Reproductive Sciences (2024) 31:3462–3474
https://doi.org/10.1007/s43032-024-01677-7

REPRODUCTIVE BIOLOGY: ORIGINAL ARTICLE

Bisphenol AF Caused Reproductive Toxicity in Rats and Cineole Co-Treatment Exhibited Protective Effect

Ahmet Uyar[1] · Mustafa Cellat[2] · Özgür Kanat[3] · Muhammed Etyemez[2] · Tuncer Kutlu[1] · Mehmet Zeki Yılmaz Deveci[4] · İlker Yavaş[5] · Müslüm Kuzu[6]

Received: 15 May 2024 / Accepted: 8 August 2024 / Published online: 19 August 2024

Abstract

Bisphenol AF (BPAF) is increasingly used and now found in products intended for human consumption. The protective effect of 1,8-cineole (CIN) against BPAF-induced reproductive toxicity was investigated. Four groups were created, with each group consisting of eight rats: control, BPAF (200 mg/kg), CIN (200 mg/kg), and BPAF + CIN groups. The results demonstrated that the BPAF group exhibited a decline in testosterone levels and a decrease in sperm parameters compared with the control. Additionally, higher levels of MDA were observed, along with lower levels of GSH and GPx activity. CAT activity also decreased slightly. Tnf-α, Nf-κB levels were significantly higher, and caspase-3 expression was elevated, while PCNA expression decreased. BPAF significantly increased tissue degeneration compared with the control. However, the BPAF + CIN group showed statistically significant improvements in sperm parameters, except for concentration. They also exhibited an increase in testosterone levels and an improvement in MDA and GSH levels compared with the BPAF group. However, GPx activity partially enhanced. Tnf-α and Nf-κB levels were significantly reduced, and caspase-3 levels declined while PCNA and Bcl-2 levels increased. The Johnsen Testicular Biopsy score showed a substantial increase. Overall, these results suggest that CIN co-treatment in rats enhanced reproductive health and exhibited antioxidant, antiapoptotic, and anti-inflammatory properties against BPAF-induced testicular damage.

Keywords Endocrine disruptor · Oxidative stress · Infertility · Natural antioxidant · Herbal medicine

Introduction

Researchers have recently turned their attention to endocrine-disrupting chemicals (EDCs), which refer to substances that interfere with the proper functioning of the endocrine system. Comprehensive research has been conducted to evaluate the impact of these substances on human health, particularly their association with reproductive disorders [1]. Analogues of bisphenol A (BPA) have been reported to have toxic effects on living beings, particularly on the endocrine and reproductive systems. The presence of trifluoromethyl (CF_3) in bisphenol AF (BPAF) creates a structural difference between the two molecules when compared to BPA. The electronegativity of the CF_3 group is substantially higher than that of the methyl (CH_3) group, as is its reactivity potential. This suggests that BPAF may be more harmful than BPA. BPAF has been detected in meat, vegetables, and shellfish due to its increased industrial use. Human exposure to bisphenols, including the accumulation of bisphenols in adult and pregnant individuals' urine, blood, and adipose tissues, is caused by absorption and contact with these chemicals [2–4]. Many polymers, including fluoropolymers, fluoroelastomers, polyamides, polyimides, polycarbonates, polyesters, and food contact polymers are produced using BPAF as a monomer [5, 6]. Although BPAF-containing fluoroelastomers have received FDA approval, [7], exposure limits and toxicological effects of BPAF are unknown. Due to their high lipophilic nature, low water solubility and volatility, and biological resilience, chemicals like BPAF are regarded as persistent and common environmental contaminants [8]. BPAF can serve as an EDC since it acts as an antagonist at low concentrations and an agonist at high concentrations for the estrogen receptors alpha and beta [9]. BPAF has been shown to have an estrogenic effect by disrupting the development of reproductive organs in chicken embryos when administered at a level of 210 nmol/g [10]. Additionally, BPAF's ability to function as an androgen and thyroid receptor antagonist in vitro has

Extended author information available on the last page of the article

been documented in several studies [11]. Exposure to BPAF is reported to negatively impact the development of mammalian testicles, raising concerns about future reproductive risks in mammals, including humans [12]. Male infertility is a multifaceted and complex condition that can result from various factors, including infections, genetics, environmental influences, and dietary factors [13].

Plants are considered an alternative in treating diseases in various parts of the world, thanks to the healing properties of the phytochemicals they contain [14]. It has been suggested that herbal remedies may mitigate the effects of toxic substances exposed due to environmental or occupational factors [15]. The primary element responsible for the therapeutic benefits of eucalyptus oils is CIN, also known as eucalyptol [16]. With an excellent pharmacokinetic profile, CIN is quickly absorbed when applied topically, orally, or via mucosal membranes. Depending on the method of administration, the drug reaches its peak plasma concentration in 1 to 3 h and can be excreted from the body through the lungs and kidneys [17]. The FDA has recognized eucalyptus essential oil as safe and non-toxic. Additionally, Europe and Japan have approved eucalyptus extracts for use as food additives. Studies have revealed that CIN is safe when taken in regular doses [18]. Animal studies involving CIN have not shown any evidence of mutagenicity, reproductive or developmental harm, or carcinogenicity. Rats administered CIN orally have exhibited a remarkably high LD_{50} (2.5 g/kg) [19]. Furthermore, eucalyptus leaf extract has been shown to positively affect the physiology of the male reproductive system [20]. No previous studies have investigated the curative effectiveness of CIN treatment against BPAF-induced reproductive toxicity. Therefore, this study aims to, for the first time, reveal the protective effects of CIN treatment against BPAF-induced reproductive damage in rats through spermatological, biochemical, histological, and immunohistochemical assessments.

Methods

Chemicals

The study made use of solvents and chemicals that were purchased from Sigma Chemical Co. CIN was obtained from Sigma-Aldric (St Louis, MO, USA). Testosterone, nuclear factor kappa B (Nf-κB) and tumor necrosis factor-alpha (Tnf-α) ELISA kits acquired from Bioassay Technology Laboratory, located in China.

Animals

This study, used healthy male *Wistar Albino* rats, with weights 200–250 g, as the subjects for the experiments. All experimental protocols adhered to ethical standards for the housing, nourishment, and utilization of laboratory animals. The rats were maintained under a consistent environment, featuring a 12 h light and 12 h dark cycle, with a constant temperature maintained at 24 ± 1 °C. Throughout the duration of the study, the rats had unrestricted access to standard pellet feed and tap water.

Animal Groups

The dose of CIN was determined as described by Lima and co-workers [21], and the experimental model of testicular toxicity in rats induced by BPAF was based on the method developed by Feng and co-workers [5]. The study was planned as 28 days in line with the previous study [22], and the groups underwent the following procedures:

Group 1 (Control): Rats in this group received a daily oral gavage of 1 ml of corn oil for a period of 28 days.
Group 2 (BPAF): Rats in this group received daily doses of BPAF (200 mg/kg b.w.) dissolved in corn oil via oral gavage for 28 days.
Group 3 (CIN): Rats were orally administered with 200 mg/kg b.w of CIN in 1 ml of saline containing 2% Tween 80 for 28 days.
Group 4 (BPAF + CIN): Rats in this group were given BPAF (200 mg/kg b.w.) in corn oil by oral gavage daily for 28 days. Additionally, the group was administered CIN once daily for 28 days via oral gavage using 1 ml of physiological saline containing 2% Tween 80 at a dose of 200 mg/kg b.w.

All treatments during the experimental process were conducted in accordance with the approval decision of the Hatay Mustafa Kemal University Local Ethics Committee for Animal Experiments, with the decision number 2022/01–02.

Preparation of Serum Samples

Under a combination of ketamine anesthesia (60 mg/kg, intramuscularly) and xylazine anesthesia (10 mg/kg, intramuscularly), blood samples were collected intracardially from all rats into serum tubes without anticoagulant 24 h after the latest treatment. Blood serum was obtained by centrifuging the blood samples at 2200 g for 15 min. Then, fresh samples were then transferred to Eppendorf tubes and testosterone levels were measured.

Preparation of Tissue Samples

After the blood collection procedure, necropsies were conducted on the rats, and testicular tissues were extracted and cleaned with physiological saline. A portion of the tissue samples that were collected were reserved for biochemical analyses. The remaining portions of the testicular tissues were preserved in 10% buffered formaldehyde for subsequent immunohistochemical and histopathological analyses.

Measurement of Sperm Parameters

Spermatological tests, including assessments of density, motility, abnormalities, and the dead/viable ratio, were conducted following the procedure outlined by Türk et al. After decapitation, a segment of the right epididymis was removed and cut into pieces. These pieces were then crushed thoroughly with forceps for two minutes in 1 ml of physiological saline (0.9% NaCl), using a scalpel and scissors. Following a 4-h incubation period, the described procedure was used to extract the spermatozoon-containing supernatant [23].

Measurement of Oxidative Stress Parameters

Testicular tissues stored at -80 °C were homogenized according to the procedure outlined in Cellat et al.'s study. A portion of this homogenate was set aside for use in the malondialdehyde (MDA) assay. The collected supernatant aliquot was evaluated for levels of glutathione (GSH), catalase (CAT), and (glutathione peroxidase) GPx activity [24]. Spectrophotometric measurement of antioxidant parameters was conducted. MDA levels were evaluated using the method outlined by Ohkawa et al. [25], while GSH levels were determined following the method outlined by Beutler and colleagues [26]. For CAT activity, the procedure described by Aebi was used [27], and for GPx activity, the method of Beutler was followed [28]. The protein levels in all samples were determined using Lowry and colleagues' method [29].

Inflammation Markers and Measurement of Testosterone Levels

Nf-κB (Catalog No: ELK5691, Detection Range: 0.32–20 ng/mL, ELK Biotehcnology, USA) and Tnf-α (Catalog No: ELK1396, Detection Range: 15.63–1000 pg/mL, ELK Biotehcnology, USA) proteins were evaluated through the ELISA method, following the commercial kit procedure to measure the level of inflammation in the tissues. The level of testosterone was also evaluated using the ELISA technique (Catalog No: ELK8314, Detection Range: 156.25–10,000 pg/mL, ELK Biotehcnology, USA). The serum samples were measured using the protocol recommended by the manufacturer of the kit.

Histopathological Examination

The procedures outlined in the study by Türk et al. were applied to tissue samples [30] that had been preserved in 10% buffered formaldehyde for 72 h. To conduct pathological examinations, testicular tissue samples were processed through alcohol and xylene series, and paraffin blocks were prepared. Sections of 4–5-micron thickness were obtained using a microtome (Leica RM 2135). Tissue samples from each rat were stained with Hematoxylin–Eosin and monitored under a light microscope [31]. The results were evaluated according to criteria of the Johnsen Testicular Biopsy Score [32].

Immunohistochemical Examination

Proliferating cell nuclear antigen (PCNA), Caspase-3, and B-cell lymphoma 2 (Bcl-2) expressions were assessed by the streptavidin-peroxidase technique (ABC) following the staining instructions supplied by the manufacturer of the kits. The methodology used was the same as described in the research conducted by Uyar et al. [33]. For each tissue sample, the following primary antibodies were used: Bcl-2 (bell-2 monoclonal antibody (DAKO A/S, Glostrup, Denmark; 1/100 dilution) monoclonal, caspase-3 (ThermoFisher Scientific, USA; 1/100 dilution) polyclonal, and PCNA (St John's Laboratory Ltd. London, UK; dilution ratio 1/100) antibody. The staining intensities of immunoreactivity in testis samples were categorized as mild (+), moderate (++), and severe (+++).

Statistical Analysis

Statistical analysis was conducted by IBM SPSS Statistics Software Version 23.0. Group means were compared using one-way ANOVA, and Tukey's test was performed to identify significant differences. The data was presented as mean ± standard deviation. Statistical significance was considered at a P-value < 0.05.

Results

CIN Co-Treatment's Effect on Sperm Parameters

To evaluate the protective effect of cineole on sperm quality, The study analyzed four parameters—concentration, sperm motility, abnormal sperm ratio, and dead/live sperm ratio.

Table 1 Spermatological and biochemical analysis results

Variables / Groups	Control	BPAF	CIN	BPAF + CIN
Motility (%)	74.38 ± 6.23^{a}	51.25 ± 8.35^{c}	77.50 ± 5.98^{a}	68.13 ± 10.33^{b}
Concentration (million/right cauda epididymis)	160.63 ± 45.70^{a}	101.25 ± 24.31^{b}	167.50 ± 52.10^{a}	131.63 ± 21.51^{c}
Dead/Live Sperm Rate (%)	18.00 ± 1.51^{c}	39.00 ± 7.37^{a}	16.25 ± 5.99^{c}	27.50 ± 3.66^{b}
Abnormal sperm rate (%)	7.38 ± 1.51^{c}	19.25 ± 3.49^{a}	8.13 ± 1.13^{c}	14.00 ± 1.51^{b}
MDA (nmol/gr prot)	4.02 ± 1.14^{c}	11.16 ± 1.79^{a}	3.61 ± 0.66^{c}	8.28 ± 1.65^{b}
GSH (nmol/gr prot)	6.92 ± 1.04^{a}	2.93 ± 1.66^{c}	7.07 ± 1.03^{a}	4.49 ± 1.85^{b}
GPx (IU/gr prot)	98.34 ± 16.46^{a}	64.68 ± 17.18^{b}	108.25 ± 26.74^{a}	76.94 ± 16.48^{b}
CAT (U/gr prot)	100.57 ± 21.85	91.43 ± 14.90	104.43 ± 12.08	97.96 ± 12.16
Tnf-α (pg/mg prot)	$169{,}69 \pm 54{,}66^{c}$	$295{,}28 \pm 38{,}38^{a}$	$162{,}45 \pm 41{,}41^{c}$	$240{,}39 \pm 51{,}13^{b}$
Nf-κB (pg/mg prot)	$1{,}60 \pm 0{,}52^{c}$	$5{,}03 \pm 1{,}02^{a}$	$1{,}77 \pm 0{,}48^{bc}$	$2{,}65 \pm 1{,}21^{b}$

Values represent mean ± SD for each group. Different superscript letters (a, b, c) within the same line show statistically significant differences ($p < 0.05$) between the groups

However, these parameters were similar between the CIN and control groups ($p \geq 0.05$). Sperm concentration, motility values decreased, while abnormal and dead/live sperm ratios increased in BPAF group compared to control. However, in comparison to the BPAF group, the CIN-treated group showed a significant improvement in all values (Table 1, $p < 0.05$).

The impact of Co-Treatment with CIN on Antioxidant Parameters

To explore the antioxidant effect of CIN co-treatment against testicular damage induced by BPAF, the current study evaluated levels of GSH and MDA and enzyme activity for CAT and GPx in testicular tissues. The data indicated that CAT enzyme activity decreased slightly in the BPAF group compared with the control, while there was a slight increase in the CIN co-treatment group compared with the BPAF group ($p > 0.05$). It was found that the levels of MDA increased, GSH decreased, and GPx activity was reduced in the BPAF group compared with the control ($p < 0.05$). However, when comparing the BPAF group to the BPAF + CIN groups, it was found that co-treatment with CIN significantly lowered MDA levels, raised GSH levels, and resulted in a slight increase in GPx activity ($p < 0.05$, Table 1).

The Impact of Co-Treatment With CIN on Inflammation Markers

To asses the impact of BPAF exposure on inflammation and the effectiveness of CIN treatment against it, testicular tissue samples were analyzed for levels of Tnf-α and Nf-κB. The findings revealed that administering BPAF increased testicular Nf-κB and Tnf-α levels compared with the control ($p < 0.05$). Both parameters significantly improved in the BPAF + CIN group compared to BPAF alone (Table 1).

Effect of CIN Co-Treatment on Testosterone Level

The study measured testosterone levels in serum samples from different groups of rats using the ELISA technique. The BPAF group had lower testosterone levels than the control group. However, testosterone levels were similar in the control and CIN groups. Interestingly, the testosterone levels significantly increased when comparing the BPAF + CIN group with the BPAF group ($p < 0.05$, Fig. 1A).

Effect of CIN Co-Treatment on Johnsen Scoring and Histopathological Findings

Macroscopic findings showed no difference in any of the experimental groups. Figure 1B presents the Johnsen Testicular Biopsy Scores of the groups. The results revealed that the CIN group and the control exhibited similar scores, while the BPAF group significantly differed from the control group. The BPAF + CIN group had higher scores than the BPAF and the control groups ($p < 0.05$). Microscopic examination revealed that the tubulus seminiferus contortus, Leydig cells, and the cells responsible for sperm production in the seminiferous tubules exhibited normal histological appearances, and a smooth spermatogenesis was observed in these tubules in the rat testicles in the control (Fig. 2A and 2B) and CIN (Fig. 2E and F) groups. Morphologically, the tubules had smooth outlines, prominent lumens, and thick walls that contained 4–6 rows of Sertoli cells and spermatogenic germinative epithelial cells.

In contrast, microscopic examination of the testicles in the BPAF group rats revealed distorted morphologies in many tubules, with degenerated basement membranes in recessed and protruding structures. Some tubules were atrophied, while in certain areas, only tubules composed of a basement membrane were present. Also, adjacent tubules were observed, the detachment of germinative cells from the basal membrane was detected, and some regions exhibited

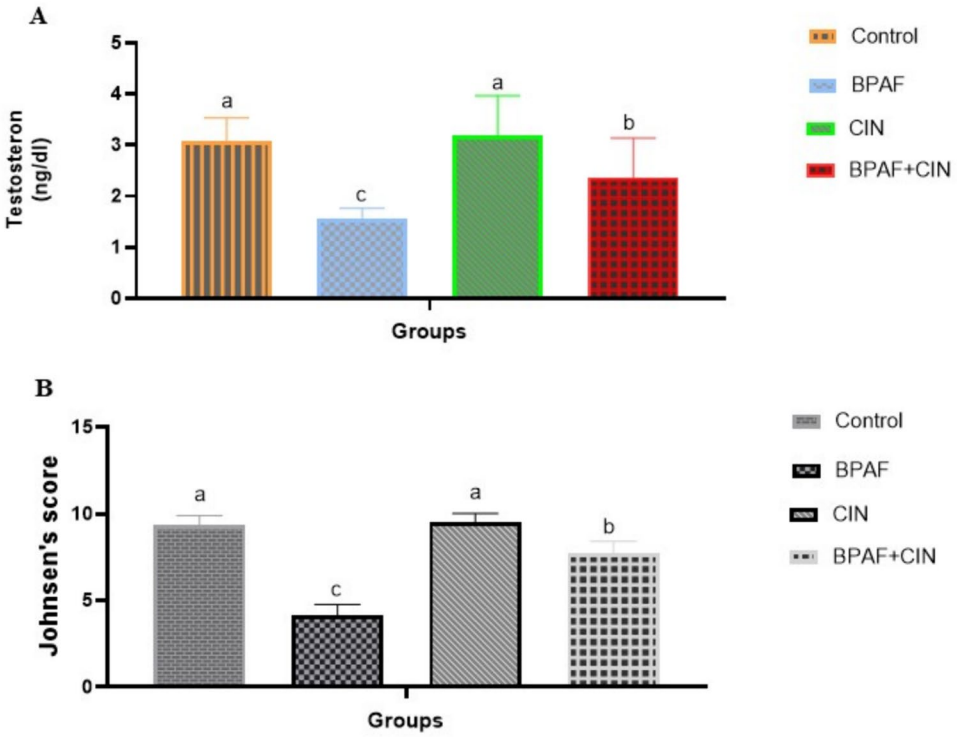

Fig. 1 **A** Serum testosterone levels, **B** Histopathologic variables in testes according to the Johnsen's score. Data represent the mean ± SD of eight rats in each group. Tukey test was used to determine the differences among the groups. Graphs (a–c) show significant differences ($p < 0.05$) among each group

significant loss or disorganization of the germinative epithelium. Detachment between spermatogonia and Sertoli cells was noted, along with germinal cell degeneration, nuclei shrinkage in some spermatogonia and Leydig cells, and a loss or reduction in spermatogenesis (Fig. 2C and D).

Regarding morphology, the tubules in the testicular tissues of the rats in the BPAF + CIN group were almost completely normal, with fewer morphological abnormalities in certain tubules and the interstitial region, which contains Leydig cells, compared with the BPAF-treated group. Continuous development of spermatogenesis, including spermatozoa production, was observed in this group. The interstitial space, including Leydig cells, appeared normal, and vascular congestion was rarely observed (Fig. 2G and H).

Effect of CIN Co-Treatment on Apoptosis Markers and Cell Proliferation

To evaluate the impact of CIN co-treatment on apoptosis and cell proliferation in testicular toxicity induced by BPAF, we determined Caspase-3, Bcl-2, and PCNA expression levels in tissues through immunohistochemistry. Microscopic representations of immunohistochemistry staining are presented in Figs. 3, 4, and 5, while Fig. 6 illustrates the immunoreactivity results using caspase-3, Bcl-2, and PCNA primary antibodies. Caspase-3 immune reactions were scarcely observed in spermatogenic cells in control and CIN group rat testicular tubules. Bcl-2 immunoreactivity was rarely. In contrast caspase-3 immunoreactivity was notably moderate/severe (++/+++) in the BPAF group and mild (+) in the BPAF + CIN group. Bcl-2 immunoreactivity was mild (+) detected in the BPAF group but was moderate/severe (++/+++) in the BPAF + CIN group. PCNA antibody staining showed intense immunoreactivity in the Control and CIN groups at all stages of spermatogenesis. PCNA immunoreactivity was moderate/severe (++/+++) in the BPAF + CIN, and mild (+) in the BPAF group. Additionally, in the interstitial region, PCNA exhibited partial positivity in Leydig cells and capillary endothelium.

Discussion

Approximately 15% of couples suffer from infertility, with male infertility accounting for 30–50% of these cases. While various factors contribute to male infertility, lifestyle choices and environmental factors also play a significant role [34]. Male infertility can be attributed to factors including endocrine disruptors like bisphenol A and phthalates, herbicides, and metal pollution, all of which have detrimental effects on sperm parameters [35]. In this study, we aimed to explore the therapeutic potential of CIN in mitigating testicular toxicity induced by BPAF.

Semen analysis is a crucial initial step in diagnosing male infertility. When the sperm concentration falls below a certain threshold, the likelihood of achieving pregnancy decreases. The diagnosis of male infertility necessitates an assessment of parameters, including sperm motility,

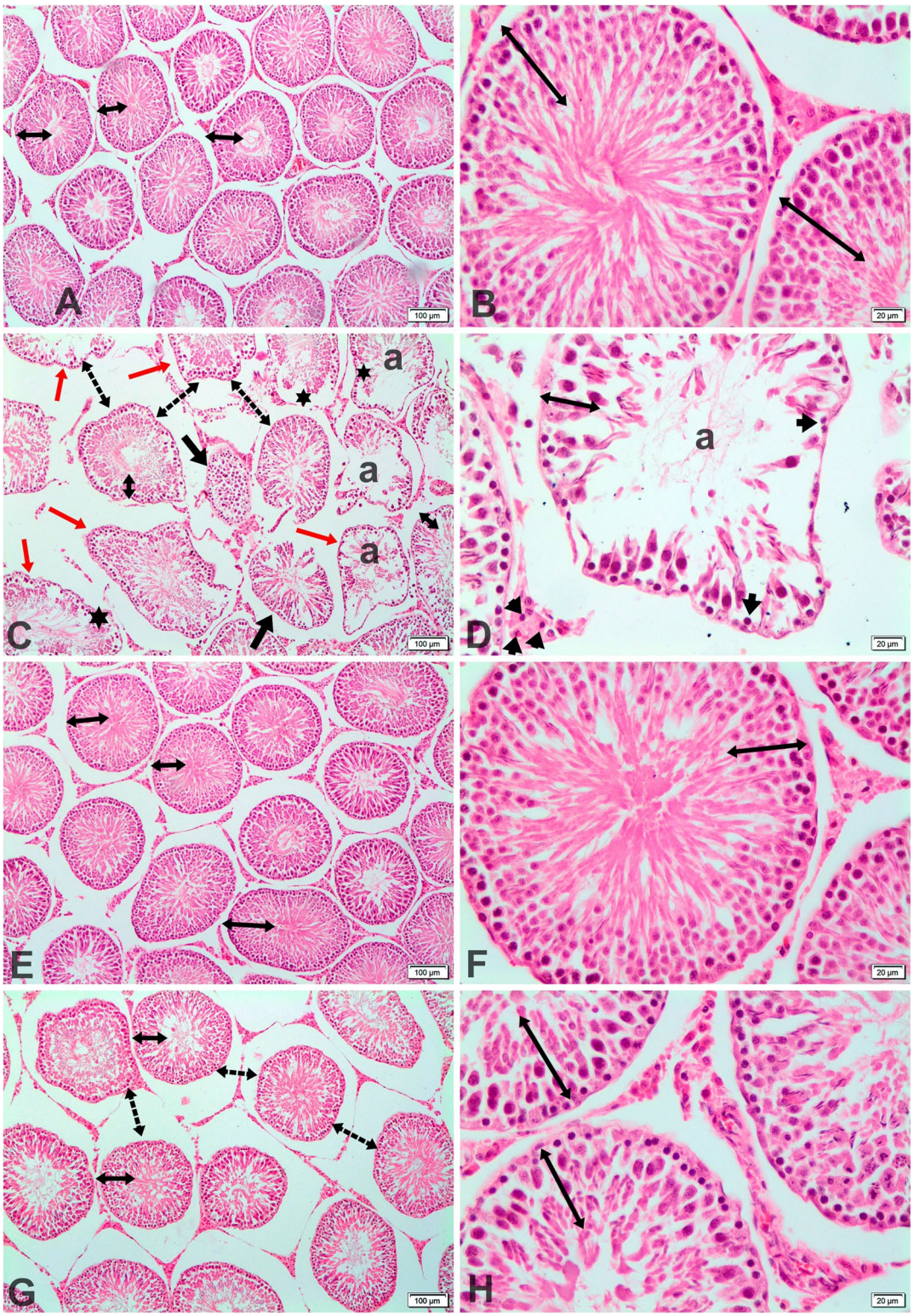

Fig. 2 Microscopical findings of the testis (H&E). **A** and **B**: Control group; Normal histological structure. **C** and **D**: BPAF group; Severe damage to the seminiferous tubules: Degenerative tubules with irregular contours and completely distorted morphological structure (Red arrow), spermatogenic cells separated from the basement membrane (star), thinning of the germinative epithelial wall/loss (two way arrow), pycnosis in germinative epithelium and Leydig cells (arrowhead), arrest in the spermatogenesis (a), increasing of intertubular distance (two way dashed arrow), atrophic tubule (black arrow), **E** and **F**: CIN group; Normal histological structure, **G** and **H**: BPAF + CIN group; Seminiferous tubule is close to normal histological structure

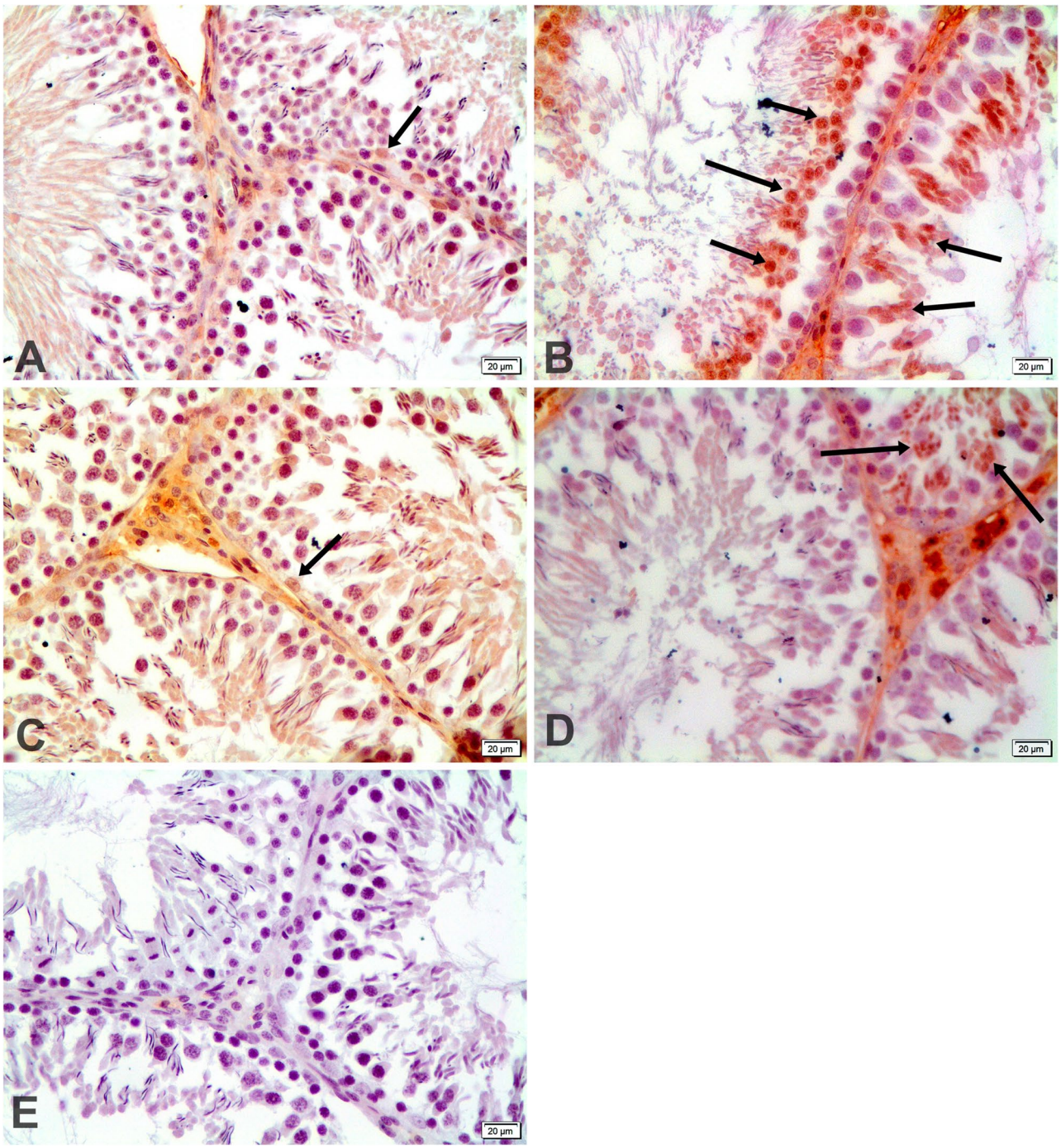

Fig. 3 Caspase-3 immunoreaktivity in seminiferous tubuli **A** Control group; rarely immunoreaktivity **B** BPAF group; moderate/severe degree **C** CIN group; rarely immunoreaktivity **D** BPAF + CIN group; slight degree **E** No staining; negative control

concentration, sperm count, motility percentage and normal forms [36]. The effect of BPAF exposure and the potential protective effect of CIN treatment on the male rat's reproductive health was determined by sperm analysis in groups. The results revealed that CIN therapy reversed the impairment in all parameters examined in the BPAF group. Previous research has also indicated that BPAF exposure negatively affects sperm counts and testicular development [37].

In separate studies, rats treated with Eucalyptus globulus extract, which contains significant amount of CIN [38], showed improvements in sperm parameters when exposed to diclofenac toxicity. This effect is believed to be mediated through the enhancement of the cell's antioxidant system,

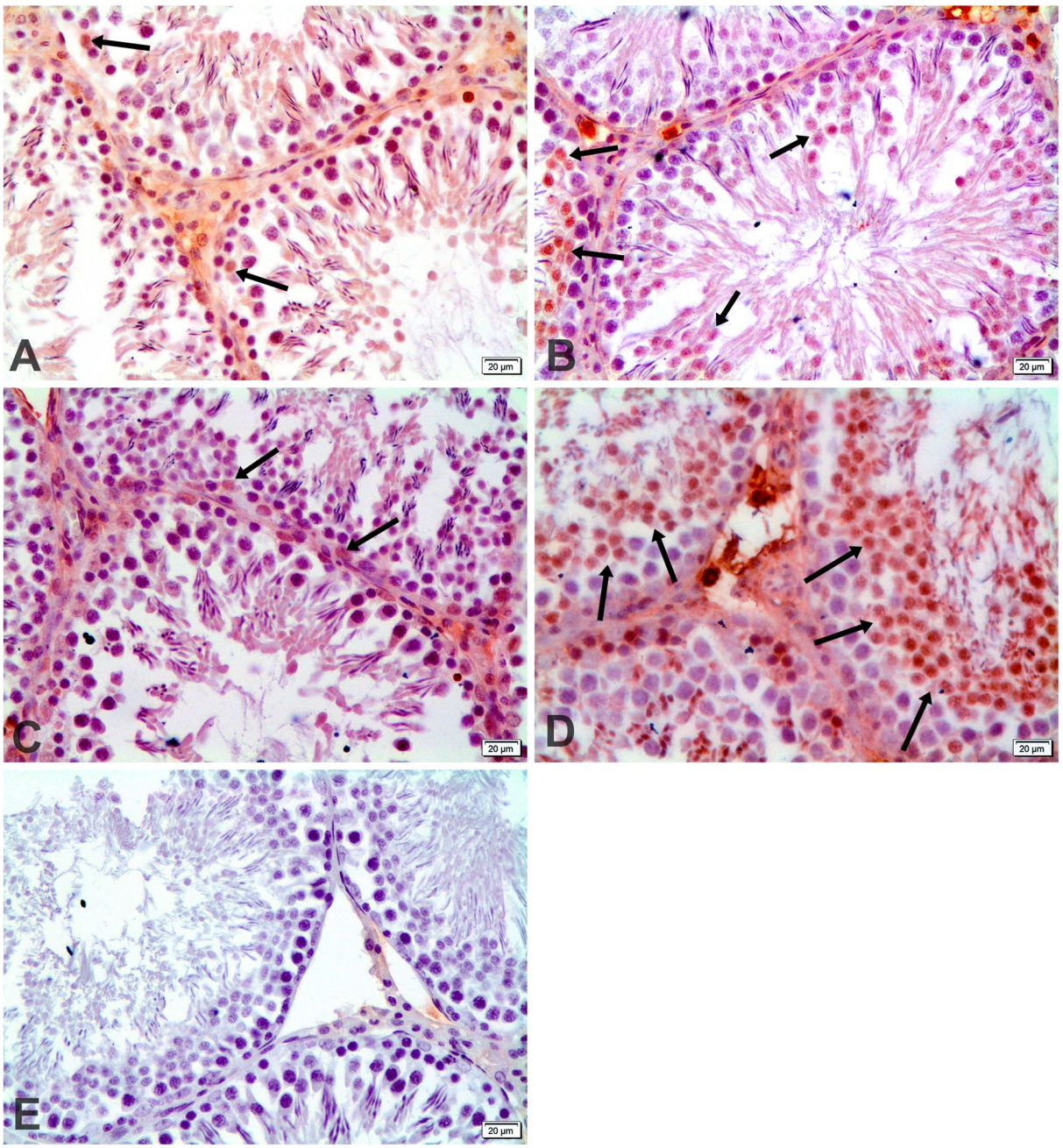

Fig. 4 Bcl-2 immunoreaktivity in seminiferous tubuli **A** Control group; rarely immunoreaktivity **B** BPAF group; slight/moderate degree **C** CIN group; rarely immunoreaktivity **D** BPAF + CIN group; moderate/severe degree **E** No staining; negative control

particularly by increasing the level of GSH [39]. Given the influence of CIN on antioxidant parameters observed in our study, it is plausible that CIN exerts its effect on sperm parameters through a similar mechanism. Compared with the BPAF-exposed group, the BPAF + CIN group reduced tubular degradation and continued normal spermatogenesis. These findings provide further support for the observed results [20], confirming the curative effects of CIN against BPAF-induced alterations in sperm parameters and reinforcing the notion of normal spermatogenesis.

Sperm cells are highly vulnerable to oxidative stress due to their abundance of polyunsaturated fatty acids in their plasma membranes [40]. An indication of oxidative stress is an increased MDA levels caused by lipid peroxidation. The elimination of hydrogen peroxide is conducted by the endogenous antioxidant defence system's GPx and CAT, while tissue damage is caused by a decrease in GSH [41].

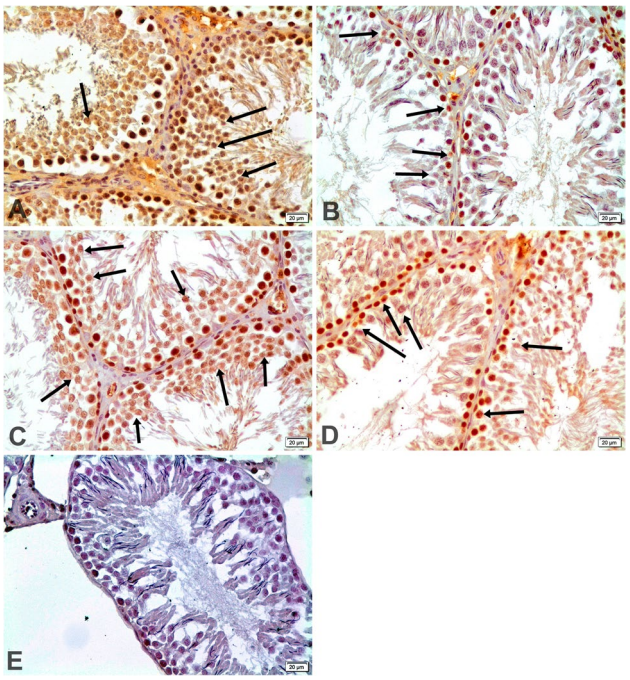

Fig. 5 PCNA immunoreaktivity in seminiferous tubuli **A** Control group; severy degree **B** BPAF group; slight degree **C** CIN group; severy degree **D** BPAF + CIN group; moderate/severe degree **E** No staining; negative control

We assessed the BPAF-induced oxidative stress in testicular tissue and the effectiveness of CIN co-treatment through measurements of MDA and GSH levels, as well as GPx and CAT activities. BPAF had a disruptive effect on these parameters in testicular tissue. A prior study reported a decrease in GPx activity in cells treated with BPAF, accompanied by increased ROS and MDA levels [42]. In a separate investigation, a strong association was found between the MDA levels and bisphenols in serum blood samples from residents of industrial districts [43]. While CIN co-treatment significantly improved MDA and GSH values, its impact on GPx activity was minimal. It has been documented that CIN, administered through the diet to fish, prevents the increase in MDA caused by crowding stress [44], and increases the GPx activity and GSH level against oxidative damage in the rats [45]. Taheri Mirghaed et al. showed that CIN therapy against ammonia-induced MDA increase in fish lowered the MDA level but had no effect on the antioxidant enzyme activities, suggesting that the antioxidant effect may be caused by its radical scavenging property [46]. It has been stated that antioxidant supplements may be beneficial in reproductive defects caused by oxidative stress [47, 48]. Other studies have also reported that antioxidant supplements have effects on improving sperm quality and reducing oxidative stress in cryopreservation processes [49]. When considering our results alongside existing literature, it can be concluded that CIN treatment has the potential to mitigate BPAF-induced oxidative stress, leveraging both its radical scavenging effect and its ability to enhance the endogenous antioxidant defence system.

Nf-κB is a transcription factor responsible for regulating the expression of certain genes, including proinflammatory cytokines and apoptotic markers [50]. Tnf-α is a 26 kDa transmembrane protein-structured cytokine that is secreted from fat cells and has a regulatory effect on the immune system [51]. We determined that Nf-κB and Tnf-α levels were significantly higher in the BPAF group's testicular tissues than in the control group. However, this increase was reduced by co-treatment with CIN. In another study, Wang et al. notified that BPAF stimulates the Nf-κB pathway and elevates Tnf-α expression in prostate tissue. They also reported that Nf-κB suppressors can mitigate the prostate-damaging effects of BPAF [52]. Furthermore, CIN exhibited its anti-inflammatory effect in acute pancreatitis by decreasing the Nf-κB expression and modulating the synthesis of cytokines such as serum IL-1β, IL-6, and Tnf-α [21]. Co-treatment with CIN exhibited anti-inflammatory effect by lowering Nf-κB and Tnf-α levels, which may reduce BPAF-induced toxicity in the testicles.

Testosterone is essential for male fertility and spermatogenesis [53]. Testosterone plays a crucial role in supporting healthy spermatogenesis, including spermiogenesis. Disruption of steroid hormone synthesis can impair sperm

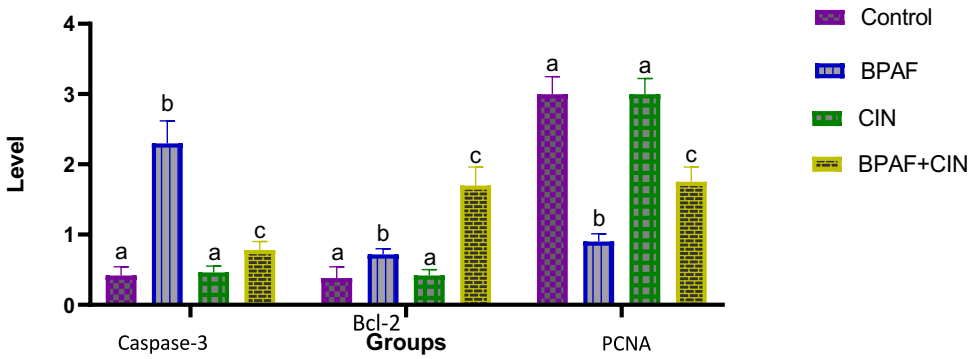

Fig. 6 Distribution of Caspase-3, Bcl-2 and PCNA immunoreactivity scores by groups. Data represent the means ± SD. of eight rats in each group. Tukey test was used to determine the differences among the groups. Graphs (a–c) show significant differences ($p < 0.05$) among each group

quality and spermatogenesis, affecting germ cell number or directly impacting germ cells [54]. Our investigation's results indicated that testosterone levels in the BPAF group were lower than the control group rats. Estrogen receptors (ERs) are abundantly expressed in the testicles and act a significant function in spermatogenesis. BPAF exposure has been reported to lead to decreased ERα levels, and these reduced testosterone levels may be partly associated with abnormalities in ER-dependent signalling [5]. A statistically significant increase in testosterone level was observed when comparing the BPAF + CIN group to the BPAF group. In a related context, it has been reported that testosterone levels increased in dose-dependent manner in rats administered an extract of the E*ucalyptus globulus*, which includes CIN and terpenes. This increase in hormone levels has been attributed to the expansion of interstitial cells, stimulated by these cells' release of testosterone [20]. Histopathological analysis revealed that the degeneration observed in the interstitial area, including Leydig cells, in the BPAF-exposed group appeared normal in the BPAF + CIN group of rats. These findings suggest that CIN may hold promise as a potential treatment for restoring the BPAF-induced reduction in testosterone.

Wu et al. reported in their study that exposure to BPAF disrupted mouse Sertoli cell architecture [38]. However, studies determining the histopathological effects of BPAF on testicular tissue are quite limited. The morphological structure of the testicular tubules in rats from the BPAF group was significantly disrupted according to the results of our study. Their basement membranes exhibited an indented and protruding structure, some tubules had atrophied, germinal showed signs of degeneration, and spermatogenesis was impaired. In contrast, when CIN co-treatment was administered, these histopathological damages were largely prevented, and the histological structure of the testis appeared almost normal.

Caspases play a central role in apoptotic processes, as their activation leads to cell death, particularly when caspase-3 is activated. Conversely, Bcl-2 stops the formation of pores in the mitochondrial membrane, which results in a protective effect against apoptosis [55]. PCNA, on the other hand, is a nuclear protein present exclusively in actively dividing cells. It recreates a crucial role in the cell cycle and DNA replication, serving as a vital indicator of the cell proliferation process's health [56]. Immunohistochemical results in this study revealed that caspase-3 expression increased while PCNA expression decreased in the testicular tissue of rats in the BPAF group compared with the control. Gyimah and colleagues reported significantly increase in caspasa-3 expression in zebrafish larvae exposed to BPAF [57]. Furthermore, the rise in caspase-3 expression was prevented in the BPAF + CIN group compared with the BPAF group, and Bcl-2 and PCNA expression increased. CIN therapy is known to protect fish from apoptosis caused by bisphenol A by reducing the decline in Bcl-2and the rise in caspase-3 levels [58]. In a study involving rats with ulcers, it was found that CIN treatment increased PCNA expression in the stomach lining cells, indicating the drug's proliferative effect [59]. These findings suggested that co-treatment with CIN alongside BPAF in rats inhibited apoptosis by reducing caspase-3 levels and increasing Bcl-2 levels in testicular tissue. Additionally, the enhanced expression of PCNA indicated induced cell division.

Conclusion

In conclusion, BPAF treatment induces reproductive damage in rats. Aside from its impact on sperm parameters, reduction in testosterone levels, and induction of inflammation and apoptosis, BPAF also disrupts testicular tissue. In contrast, co-treatment with CIN exhibited anti-apoptotic and anti-inflammatory effects, alongside the regulation of testosterone levels, increased cell proliferation, and improvements in sperm parameters. Furthermore, the majority of the histological deteriorations were mitigated. These findings suggest that CIN might offer an alternative for the treatment of reproductive damage caused by exposure to BPAF. However, further research is needed to elucidate the mechanism underlying this therapeutic effect, explore optimal dosages and durations of treatment, and investigate its effects on various pathways.

Funding The Scientific Research Projects Coordination Unit of Hatay Mustafa Kemal University supported this study [Project No. 22.GAP.006].

Data Availability All data generated is included in this publication.

Code Availability Not applicable.

Declarations

Ethics Approval All treatments during the experimental process were conducted in accordance with the approval decision of the Hatay Mustafa Kemal University Local Ethics Committee for Animal Experiments, with the decision number 2022/01–02.

Consent to Participate Not applicable.

Conflict of Interests The authors declared no conflict of interest.

Disclaimer The content is solely the responsibility of the authors.

References

1. Madore MP, Sakaki JR, Chun OK. Protective effects of polyphenols against endocrine disrupting chemicals. Food Sci Biotechnol. 2022;31(8):905–34. https://doi.org/10.1007/s10068-022-01105-z.
2. Tvrdý V, Dias P, Nejmanová I, Carazo A, Jirkovský E, Pourová J, Fadraersada J, Moravcová M, Peterlin Mašič L, Sollner Dolenc M, Mladěnka P. The effects of bisphenols on the cardiovascular system ex vivo and in vivo. Chemosphere. 2023;2023(313):137565. https://doi.org/10.1016/j.chemosphere.2022.137565.
3. Meng Z, Tian S, Yan J, Jia M, Yan S, Li R, Zhang R, Zhu W, Zhou Z. Effects of perinatal exposure to BPA, BPF and BPAF on liver function in male mouse offspring involving in oxidative damage and metabolic disorder. Environ Pollut. 2019;2019(247):935–43. https://doi.org/10.1016/j.envpol.2019.01.116.
4. Lei B, Sun S, Xu J, Feng C, Yu Y, Xu G, Wu M, Peng W. Low-concentration BPAF- and BPF-induced cell biological effects are mediated by ROS in MCF-7 breast cancer cells. Environ Sci Pollut Res. 2018;25:3200–8. https://doi.org/10.1007/s11356-017-9709-7.
5. Feng Y, Yin J, Jiao Z, Shi J, Li M, Shao B. Bisphenol AF may cause testosterone reduction by directly affecting testis function in adult male rats. Toxicol Lett. 2012;211(2):201–9. https://doi.org/10.1016/j.toxlet.2012.03.802.
6. Chen S, Li X, Li H, Yuan S, Li J, Liu C. Greater toxic potency of bisphenol AF than bisphenol A in growth, reproduction, and transcription of genes in Daphnia magna. Environ Sci Pollut Res. 2021;28:25218–27. https://doi.org/10.1007/s11356-020-12153-5.
7. Shi J, Jiao Z, Zheng S, Li M, Zhang J, Feng Y, Yin J, Shao B. Long-term effects of Bisphenol AF (BPAF) on hormonal balance and genes of hypothalamus-pituitary-gonad axis and liver of zebrafish (Danio rerio), and the impact on offspring. Chemosphere. 2015;2015(128):252–7. https://doi.org/10.1016/j.chemosphere.2015.01.060.
8. Song M, Liang D, Liang Y, Chen M, Wang F, Wang H, Jiang G. Assessing developmental toxicity and estrogenic activity of halogenated bisphenol a on zebrafish (Danio rerio). Chemosphere. 2014;2014(112):275–81. https://doi.org/10.1016/j.chemosphere.2014.04.084.
9. Li Y, Burns KA, Arao Y, Luh CJ, Korach KS. Differential estrogenic actions of endocrine-disrupting chemicals bisphenol a, bisphenol af, and zearalenone through estrogen receptor α and β in vitro. Environ Health Perspect. 2012;120(7):1029–35. https://doi.org/10.1289/ehp.1104689.
10. Mentor A, Wann M, Brunström B, Jönsson M, Mattsson A. Bisphenol AF and bisphenol f induce similar feminizing effects in chicken embryo testis as bisphenol a. Toxicol Sci. 2020;178(2):239–50. https://doi.org/10.1093/toxsci/kfaa152.
11. Skledar DG, Carino A, Trontelj J, Troberg J, Distrutti E, Marchianò S, Tomašič T, Zega A, Finel M, Fiorucci S, Mašič LP. Endocrine activities and adipogenic effects of bisphenol AF and its main metabolite. Chemosphere. 2019;2019(215):870–80. https://doi.org/10.1016/j.chemosphere.2018.10.129.
12. Li Y, Xiong Y, Lv L, Li X, Qin Z. Effects of low-dose bisphenol AF on mammal testis development via complex mechanisms: alterations are detectable in both infancy and adulthood. Arch Toxicol. 2022;96:3373–83. https://doi.org/10.1007/s00204-022-03377-0.
13. Barratt CLR, Björndahl L, De Jonge CJ, Lamb DJ, Martini FO, McLachlan R, Oates RD, van der Poel S, John BS, Sigman M, Sokol R, Tournaye H. The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance—challenges and future research opportunities. Hum Reprod Update. 2017;23(6):660–80. https://doi.org/10.1093/humupd/dmx021.
14. Taskin T, Kahvecioglu D, Turkoglu A, Dogan A, Kuzu M, Turkoğlu A. In vitro biological activities of different extracts from alcea dissecta. Clin Exp Health Sci. 2022;12(1):53–60. https://doi.org/10.33808/clinexphealthsci.787845.
15. Dorostghoal M, Seyyednejad SM, Nejad MNT. Beneficial effects of Cichorium intybus L. extract on oxidative status and reproductive parameters in male Wistar rats: an experimental study. Int J Reprod Biomed. 2019;17(6):425–34. https://doi.org/10.18502/ijrm.v17i6.4814.
16. Salehi B, Sharifi-Rad J, Quispe C, Llaique H, Villalobos M, Smeriglio A, Trombetta D, Ezzat SM, Salem MA, Zayed A, Salgado Castillo CM, Yazdi SE, Sen S, Acharya K, Sharopov F, Martins N. Insights into Eucalyptus genus chemical constituents, biological activities and health-promoting effects. Trends Food Sci Technol. 2019;2019(91):609–24. https://doi.org/10.1016/j.tifs.2019.08.003.
17. Dey B, Mitra A. Chemo-profiling of eucalyptus and study of its hypoglycemic potential. World J Diabetes. 2013;4(5):170–6. https://doi.org/10.4239/wjd.v4.i5.170.
18 Silva SM, Abe SY, Murakami FS, Frensch G, Marques FA, Nakashima T. Essential oils from different plant parts of eucalyptus cinerea F. Muell. ex Benth. (Myrtaceae) as a source of 1,8-cineole and their bioactivities. Pharmaceuticals. 2011;4(12):1535–50.
19. Bhowal M, Gopal M. Eucalyptol: Safety and pharmacological profile. J Pharm Sci. 2015;5(4):125–31. https://doi.org/10.5530/rjps.2015.4.2.
20. Widjaja G, Ibrahim NJ, Hadi SJ, Cababat F, Jalil AT, Al-Charak AGH, Yasin G, Sultan MQ, Fakri Mustafa Y. The effect of eucalyptus globulus hydroalcoholic extract on LH, FSH and testosterone concentrations and sperm morphology. Arch Razi Inst. 2023;78(1):115–23. https://doi.org/10.22092/ARI.2022.359470.2426.
21. Lima PR, De Melo TS, Carvalho KMMB, De Oliveira ÍB, Arruda BR, De Castro Brito GA, Rao VS, Santos FA. 1,8-cineole (eucalyptol) ameliorates cerulein-induced acute pancreatitis via modulation of cytokines, oxidative stress and NF-κB activity in mice. Life Sci. 2013;92(24–26):1195–201. https://doi.org/10.1016/j.lfs.2013.05.009.
22 Karnam SS, Ghosh RC, Mondal S, Mondal M. Evaluation of subacute bisphenol – A toxicity on male reproductive system. Vet World. 2015;8(6):738–44.
23. Türk G, Ateşşahin A, Sönmez M, Yüce A, Çeribaşi AO. Lycopene protects against cyclosporine a-induced testicular toxicity in rats. Theriogenology. 2007;67(4):778–85. https://doi.org/10.1016/j.theriogenology.2006.10.013.
24. Cellat M, Kuzu M, İşler CT, Etyemez M, Dikmen N, Uyar A, Gökçek İ, Türk E, Güvenç M. Tyrosol improves ovalbumin (OVA)-induced asthma in rat model through prevention of airway inflammation. Naunyn Schmiedebergs Arch Pharmacol. 2021;394:2061–75. https://doi.org/10.1007/s00210-021-02117-y.
25. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351–8. https://doi.org/10.1016/0003-2697(79)90738-3.
26. Beutler E, Dubon O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med. 1963;61:882–8.
27. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121–6. https://doi.org/10.1016/S0076-6879(84)05016-3.
28. Beutler E (1975) Red cell metabolism: a manual of biochemical methods. Newyork: Grune Strottan
29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurument with the folin phenol geagent. J Biol Chem. 1951;193(1):265–75.
30. Türk E, Tekeli IO, Özkan H, Uyar A, Cellat M, Kuzu M, Yavas I, Yegani AA, Yaman T, Güvenç M. The protective effect of esculetin against aluminium chloride-induced reproductive toxicity in rats. Andrologia. 2021;53:e13930. https://doi.org/10.1111/and.13930.

31. Luna L. Manual of histologic staining methods of the Armed Forces Institute of Pathology. New York: McGraw-Hill Book Co.; 1968.
32. Johnsen SG. Testicular biopsy score count – a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Horm Res Paediatr. 1970;1(1):2–25. https://doi.org/10.1159/000178170.
33. Uyar A, Cellat M, Kanat Ö, Etyemez M, Kutlu T, Deveci MYZ, Yavaş İ, Kuzu M. Carvacrol showed a curative effect on reproductive toxicity caused by Bisphenol AF via antioxidant, anti-inflammatory and anti-apoptotic properties. Reprod Toxicol. 2023;121:108456. https://doi.org/10.1016/j.reprotox.2023.108456.
34. Biggs SN, Kennedy J, Lewis SL, Hearps S, O'Bryan MK, McLachlan R, von Saldern S, Chambers G, Halliday J. Lifestyle and environmental risk factors for unexplained male infertility: study protocol for Australian Male Infertility Exposure (AMIE), a case-control study. Reprod Health. 2023;20(32):1–9. https://doi.org/10.1186/s12978-023-01578-z.
35. Mima M, Greenwald D, Ohlander S. Environmental toxins and male fertility. Curr Urol Rep. 2018;19(50):1–8. https://doi.org/10.1007/s11934-018-0804-1.
36. Nallella KP, Sharma RK, Aziz N, Agarwal A. Significance of sperm characteristics in the evaluation of male infertility. Fertil Steril. 2006;85(3):629–34. https://doi.org/10.1016/j.fertnstert.2005.08.024.
37. Li Y, Xiong Y, Lv L, Li X, Qin Z. Effects of low-dose bisphenol AF on mammal testis development via complex mechanisms: alterations are detectable in both infancy and adulthood. Arch Toxicol. 2022;96(12):3373–83. https://doi.org/10.1007/s00204-022-03377-0.
38. Wu D, Huang CJ, Jiao XF, Ding ZM, Zhang SX, Miao YL, Huo LJ. Bisphenol AF compromises blood-testis barrier integrity and sperm quality in mice. Chemosphere. 2019;237:124410. https://doi.org/10.1016/j.chemosphere.2019.124410.
39. Čmiková N, Galovičová L, Schwarzová M, Vukic MD, Vukovic NL, Kowalczewski PŁ, Bakay L, Kluz MI, Puchalski C, Kačániová M. Chemical composition and biological activities of eucalyptus globulus essential oil. Plants. 2023;12(5):1–18. https://doi.org/10.3390/plants12051076.
40. Mousa AA, Elweza AE, Elbaz HT. Tahoun EAE aziz, Shoghy KM, Elsayed I, Hassan EB. Eucalyptus Globulus protects against diclofenac sodium induced hepatorenal and testicular toxicity in male rats. J Tradit Complement Med. 2020;10(6):521–8. https://doi.org/10.1016/j.jtcme.2019.11.002.
41 Cellat M, İşler CT, Uyar A, Kuzu M, Aydın T, Etyemez M, Türk E, Yavas I, Güvenç M. Protective effect of Smilax excelsa L pretreatment via antioxidant, anti-inflammatory effects, and activation of Nrf-2/HO-1 pathway in testicular torsion model. J Food Biochem. 2022;46:e14161. https://doi.org/10.1111/jfbc.14161.
42. Yan B, Lu G, Wang R, Kang S, Huang C, Wu H, Yong Q. Protective effects of lignin fractions obtained from grape seeds against bisphenol AF neurotoxicity via antioxidative effects mediated by the Nrf2 pathway. Front Chem Sci Eng. 2023;7(7):976–89. https://doi.org/10.1007/s11705-022-2237-0.
43. Gao C, He H, Qiu W, Zheng Y, Chen Y, Hu S, Zhao X. Oxidative stress, endocrine disturbance, and immune interference in humans showed relationships to serum bisphenol concentrations in a dense industrial area. Environ Sci Technol. 2021;55(3):1953–63. https://doi.org/10.1021/acs.est.0c07587.
44. Taheri Mirghaed A, Hoseini SM, Ghelichpour M. Effects of dietary 1,8-cineole supplementation on physiological, immunological and antioxidant responses to crowding stress in rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol. 2018;81:182–8. https://doi.org/10.1016/j.fsi.2018.07.027.
45. Ciftci O, Ozdemir I, Tanyildizi S, Yildiz S, Oguzturk H. Antioxidative effects of curcumin, β-myrcene and 1,8-cineole against 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced oxidative stress in rats liver. Toxicol Ind Health. 2011;27(5):447–53. https://doi.org/10.1177/0748233710388452.
46. Taheri Mirghaed A, Fayaz S, Hoseini SM. Effects of dietary 1,8-cineole supplementation on serum stress and antioxidant markers of common carp (Cyprinus carpio) acutely exposed to ambient ammonia. Aquaculture. 2019;509:8–15. https://doi.org/10.1016/j.aquaculture.2019.04.071.
47 Bhattacharya I, Sen Sharma S, Majumdar SS. Etiology of Male Infertility: an Update. Reprod Sci. 2024;31:942–65.
48. Walke G, Gaurkar SS, Prasad R, Lohakare T, Wanjari M. The Impact of Oxidative Stress on Male Reproductive Function: Exploring the Role of Antioxidant Supplementation. Cureus. 2023;15(7):e42583. https://doi.org/10.7759/cureus.42583.
49. Meli R, Monnolo A, Annunziata C, Pirozzi C, Ferrante MC. Oxidative Stress and BPA Toxicity: An Antioxidant Approach for Male and Female Reproductive Dysfunction. Antioxidants. 2020;9:405. https://doi.org/10.3390/antiox9050405.
50. Kuzu M, Kandemir FM, Yıldırım S, Çağlayan C, Küçükler S. Attenuation of sodium arsenite-induced cardiotoxicity and neurotoxicity with the antioxidant, anti-inflammatory, and antiapoptotic effects of hesperidin. Environ Sci Pollut Res. 2020;28:10818–31. https://doi.org/10.1007/s11356-020-11327-5.
51 Çibuk S, Yılmaz HC, Mert H, Mis L, Yörük M, Mert N. Effect of Bisphenol-A on Oxidative Status and Certain Inflammatory Markers in Rats: Experimental Study. Turkiye Klinikleri J Vet Sci. 2023;14(2):39–46. https://doi.org/10.5336/vetsci.2023-98134.
52. Wang K, Huang D, Zhou P, Su X, Yang R, Shao C, Ma A, Wu J. Individual and Combined Effect of Bisphenol A and Bisphenol AF on Prostate Cell Proliferation through NF-κB Signaling Pathway. Int J Mol Sci. 2022;23(20):12283. https://doi.org/10.3390/ijms232012283.
53. Asadi M, Rahmani M, Samadi A, Hesari AK. Protective Effect of Low-Volume High-Intensity Interval Training on Aspirin-Induced Reproductive Impairments in Adult Male Wistar Rats. Reprod Sci. 2024;31:393–403. https://doi.org/10.1007/s43032-023-01369-8.
54. Liu XX, Wang ZX, Liu FJ. Chronic exposure of BPA impairs male germ cell proliferation and induces lower sperm quality in male mice. Chemosphere. 2021;262:127880. https://doi.org/10.1016/j.chemosphere.2020.127880.
55. Kuzu M, Kandemir FM, Yildirim S, Kucukler S, Caglayan C, Turk E. Morin attenuates doxorubicin-induced heart and brain damage by reducing oxidative stress, inflammation and apoptosis. Biomed Pharmacother. 2018;106:443–53. https://doi.org/10.1016/j.biopha.2018.06.161.
56. Duan S, Jiang X, Li J, Fu M, Li Z, Cheng Y, Zhuang Y, Yang M, Xiao W, Ping H, Xie Y, Xie X, Zhang X. The RXFP2-PLC/PKC signaling pathway mediates INSL3-induced regulation of the proliferation, migration and apoptosis of mouse gubernacular cells. Cell Mol Biol Lett. 2023;28(16):1–14. https://doi.org/10.1186/s11658-023-00433-0.
57. Gyimah E, Zhu X, Zhang Z, Guo M, Xu H, Mensah JK, Dong X, Zhang Z, Gyimah GNW. Oxidative Stress and Apoptosis in Bisphenol AF–Induced Neurotoxicity in Zebrafish Embryos. Environ Toxicol Chem. 2022;41(9):2273–84. https://doi.org/10.1002/etc.5412.
58. Chen L, Tao D, Yu F, Wang T, Qi M, Xu S. Cineole regulates Wnt/β-catenin pathway through Nrf2/keap1/ROS to inhibit bisphenol A-induced apoptosis, autophagy inhibition and immunosuppression of grass carp hepatocytes. Fish Shellfish Immunol. 2022;131:30–41. https://doi.org/10.1016/j.fsi.2022.09.067.
59. Rocha Caldas GF, Oliveira ARDS, Araújo AV, Lafayette SSL, Albuquerque GS, Silva-Neto JDC, Costa-Silva JH, Ferreira F, Da Costa JGM, Wanderley AG. Gastroprotective Mechanisms

of the Monoterpene 1,8-Cineole (Eucalyptol). PLoS ONE. 2015;10:e0134558. https://doi.org/10.1371/journal.pone.0134558.

Authors and Affiliations

Ahmet Uyar[1] · Mustafa Cellat[2] · Özgür Kanat[3] · Muhammed Etyemez[2] · Tuncer Kutlu[1] · Mehmet Zeki Yılmaz Deveci[4] · İlker Yavaş[5] · Müslüm Kuzu[6]

✉ Ahmet Uyar
ahmetuyar@mku.edu.tr

Mustafa Cellat
mcellat@mku.edu.tr

Özgür Kanat
vetstorm@gmail.com

Muhammed Etyemez
metyemez82@gmail.com

Tuncer Kutlu
tuncerkutlu83@gmail.com

Mehmet Zeki Yılmaz Deveci
mzydeveci@mku.edu.tr

İlker Yavaş
iyavas@mku.edu.tr

Müslüm Kuzu
muslumkuzu@karabuk.edu.tr

[1] Department of Pathology, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, 31060 Hatay, Türkiye

[2] Department of Physiology, Hatay Mustafa Kemal University, Hatay, Türkiye

[3] Department of Pathology, Necmettin Erbakan University, Konya, Türkiye

[4] Department of Surgery, Hatay Mustafa Kemal University, Hatay, Türkiye

[5] Department of Reproduction and Artificial Insemination, Mustafa Kemal University, Hatay, Türkiye

[6] Department of Nutrition and Dietetics, Karabuk University, Karabuk, Türkiye