}

BPA and NP may substantially decrease sex hormone activity and induce infertility in both genders.⁸ A decrease in serum cortisol levels and increased levels of hormones like progesterone, estrogen, and luteinizing hormone were observed due to BPA damage.⁹ It has been found that female exposure to BPA changes the endometrial wall's thickness.⁹ BPA can also suppress the immune system and reduce T cells and antioxidant genes. These endocrine disruptors have serious genotoxic and cytotoxic effects, and there is an urgent need to detect these effects and develop preventive methods.¹⁰

The antioxidant systems have become unbalanced due to BPA and NP, particularly regarding the reduced activity of antioxidant enzymes and genes, the oxidation of proteins and lipids, and the subsequent production of free radicals that lead to cell oxidative stress.¹¹

According to investigations, even at low concentrations, BPA and NP can impair the male reproductive organs. These consequences include lower sperm production and motility, apoptosis of Leydig cells and germ cells, testis weights, and delayed growth and maturation.¹²⁻¹⁴ The chemical structure of BPA can cause harmful effects by allowing it to bind to nuclear estrogen receptors, resulting in changes in cell proliferation, apoptosis, or migration. In addition, it has been reported that exposure to both adversely affects the architecture of the testicular tissue, intracellular antioxidants, and immune-inflammatory mediators.¹³

Various environmental factors may cause dysfunction in tissues by inducing apoptosis and necroptosis. RIPK1 plays a role as a regulator in both cell death pathways. Studies have shown that one of the essential physiological functions of necroptosis is to encourage testicular aging in mice.¹⁵ Although the role of apoptosis in testicular tissue in EDC exposure has been frequently investigated in the literature, it has not yet been determined whether RIPK1 plays a role in testicular pathology. Therefore, in this study, we evaluated the immunohistochemical effects of RIPK1 on the mechanism of damage to testicular tissue in rats applied to various doses of BPA and NP.

METHODS

Animals and Study Groups

For this study, 42 male Wistar albino rats (10 weeks old, 250±50 g) were used. Kastamonu University Animal Experiments Local Ethics Committee approved the experimental protocol (Date: 01.08.2023 and Number: 26). ARRIVE guidelines were followed for all animal research. The study groups and the procedures applied to the groups are given in **Table 1**. BPA and NP were dissolved with minimal dimethyl sulfoxide (DMSO) and mixed with corn oil. The control group received the same amount of DMSO-containing corn oil. Mix (low) group (25 mg/kg) and Mix (high) group (100 mg/kg) were given equal amounts of each of BPA and NP. All agents were administered orally by gastric tube as 4 mL/kg.¹⁶

Table 1. Study groups.

Group	Compound	Dose
Control	Corn oil	5 ml/kg
BPA (Low)	BPA (Bisphenol A, Sigma-aldrich 239658)	25 mg/kg
NP (Low)	NP (4-Nonylphenol, Acros organics, 41624001)	25 mg/kg
Mix (Low)	BPA+NP	25 mg/kg
BPA (High)	BPA	100 mg/kg
NP (High)	NP	100 mg/kg
Mix (High)	BPA+NP	100 mg/kg

After 21 days of the experimental procedure, the rats were anesthetized, and testis tissues were removed for the immunohistochemical analysis.

Tissue Preparation

Testis tissues were fixed in Bouin's solution for 24 hours. Tissues were dehydrated and then cleaned using an ethyl alcohol series in rising strength and xylene after fixation. After that, paraffin was used to embed the testicular tissues. The paraffin blocks were then sectioned using a rotary microtome (Leica, RM2525, Germany) to a thickness of 5 µm and taken on poly-lysine covered slides.

Immunohistochemical Analysis

The streptavidin-biotin-peroxidase complex method was used for immunohistochemical staining. Sections taken were deparaffinized, then citrate buffer (10 mM, pH 6.0; 10X-T0050; Diapath) was used for heat-induced antigen retrieval after tissues were left at room temperature for 20 minutes and then rinsed with phosphate-buffered saline (PBS). Using 3% hydrogen peroxide, the endogenous peroxidase activity in the tissue was inhibited. The UltraV block solution was then incubated with the sections and incubated with RIPK1 (17519-1-AP, Proteintech, 1/100) antibody overnight at four °C. Slides were treated with a biotinylated secondary antibody (Thermo Scientific TL-125-PB) after being rinsed with PBS. The antibody binding sites were visualized using AEC chromogen (TA-060-HA, Thermo Fisher Scientific, LabVision Corporation). Gill's hematoxylin was used for counterstaining for 2 min, and then stained sections were evaluated under a light microscope (Zeiss Axiolab 5, Jena, Germany).¹⁷

Immunohistochemical Evaluation and H- Score

RIPK1 immunoreactivity was evaluated by scoring the positive staining density in sections. Antibody staining in the plasma membrane, cytoplasm, and nucleus was accepted as an evaluation criterion for positive staining. Staining density in the sections scored as (-); negative, (+); weak, (++) medium, (+++); intense staining.^{17,18} In addition, the H-score obtained by multiplying the staining intensity and the percentage of positive cells was calculated. The expression level of the RIPK1 antibody was statistically compared according to the mean value of the obtained H-score.¹⁷

Statistical Analysis

The statistical analyses were performed using the SPSS 26.0 (IBM SPSS Statistics, IBM Corporation, Chicago, IL) software for MAC. The data were analyzed for normality using the Shapiro-Wilks test. One-way ANOVA (Tukey's post-hoc test) was used to examine the data, and the p<0.05 value was considered significant.

RESULTS

RIPK1 Immunohistochemistry and H-Score

No positive reaction was detected in the negative control. The immunoreactivity of RIPK1 was weakly positive in the Control group's round spermatids and moderate in interstitial cells. The strong positive immunoreactivity of RIPK1 was detected in round spermatids cells in the low mix dose, high dose of BPA and NP, and high mix group. Also, round spermatids had moderate immunoreactivity in the low dose of BP and NP groups (Table 2). Weak RIPK1 immunoreactivity was observed in elongated spermatids in all groups other than the Control groups. Interstitial cells of BPA low and high doses were shown weak positive when comparing the other NP and Mix groups (Fig 1).

The H score of the high dose of BPA, NP, and their mix group was significantly higher than the Control and their low dose groups ($p < 0.01$) (Fig 2).

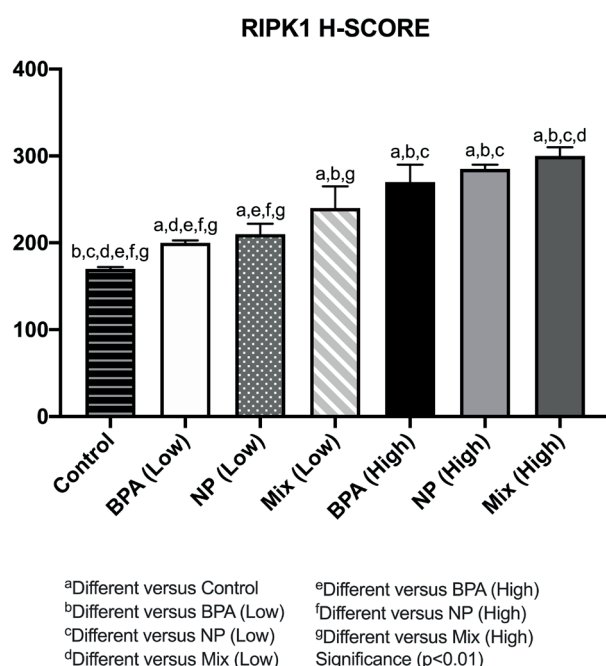


Fig. 2. Statistical analysis of the H-score of RIPK1 staining. The H-score of the mix low dose and high-dose groups was significantly higher than that of the Control groups ($p < 0.01$). The data were displayed as mean $\pm$ SD.

Parameters	Negative Control	Control	BPA (Low)	NP (Low)	Mix (Low)	BPA (High)	NP (High)	Mix (High)
Spermatogonium	-	-	-	-	-	-	-	-
Primer spermatocyte	-	-	-	-	-	-	-	-
Sekonder spermatocyte	-	-	-	-	-	-	-	-
Elongated spermatids	-	-	+	+	+	+	+	+
Round spermatids	-	+	++	++	+++	+++	+++	+++
Spermatozoon	-	-	-	-	-	-	-	-
Sertoli cells	-	-	-	-	-	-	-	-
Interstitial cells	-	-	+	-	-	+	-	-
Peritubular myoid cells	-	-	-	-	-	-	-	-

-: No staining; +: weak positive; ++: moderate positive; +++: strong positive.

DISCUSSION

It has been established that exposure to hazardous environmental pollutants harms the reproductive system. Previous research has demonstrated that exposure to BPA and NP impairs sperm cell integrity and daily sperm production.¹⁹ Among the studies performed, significant increases in TNF Alpha, TNF Beta, and Caspase 3 levels were observed in BPA and NP exposure, and histological changes such as decreased spermatozoa in the lumen of seminiferous tubules and impaired germinal epithelium were detected.¹⁶ In the present study, we found that different doses of these EDCs immunohistochemically altered the expression level of RIPK1 in testicular tissue. We used low and high dosages of BPA, NP, and their combined forms in a rat model. When comparing the high-dose group to the control and low-dose BPA and NP in testis, we found that the H-score of testicular RIPK1 immunoreactivity in both individual and mixed groups was considerably higher in the high-dose group. In this study, we dissolved the substances using DMSO, even in minimal amounts, it is known that DMSO is toxic to germ cells, so the weak immunoreactivity we detected in the control group may be the effect of DMSO. A pure control group is needed to prove this. In the BPA and NP treatment groups, it was

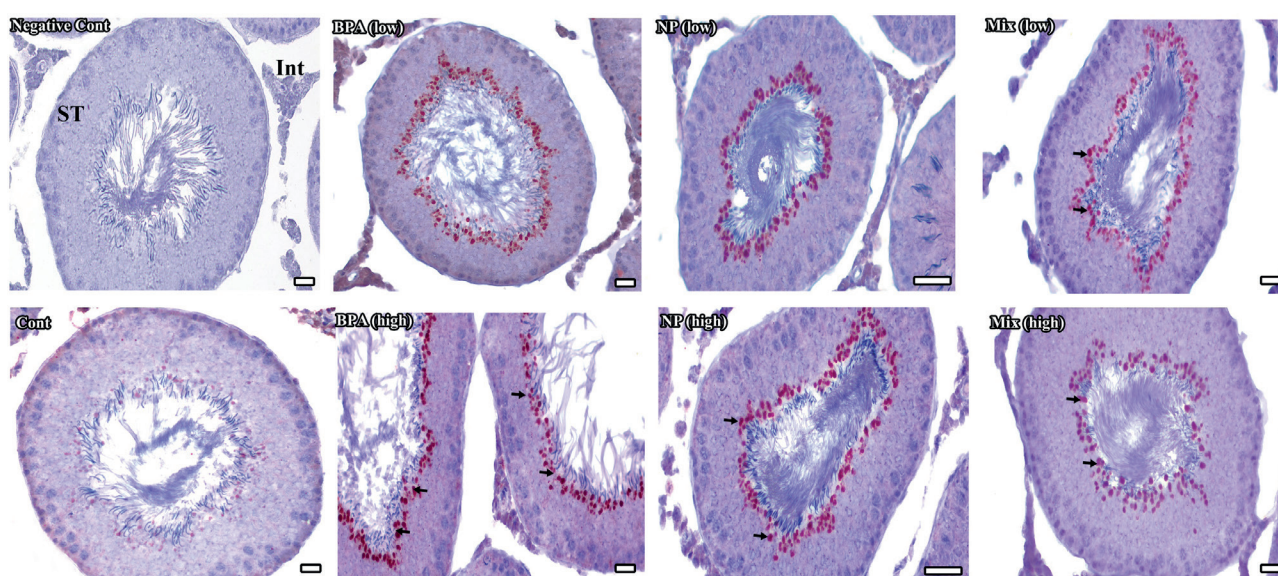


Fig 1. Analysis of RIPK1 expression by immunohistochemistry (IHC) in testicular tissues. Representative staining of RIPK1 in the negative control group is negative, and the control group is weakly positive in round spermatids. Strong positive staining was observed in round spermatids in the low mix dose, high dose of BPA and NP, and high mix group. Strongly positive staining cells (arrow); seminiferous tubules (ST); interstitial cells (Int). The scale bar shows 40 μ m for all panels.

determined that round spermatids' key player of apoptosis and necroptosis was overactivated. These findings suggest that the testis is damaged by RIPK1 activation against these chemicals. In a study similar to our findings, RIPK1-mediated apoptotic signaling pathway activation was detected, and it was found that it induced cell apoptosis and caused male reproductive toxicity.²⁰

In various studies, BPA and NP have been found to cause biochemical and histological changes in testicular tissue.²¹ Studies have noted apoptosis in germ cells, Sertoli and Leydig cells, and vacuolation in seminiferous tubules.^{16,22,23} In a study, apoptotic formations and a significant increase in serum caspase three levels were detected in the germinal cells of rats exposed to 100 mg/kg doses of BPA, NP, and their mixture.¹⁶

According to one study, BPA and NP have harmful effects that are dose-dependent on several parameters, including sperm toxicity, testicular weight, and prostate gland weight.¹³ Studies confirmed that this substance causes oxidative stress by lowering SOD, CAT levels, and GPx in different tissues.^{16,24}

In an ultrastructural study, enlarged intracellular spaces, cytoplasmic vacuoles, and apoptotic bodies were detected in round spermatids in testicular tissue treated with 12.5 µg/kg and 25 µg/kg BPA.²⁵

In a different study, exposure to BPA at doses of 160, 480, and 960 mg/kg/day, mice's germ cells, and Leydig cells showed signs of apoptosis and increased expression of Fas/FasL and caspase-3.¹⁴

Our research found that RIPK1 was strongly expressed in round spermatids, mainly at mixed low doses and high doses. None of the studies to date have examined the role of RIPK1 as a mechanism of the adverse effects of EDCs in testicular tissue. Nevertheless, it was discovered that SH-SY5Y cells treated with low doses of BPA exhibited RIPK1-mediated necroptosis.²⁶ Weak positive staining of interstitial cells was observed in both low and high doses of BPA. RIPK1 activation may affect Leydig cells, accompanying the endocrine-disrupting effects of BPA.

Different studies have determined that RIPK1 activation can affect sperm quality by causing necroptosis activation together with RIPK3 and MLKL.^{27,28} Additionally, a study found that necroptosis in mice's testicles may facilitate the male reproductive system's deterioration due to aging.¹⁵ Another study revealed that semen quality was reduced in bilateral varicocele and that activating RIPK1, RIPK3, and HSP 90 was effective.²⁷ A study it was shown that SnS2 nanoflowers increased the necroptosis-related RIPK1-RIPK3-MLKL signal in testicular tissue by inducing oxidative stress.²⁸

As in these studies, RIPK1 activation, which we detected in our study, may adversely affect sperm quality and number motility. Further studies are needed to prove this. However, when the death signal is activated, RIPK1 and RIPK3 collaborate to phosphorylate the latter, which is essential for initiating necroptosis. RIPK3 stimulation also phosphorylates MLKL, which drives necroptosis.²⁹ Therefore, to be able to talk about definite necroptotic cell death, RIPK3 and MLKL levels should also be evaluated in future studies. Another interesting finding in this study is that RIPK1 is expressed only in round and elongated spermatids in the seminiferous tubule epithelium. The reason why these cells are more prone to RIPK1-mediated death than other-stage germ cells or Sertoli cells remains to be investigated.

CONCLUSION

In conclusion, our study found that RIPK1 levels increased in round spermatids at different doses of BPA and NP. Since RIPK1 has an essential place in the necroptotic death pathway and in the apoptotic death pathway, its varying levels may cause changes that may affect testicular function.

ETHICAL DECLARATIONS

Ethics Committee Approval: Kastamonu University Animal Experiments Local Ethics Committee approved the experimental protocol (Date: 01.08.2023, Decision No: 26).

Informed Consent: Note applicable.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

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