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Vitamin E fails to prevent bisphenol S induced testicular damage in diabetic rats

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Abstract

Bisphenol S (BPS) is a recognized environmental contaminant that harms reproductive organs and fertility, affecting human health worldwide. Over the last few decades, the search for a compound that mitigates its harmful effects has increased. Vitamin E has not been evaluated in diabetic rats as a study model. We assessed for the first time the use of Vitamin E as a potential ameliorant compound. We used 26 Wistar rats, and assigned it randomly in five groups: 1) healthy rats (Ctrl, n=6); 2) diabetic rats without treatment (Ctrl-D, n=5); 3) diabetic rats treated with Vitamin E (100 mg/kg bw/day, VitE-D, n=5); 4) diabetic rats treated with BPS (100 mg/kg bw/day, BPS-D, n=5); and, 5) diabetic rats receiving a combination of Vitamin E (100 mg/kg bw/day) and BPS (100 mg/kg bw/day) (VitE+BPS-D, n=5). All doses were administered orally (p.o.). We evaluated its effect on serum estradiol and testosterone levels, testis cellular apoptosis, antioxidant enzyme activity, and sperm and testicular histologic characteristics. BPS increases oxidative stress, promotes cell apoptosis, provokes structural changes in seminiferous tubules, and negatively affects spermatogenesis and sperm quality. As a result of our study, co-administration of Vitamin E did not reduce the negative impact provoked by BPS; indeed, in some cases, the vitamin exacerbated the

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injury. The beneficial effects of VitE on testosterone serum levels were nullified when combined with BPS. Our results show the dangers of BPS to male reproductive health in the diabetes model and stress the necessity for improved strategies to mitigate its deleterious impacts.

Keywords: Contaminant environmental, Bisphenol S, Diabetes mellitus, Oxidative stress, Vitamin E (α-tocopherol), Reproductive toxicity, Blood-testis barrier.

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1. Introduction

Several synthetic industrial chemicals that act as endocrine disruptors (EDCs) and adversely affect human and animal reproductive health have been introduced into the environment [1]. Among the EDCs released worldwide is bisphenol-A (BPA), the most widely used industrial chemical in the production of polycarbonate and epoxy resins [2,3]. In recent years, governmental organizations worldwide have advertised BPA as harmful and toxic to metabolism, the cardiovascular system, cancer, and reproductive fields [4]. Moreover, there is a relationship between the ingestion of bisphenols and the induction of diabetes mellitus (DM) [5].

As EDCs, BPA administration influences reproductive and metabolic systems in female and male rodents [2,6]. Rodents' oral exposure to BPA at 0.5, 5, or 50 mg/kg body weight (bw)/day (d) produced

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spermatogenesis impairment, decreased sperm count, and motility [7]. In another study by Xu et al. (2019), after administering 12-week-old male ICR mice 5000 µg/kg bw/d of BPA subcutaneously for 5 days, they observed hyperinsulinemia and glucose intolerance [8]. Bansal et al. (2019) reported that after orally administering 10 or 10,000 µg/kg bw/d of BPA to female and male C57BL/6 J mice two weeks before mating, the female offspring exhibited glucose intolerance [9]. Because of these harmful effects, some countries have restricted the use of BPA [10,11].

Following the BPA restrictions in 2012, BPA-free products were introduced in the European market, and bisphenol S (BPS) replaced BPA in the plastics industry, with products labeled as "BPA-free." BPS is in relatively high concentrations in various products, such as food containers, personal care products, and shopping receipts [12]. It has structural and chemical properties similar to those of BPA; therefore, considerable scientific effort has been devoted to investigating its safety [13]. Although BPS has been proposed as a BPA replacement, some studies have demonstrated that it also produces harmful effects [14]. In a study, after administering 100 mg/kg bw/d of BPS to male diabetic rats for 30 days, significant decreases in body weight and pancreatic degeneration were observed [15]. In another study by Mandrah et al. in male rats, after oral gavage administration of 0.05, 0.5, or 5 mg/kg bw/d of BPS for 90 days, significant increases in blood glucose, alterations in

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glycolysis and, and decreased gluconeogenesis were observed, thereby reducing insulin response [16].

The development of reproductive and metabolic disorders is associated with oxidative stress [17]. A recent study showed that insulin resistance, the main factor in the pathogenesis of diabetes, was the strongest metabolic component associated with oxidative stress [18]. Several studies have shown that BPS alters the endogenous antioxidant system [19]. In RWPE-1 prostate epithelial cells, incubation with 600 μM of BPS or BPF for 24 h provoked imbalances in the levels of oxidative stress markers, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) activities, as well as in glutathione levels and total antioxidant capacity compared to unexposed cells [20].

The relationship between BPS ingestion and oxidative stress production affects not only metabolic diseases such as DM but also reproductive parameters. Therefore, finding an antioxidant compound that can protect the reproductive system is necessary. Vitamins and melatonin have been used as protective compounds against bisphenol-induced reproductive toxicity [21]. Vitamins, such as vitamin E (VitE), protect the cell membrane from oxidation, exhibit potent antioxidant and cholesterol-lowering properties, serve as important signaling molecules, and changes in their levels, or their loss due to oxidation, are key cellular events to which cells respond [22]. Although results on the effects of VitE in DM models are inconclusive, reports indicate that administration of

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VitE for three weeks reduced blood glucose levels in Wistar rats with DM1 [23,24]. Therefore, this study aims to investigate whether VitE ameliorates the reproductive toxic effect of BPS in diabetic rats.

2. Materials and methods

2.1. Chemicals

Bisphenol S (BPS; Sigma–Aldrich Inc., Mexico, CAS No. 80-09-1; purity 99%) was dissolved in Merainsa® olive oil without antioxidants (vehicle), purchased from local commercial suppliers. Vitamin E (α-tocopherol; Sigma–Aldrich Inc., Mexico, CAS No. 10191-41-0; purity 100%) and streptozotocin (STZ; Sigma–Aldrich Inc., Mexico, CAS No. 18883-66-4; purity ≥95% by HPLC) were used as described. Ketamine (PISA®, Mexico) and xylazine (PISA®, Mexico) were used for anesthesia. The semen collection kit (DESEGO®, Ferti México) was employed. Additionally, the following reagents were used: EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co.), Alexa Fluor 488 (Invitrogen Thermo Fisher Scientific, Waltham, MA, USA, A-11034), VECTASHIELD® Antifade Mounting Medium with DAPI (H-1200-10, Vector), In Situ Cell Death Detection Kit (Sigma-Aldrich, USA), and Proteinase K (Sigma, St. Louis, MO, USA).

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2.2 Animals

Twenty-six adult male Wistar rats (250-300 g) from the Animal Facility of the Instituto de Fisiología Celular of the Universidad Nacional Autónoma

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de México (UNAM) in Mexico City were used for the experiment. We pre-planned group sizes (Control n=6; each treatment n=5), ensuring adequate tissue/sample yield per endpoint under a minimal-use policy. The Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the School of Chemistry, UNAM, Mexico, approved the experimental procedures (Trade number: FQ/CICUAL/467/22). All experimental methods were carried out in accordance with Mexican legislation NOM-062-ZOO-1999. We confirm that all experiments were performed in accordance with relevant guidelines and regulations, including the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

The experimental rats were housed in polycarbonate cages with stainless steel covers in a temperature-controlled room maintained at 20°C, featuring a 12-h light/dark cycle and a 50 ± 10% relative humidity. Before the experiment began, rats were acclimatized for 14 days. The rats were provided ad libitum access to water and standard laboratory chow BIO-DIETA-LAB 7300 (ABENE®, Mexico). The guaranteed nutritional composition was as follows: raw protein: 23.5% min, crude fat: 6% min, raw fiber: 4% max, ashes: 8% max, humidity: 12% max, nitrogen-free extract: 46.5%. All histological evaluations, BTB, TUNEL, and spermogram analyses were performed under blinded conditions. The researchers code-labeled the samples with numbers during sample collection. During evaluation, the samples were assessed numerically,

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and only until data analysis the results of each animal was situated in its corresponding group.

2.3 Experimental Design

A randomized and blind experiment was conducted. Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ at a 45 mg/kg bw dose. To confirm the diagnosis of diabetes, blood glucose levels were assessed one and two days after STZ administration using a glucometer (OneTouch® UltraMini® Johnson & Johnson) and test strips (same brand as the glucometer) after 4 h of fasting. Rats with blood glucose levels exceeding 200 mg/dL were classified as diabetic. BPS and/or VitE administration started 7 days after STZ injection. Doses were administered orally (p.o.) daily for 30 days between 9:00 and 11:00 am. The experimental animals were divided into five groups:

Ctrl (n=6): Healthy rats, administered with olive oil

Ctrl-D (n=5): Diabetic rats, administered with olive oil

VitE-D (n=5): Diabetic rats, administered with VitE (100 mg/kg bw/d)

BPS-D (n=5): Diabetic rats, administered with 100 mg/kg bw/d of BPS diluted in olive oil

VitE+BPS-D (n=5): Diabetic rats were administered with VitE (100 mg/kg bw/d) 30 min before administering 100 mg/kg bw/d of BPS diluted in olive oil. The administration of VitE before BPS was intended to facilitate intestinal uptake, avoid direct luminal co-mixing, and allow

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tissue distribution before BPS exposure, consistent with antioxidant pretreatment in rodent toxicology, which assesses the antioxidant protective effect [25-27]. Moreover, in rats, liquid gavage empties from the stomach and reaches the small intestine within ~10–30 min [28].

The selected dose of 100 mg/kg bw/d of BPS in this study was determined from our previous findings, which showed that this dose led to adverse effects on reproductive variables in Wistar rats during chronic administration over 15 weeks (work in progress). Additionally, administering BPS to adult male Sprague-Dawley rats at 30, 60, and 120 mg/kg bw daily for 30 days resulted in elevated serum glucose, total cholesterol, and triglyceride levels [29]. Additionally, a 100 mg/kg bw/d dosage of VitE protected against the changes induced by bisphenols in antioxidant enzymes and liver damage [30,31].

The animals were weighed every seven days, and euthanasia was performed following the protocol outlined by Al-Mousawi et al. [32]. Under anesthesia, using Ketamine (PISA®, México) at a dose of 50 mg/kg bw, combined with Xylazine (PISA®, México) at 7 mg/kg bw, administered intraperitoneally (i.p.). Animals were sacrificed by decapitation one day after the end of the treatment.

Blood samples were collected in test tubes immediately post-euthanasia, and serum was obtained by centrifugation in a Beckman J221 centrifuge with a JA-18.1 rotor, at 169 g for 15 minutes at 4°C. The serum samples were stored in Eppendorf tubes at −80°C until further use for evaluation

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of testosterone and estradiol levels by electrochemiluminescence immunoassay (ECLIA) using Elecsys Estradiol III (cobas® Roche Diagnostics GmbH) and Elecsys Testosterone II (cobas® Roche Diagnostics GmbH) according to the manufacturer's protocol. At euthanasia, the testis was obtained and fixed in 4% w/v paraformaldehyde in phosphate-buffered saline (PBS). Before technicians, people who recorded data randomly numbered the samples, and researchers analyzed them. The sample results were grouped for statistical analysis. A timeline of the experimental design is shown in Figure 1.

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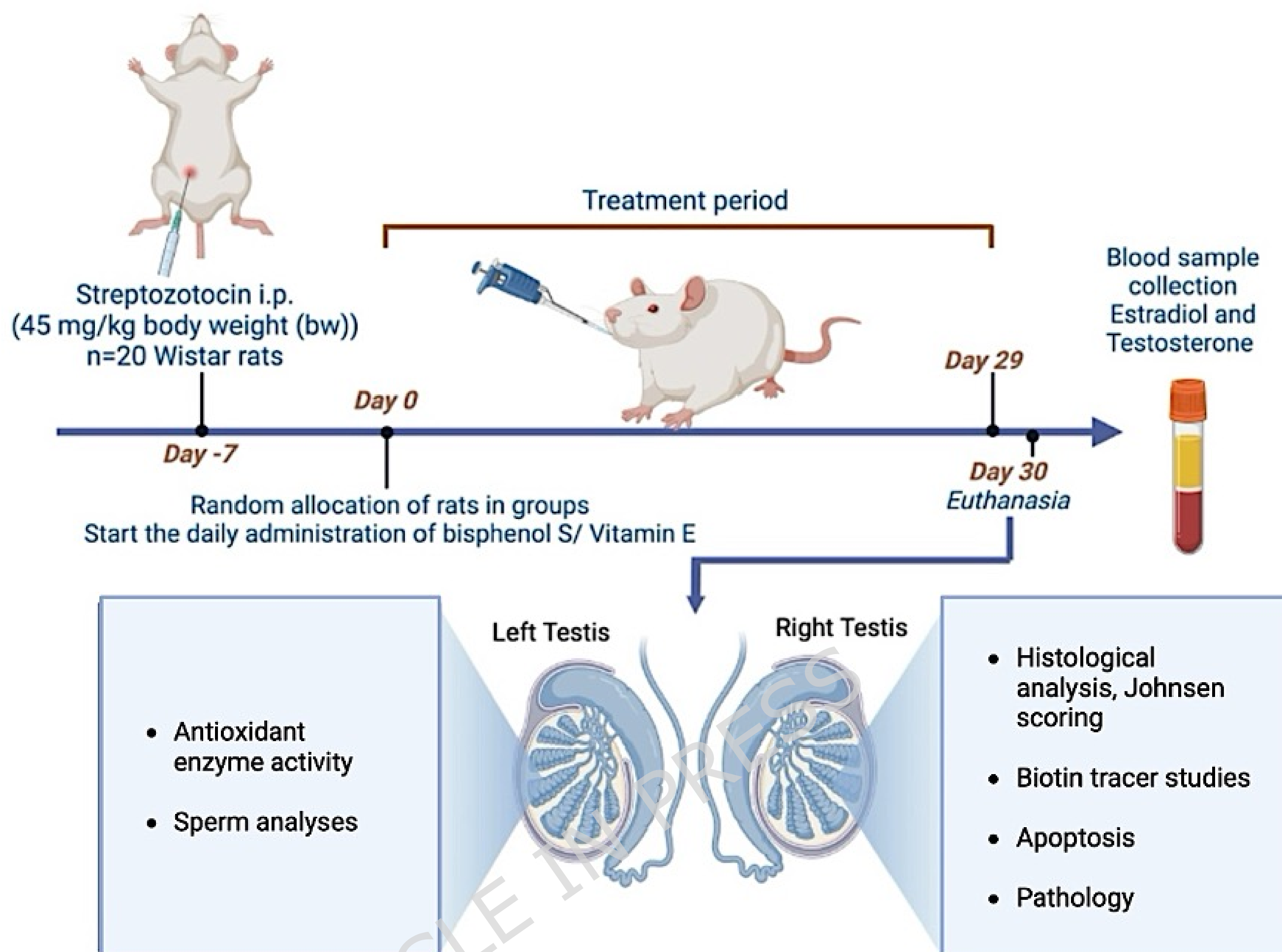

Figure 1. Experimental design to investigate the impact of bisphenol S (BPS) and vitamin E (VitE) on diabetic Wistar rats, encompassing apoptosis via terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and hormonal, antioxidant, sperm, and histological assessments following 29 days of treatment.

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2.4. Testis antioxidant enzyme activity

The activities of GPx, GR, glutathione S-transferase (GST), SOD, and catalase (CAT) in rat testis were assessed in triplicate according to the protocol previously described, which serves as our laboratory's general operating procedure [33]. GPx activity was determined by monitoring the decrease in NADPH absorbance at 340 nm in a coupled assay containing

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hydrogen peroxide (H_2O_2), glutathione (GSH), and GR. We read for one minute every three minutes. Results were expressed in U/mg protein, where 1 U is the quantity of enzyme that oxidizes 1µmol of NADPH per minute [34]. GR activity was measured by monitoring the decrease in NADPH absorbance at 340 nm in a reaction mixture containing oxidized glutathione (as the substrate). We read for one minute every three minutes. Results were expressed in U/mg protein, where 1 U is the quantity of enzyme that oxidizes 1µmol of NADPH per minute [35]. GST activity was assessed by measuring the formation of GSH/1-chloro-2,4-dinitrobenzene (CDNB) complexes at 340 nm. We did the reading for three minutes each minute. Results were expressed as U/mg protein, where 1 U is the quantity of GST enzyme conjugates required to catalyze the reaction of 1 µmol of CDNB with GSH per minute [33] [36]. CAT activity was determined by following the decrease of H_2O_2 at 240 nm [37]. SOD activity was evaluated at 560 nm by following the inhibition of nitroblue tetrazolium reduction with superoxide anion generated by the xanthine/xanthine oxidase system. We read every 15 seconds. Results were expressed in k/mg protein, where k is the first-order reaction constant of the reaction (as a catalase activity unit): $2H_2O_2 + CAT \rightarrow 2H_2O + O_2$ [38,39].

2.5. Testis histological analysis

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After fixation with 4% paraformaldehyde, the samples were dehydrated through increasing ethanol concentrations, cleared with xylene, and embedded in paraffin. Testis tissues were sectioned at 5 µm thickness and mounted on glass slides previously treated with poly-L-lysine. The sections were then dewaxed in xylene, gradually rehydrated by lowering the ethanol concentration, and stained with hematoxylin and eosin for histological analysis. We obtained twenty photos of each animal's sample at 40x and used them to evaluate the seminiferous tubules using Johnsen scoring, morphometry, and histological analysis [40].

The seminiferous tubules were classified into three intervals according to the seminiferous epithelial cycle stages: i) Stage I to VII, ii) Stage VIII - early IX, and iii) Late Stage IX – XIV. The seminiferous tubule area (STA) was measured using ImageJ software (National Institutes of Health; NIH) [41]. The seminiferous epithelium area (SEA) was determined by subtracting the internal or luminal area from the STA.

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2.6. Johnsen scoring of the seminiferous tubules

To evaluate the spermatogenesis quality, the histological findings were recorded and interpreted using the Johnsen method [40], which consists of the examination of cross-sectional testis cuts and scoring of seminiferous tubules based on the following score:

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10. Indicates complete stages of spermatogenesis with many spermatozoa, an organized germinal layer of even thickness, and an open lumen.

9. Denotes numerous spermatozoa with an irregular germinal layer and some sloughing or lumen destruction.

8. Reflects only a few spermatozoa.

7. Indicates no spermatozoa but many spermatids.

6. Shows no spermatozoa and fewer than five spermatids.

5. No spermatozoa or spermatids, but many spermatocytes.

4. No spermatozoa or spermatids, but fewer than five spermatocytes.

3. Spermatogonia are the only germ cells present.

2. No germ cells, but Sertoli cells are present.

1. No cells in the tubule section

The value on the Johnsen scale is determined by assigning a score per seminiferous tubule, with a maximum of 10 and a minimum of 1. This indicates that a tubule with a value of 10 is fully developed (mature), whereas a value of 1 indicates it is not ripe.

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2.7 Sperm analyses

Semen analysis was performed according to the World Health Organization (WHO) operational procedures, adapted for rodents [42]. For sperm concentration, the left epididymal tail was removed and immediately placed into a 5 cm plate containing 1 ml pre-warmed PBS

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(37 °C) to assess sperm concentration using a hemocytometer with 10 µl of the diluted sperm solution (the PBS suspension containing sperm was diluted 1:9 with a 2% formalin fixative) within ≤ 60 min. Results are reported per group as the mean of all animals. Progressive motility was assessed by examining at least five non-overlapping microscopic fields per sample at 400× on a pre-warmed stage. Progressive motility was evaluated by reviewing at least five non-overlapping microscopic fields, and we report it per group as the mean across five fields from all animals. Sperm death was assessed by eosin–nigrosin staining; the percentage of dead sperms was recorded per animal, and we reported the group mean. To determine sperm morphology, we use the Esperma form kit (DESEGO®, Ferti México) according to the manufacturer's instructions and evaluated the strict qualitative criteria [43]. (Tygerberg/WHO) adapted to rodents, scoring ≥200 sperm per animal on stained smears (100× oil). We recorded the total number of defect categories (loose head, bent and loose tail, and cytoplasmic drop amount) [43].

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2.8 Blood-testis barrier integrity assay

The permeability of the blood-testis barrier (BTB) was assessed using a biotin tracer, following the protocol proposed by Meng et al. [44]. We exposed the rat’s right testes under anesthesia, and a small opening was gently created in the tunica albuginea. 50 µl of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co.), a membrane-impermeable

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biotinylation reagent, freshly diluted in PBS containing 1 mM $CaCl_2$, was injected into the interstitium. After 30 minutes, the animals were euthanized, and their testes were immediately removed and fixed in 4% w/v paraformaldehyde in phosphate-buffered saline. The samples were dehydrated through increasing ethanol concentrations, cleared with xylene, and embedded in paraffin. The samples were sectioned at 5 µm on the microtome. Rehydrated sections were microwaved (two cycles of 10 min each) in 0.01M sodium citrate, pH 6, and cooled to room temperature. After rinsing in PBS, the slides were incubated in 0.5% Triton X-100, and non-specific immunoglobulin binding was blocked by incubating sections in 5% bovine serum albumin (BSA) for 30 min. The sections were incubated for 2 h at room temperature with streptavidin conjugated to Alexa Fluor 488 [Invitrogen Thermo Fisher Scientific (Waltham, MA, EUA), A-11034] at a 1:100 dilution, as described by Meng et al. [44]. After three washes with PBS, the samples were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (H-1200-10, Vector).

The distribution of Alexa Fluor 488 (green fluorescence) in the seminiferous epithelium among tubules was monitored by fluorescence microscopy using a Nikon Eclipse E600 fluorescent microscope. In the negative control, no EZ-Link Sulfo-NHS-LC-Biotin was administered to the animal, or the sections were not incubated with streptavidin conjugated to Alexa Fluor 488.

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We used ImageJ software to evaluate the damage grade in the BTB. To obtain semiquantitative data for the assessment of BTB integrity and statistical analysis, the ratio between the distance (D) traveled by the fluorescence signal from the basement membrane in a seminiferous tubule (D_{Signal}) and the radius of the tubule (D_{Radius}) was obtained for each tubule [45,46]. Approximately 50 randomly selected tubules from each testis of n = 3 rats (i.e., 150 tubules in total) were analyzed for every treatment versus control groups.

2.9 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

To localize and count apoptotic cells within the seminiferous tubules, TUNEL staining was performed according to the manufacturer's protocol for the In Situ Cell Death Detection Kit (Sigma-Aldrich, USA). Briefly, paraffin-embedded tissue sections (5 µm) were deparaffinized at 60°C for 30 min. The tissue was cleared with xylene and rehydrated in a graded series of alcohols. The sections were treated with 1 µg/ml proteinase K (Sigma, St. Louis, MO, USA) for 30 minutes at room temperature and permeabilized with 0.5% Triton X-100 for 2 minutes. After rinsing in PBS thrice for 5 min each, the sections were incubated with a TUNEL reaction mixture in a humidified chamber at 37°C for 1 hour. Subsequently, the slides were washed thrice for 5 min each in PBS and were mounted with VECTASHIELD® Antifade Mounting Medium

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with DAPI (H-1200-10, Vector). The sections were visualized using fluorescence microscopy using a Nikon Eclipse E600 microscope. TUNEL-positive cells from 60-70 seminiferous tubules per animal were counted from different fields to evaluate apoptosis in the testicular cells.

2.10 Histopathological evaluation

To evaluate the damage in the seminiferous tubule, we used the items reported by Vigueras-Villaseñor [47]. All the tissue on the slide was observed and assessed by sliding the slide in a zigzag, first with the 4x objective, followed by 10x and 40x. In some specific cases, 100x magnification was used.

- Item 1: Folding of the basal lamina, cell desquamation.
- Item 2: Epithelial vacuolization, multinucleated cells, pyknosis.
- Item 3: Tubules without spermatids.
- Item 4: Tubules without spermatocytes.
- Item 5: Tubules without spermatogonia.
- Item 6: Absence of all types of cells.

For each item, we assess the grade of damage: -: null; +: scarce; ++: moderate; +++: severe.

2.11 Statistical Analyses

We used the Student T-test to evaluate differences in continuous data between groups as pre-established contrasts; we used the Benjamini–

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Hochberg False Discovery Rate (FDR) procedure to control the multiplicity of a priori pairwise contrasts. We report FDR-adjusted p-values. Kruskal-Wallis test for the Johnsen Index, using Prism 8.0.1 (GraphPad, Software San Diego, CA, USA). A p-value $\leq$ 0.05 was considered statistically significant. Data are presented as mean $\pm$ standard error of the mean (SEM) unless specified.

3. Results

3.1 Testis antioxidant enzyme activity

There were no significant differences in antioxidant enzyme activity between the Ctrl and Ctrl-D groups, indicating that diabetes induction did not affect the activity of the studied enzymes in the testis (Figure 2). A significant reduction in GPx activity was observed in the VitE-D group vs. Ctrl (*p ≤ 0.05) and the BPS-D group vs. Ctrl (*p ≤ 0.05) and Ctrl-D groups ($^{\#}$p ≤ 0.05). Therefore, exposure to BPS and Vit-E reduced GPx activity (Figure 2A).

The VitE-D did not modify GR activity compared to the control group; therefore, its administration did not affect GR activity. BPS administration enhanced GR activity, with a significant rise in the VitE+BPS-D group compared to the VitE-D group (&p ≤ 0.05). Thus, BPS exposure in diabetic rats increased GR activity, despite VitE co-treatment (Figure 2B).

The Ctrl and Ctrl-D groups showed comparable GST activity levels, with no significant differences. A decrease in GST activity was observed in the

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VitE-D group compared with the Ctrl-D group ($^{\#}p \leq 0.05$). The VitE+BPS-D group showed activity similar to that of the Ctrl, Ctrl-D, and BPS-D groups. These results suggest that VitE treatment reduces GST activity, but not when co-administered with BPS (Figure 2C).

The Ctrl and Ctrl-D groups show the highest SOD activity levels, with no significant difference between the two. A significant decrease in SOD activity was observed in the VitE-D, BPS-D, and VitE+BPS-D groups vs. Ctrl-D ($^{\#}p \leq 0.05$). These results suggest that BPS-D and VitE exposure, alone or combined, inhibit SOD activity (Figure 2D).

The Ctrl and Ctrl-D groups showed comparable CAT activity levels with no significant differences. A reduction in CAT activity was seen in the BPS-D group compared with the Ctrl and Ctrl-D groups, although there were no significant differences. A decrease in CAT activity was observed in the VitE-D group vs. Ctrl and Ctrl-D groups ($^{*,\#}p \leq 0.05$). In contrast, a pronounced reduction in CAT activity was observed in the VitE+BPS-D group vs. Ctrl and Ctrl-D ($^{**,\#\#}p \leq 0.01$) groups. These results suggest an additive effect of BPS-D exposure and VitE supplementation. The findings indicate that BPS-D exposure significantly diminishes CAT activity, and the addition of VitE does not mitigate, but rather exacerbates this reduction (Figure 2).

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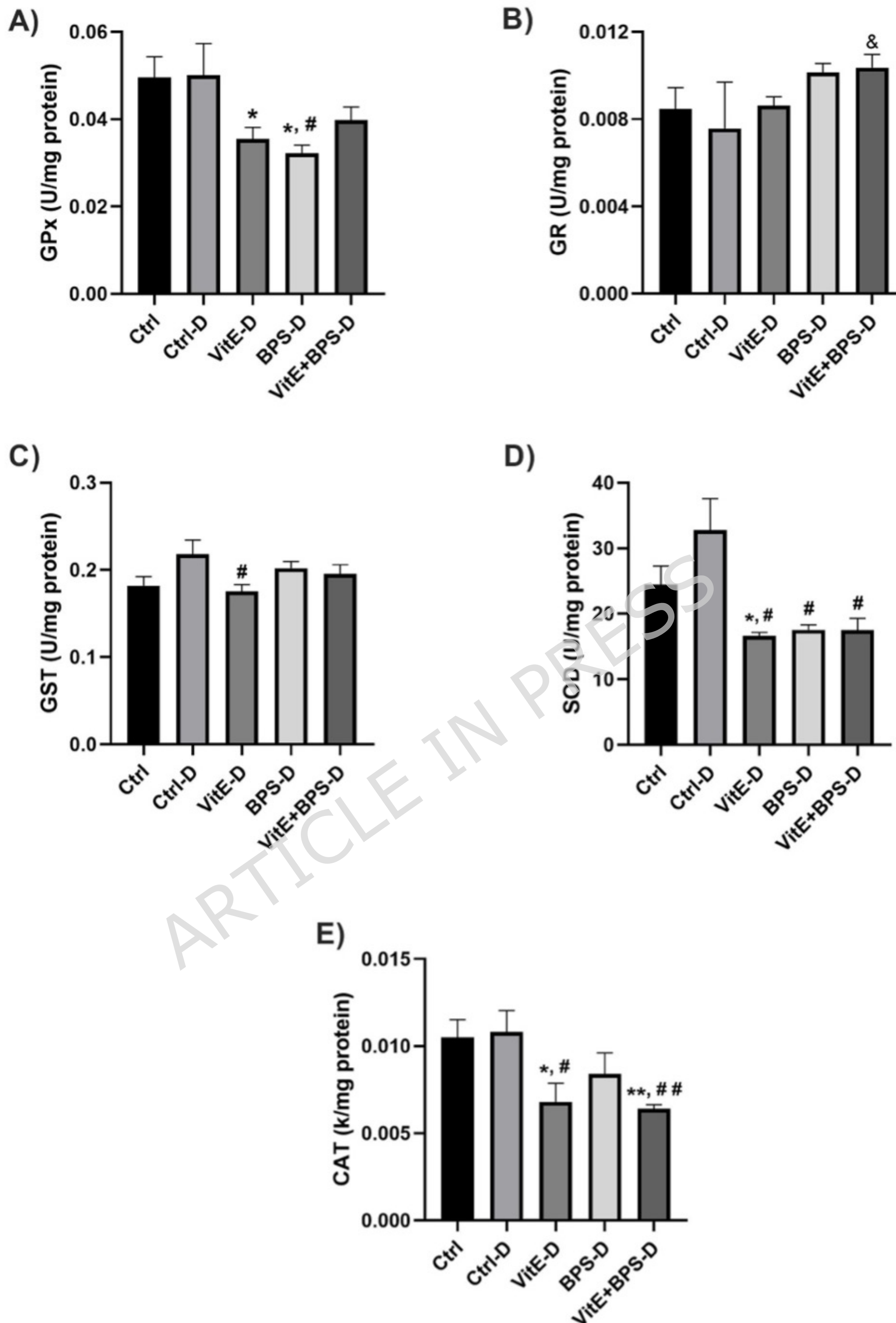

Figure 2. Antioxidant enzyme activity levels in different experimental groups: **A)** GPx (Glutathione peroxidase), **B)** GR (Glutathione reductase), **C)** GST (Glutathione S-transferase), **D)** SOD (Superoxide dismutase), and **E)** CAT

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(Catalase). Results are presented as mean ± standard error of the mean (SEM). Reduction of GPx activity was observed in the VitE-D group vs. Ctrl (*$p \leq 0.05$) and the BPS-D group vs. Ctrl (*$p \leq 0.05$) and Ctrl-D groups ($^{\#}p \leq 0.05$). GR activity was increased in the VitE+BPS-D group compared to the VitE-D group ($^{\&}p \leq 0.05$). Enhanced GR activity in the VitE+BPS-D group compared to the VitE-D group ($^{\&}p \leq 0.05$). A decrease in GST activity was observed in the VitE-D group compared with the Ctrl-D group ($^{\#}p \leq 0.05$). SOD reduced activity was observed in the VitE-D, BPS-D, and VitE+BPS-D groups vs. Ctrl-D ($^{\#}p \leq 0.05$). A decrease in CAT activity was observed in the VitE-D group vs. Ctrl and Ctrl-D groups ($^{*, \#}p \leq 0.05$), and in the VitE+BPS-D group vs. Ctrl and Ctrl-D ($^{**, \#\#}p \leq 0.01$) groups.

3.1 Effect of BPS and VitE+BPS on rat testis apoptosis

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was used to detect cellular DNA fragmentation as a marker of apoptotic cell death. The ratio of apoptotic cells to seminiferous tubules in the Ctrl group was 0.38 ± 0.09 (Figure 3). A significant increase in this ratio (*$p \leq 0.05$) was observed in all diabetic animals (Ctrl-D, VitE-D, BPS-D, and VitE+BPS-D) as compared to the Ctrl group (Figure 3). In addition, VitE+BPS-D showed a significant increase compared to the VitE-D group ($^{\&}p \leq 0.05$). These results suggest that diabetic animals exhibit a higher number of apoptotic cells in the testis than Ctrl animals. The administration of VitE + BPS further increases apoptotic cell numbers compared with VitE alone in diabetic rats (Figure 3).

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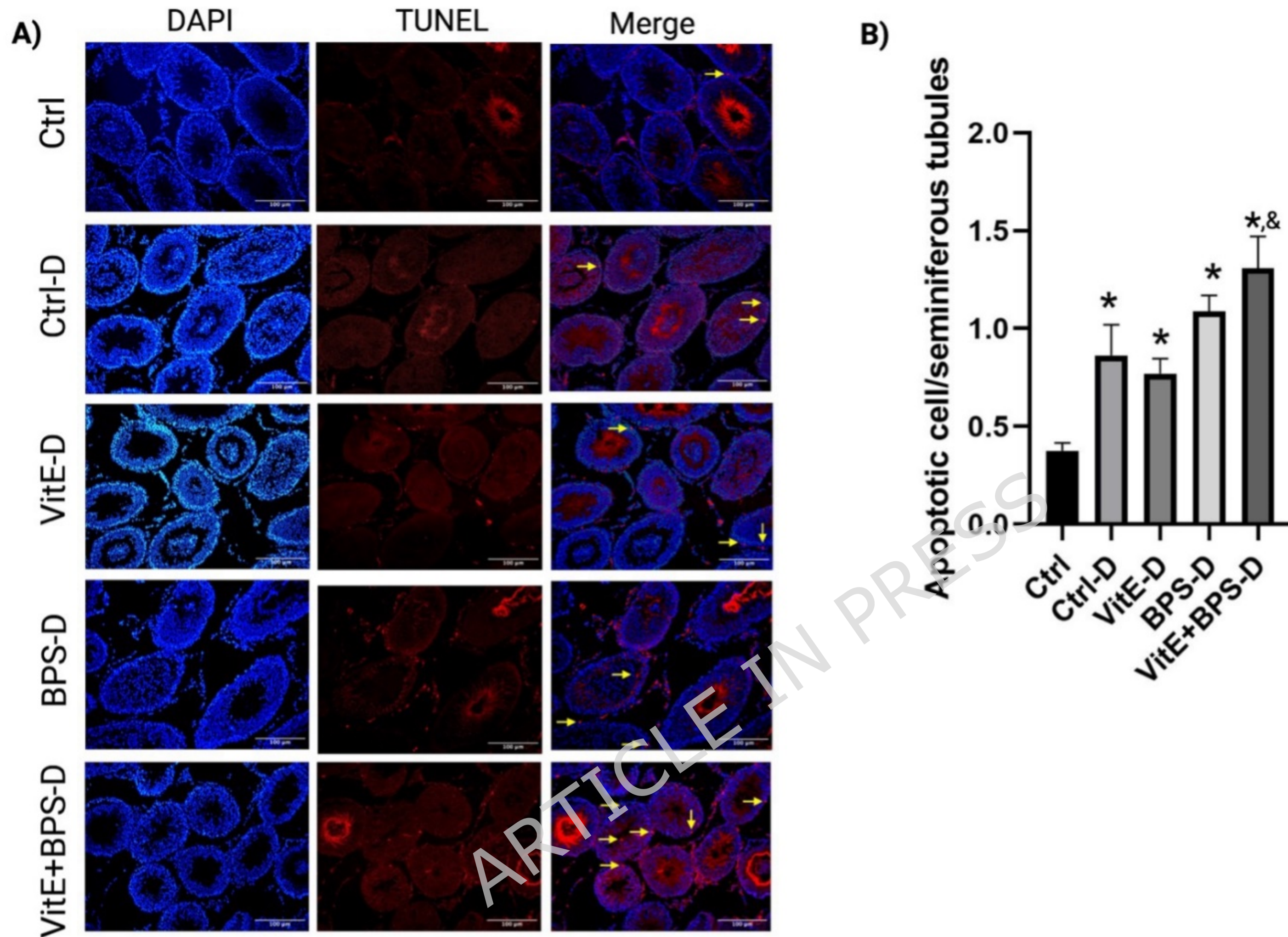

Figure 3. A) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was used to detect DNA fragmentation as a marker of apoptotic cell death. 4′,6-diamidino-2-phenylindole (DAPI) staining was used to detect all nuclei. Bar = 100 m.**B)** The graph shows the number of apoptotic cells per seminiferous tubule in the different experimental groups. Ctrl-D, VitE-D, BPS-D, and VitE+BPS-D groups vs. Ctrl group (*$p < 0.05$). VitE+BPS-

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apoptosis-positive cells.

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3.2 Sperm analysis

The Ctrl group exhibited the highest sperm concentration (122.5 ± 31.26 million sperm/ml). The sperm concentrations of the Ctrl-D and VitE-D groups were like those of the Ctrl group. Notably, the sperm concentration of the animals of the BPS-D group was significantly lower than that of the Ctrl group (*p ≤ 0.05) but not significantly different from that of the Ctrl-D group. Finally, the VitE + BPS-D group displayed the lowest sperm concentration, significantly lower than the Ctrl and VitE-D groups ($^{\&}p \leq 0.05$). The data suggest that BPS exposure significantly reduces sperm concentration, and VitE treatment does not counteract this effect when BPS is present (Table 1). The percentages of motile and dead sperm were similar across all groups, indicating that BPS or VitE treatment did not affect these parameters.

The morphological defects observed in sperm included loose heads, most frequently reported in the VitE+BPS-D group (total number: 4), while they were almost absent in other groups. Bent tails were predominantly observed in the VitE-D group (total number: 28), whereas loose tails were most frequent in the Ctrl-D group (total number: 14). Proximal cytoplasmic droplets were higher in the Ctrl group (total number: 3) and distal cytoplasmic droplets were higher in the BPS-D group (total number: 12). (Table 1).

Table 1. Sperm parameters were evaluated in control and diabetic male rats, which were administered daily for 29 days with 100 mg/kg bw/d of VitE (VitE-D group), 100 mg/kg bw/d of BPS (BPS-D group), or both (VitE+BPS-D group).

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	Concentration (mill/ml)	Progressive Motility (%)	Death (%)	No. loose head	No. bent tail	No. loose tail	No. cytoplasmic drop	
							Proximal	Distal
Ctrl, (n=6)	122.5 ± 31.26	59.17 ± 18.00	5.67 ± 2.25	0	27	1	3	7
Ctrl-D, (n=5)	91 ± 49.55	55.00 ± 15.81	11.80 ± 4.82	3	2	14	0	0
VitE-D (n=5)	106 ± 47.22	67.00 ± 13.96	7.80 ± 3.03	3	28	3	0	1
BPS-D (n=5)	65 ± 45.41*	58.00 ± 32.52	9.80 ± 6.87	0	25	1	0	12
VitE+ BPS-D (n=5)	42 ± 25.15*&	57.50 ± 38.62	6.50 ± 4.80	4	21	9	0	7

Concentration, motility, and death are presented as mean ± SD. No., the number observed. BPS-D and VitE+BPS-D $^{*}p < 0.05$ vs. Ctrl group, VitE+BPS-D $^{\&}p<0.05$ vs. VitE group.

3.3 Hormone Analyses

All diabetic animals showed increased estradiol levels compared to the Ctrl group, but the difference was not significant (Figure 4A). The Ctrl and Ctrl-D groups exhibited serum testosterone levels of approximately 3 ng/mL. The VitE-D group showed no significant increase in testosterone serum levels, reaching 8 ng/mL, the highest among the groups. The BPS-D group also displays elevated serum levels, but lower than those of the VitE-D group. The VitE+BPS-D group demonstrated significantly lower testosterone serum levels than the VitE-D group ($^{\&\&}p<0.01$) (Figure 4B).

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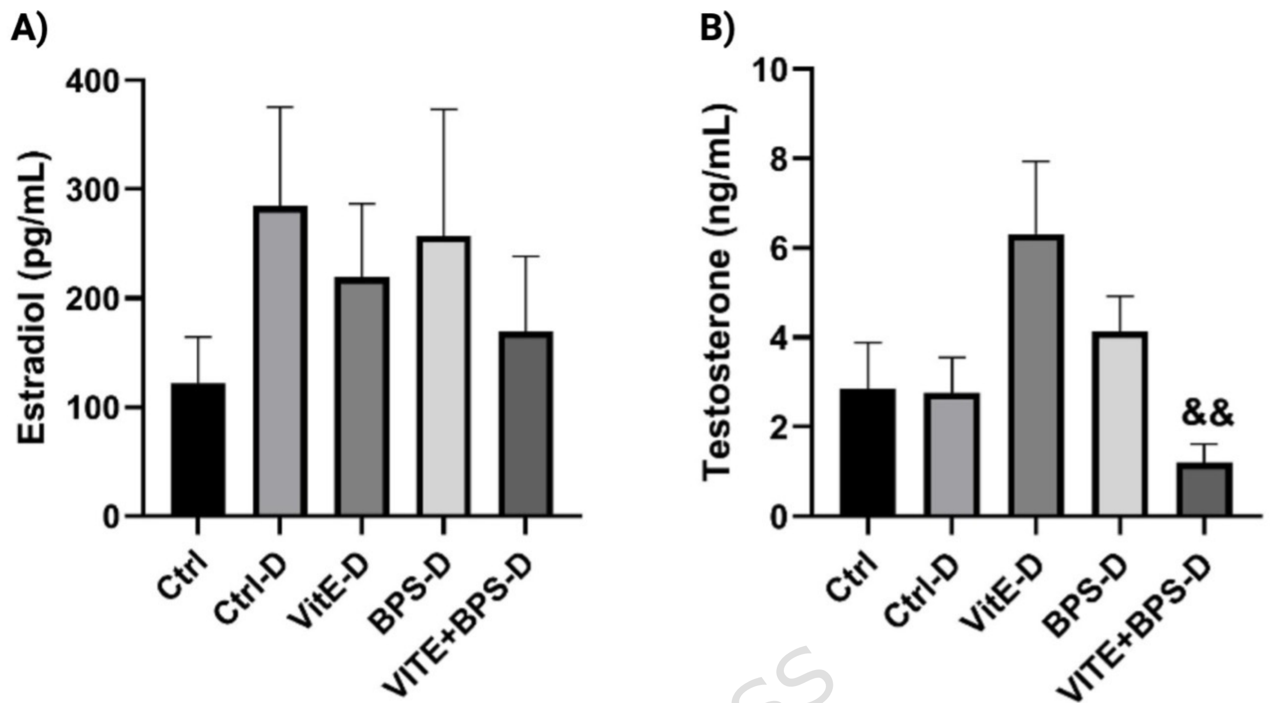

Figure 4. Serum hormone levels in the different experimental groups. **A)** Estradiol levels (pg/mL). **B)** Testosterone levels (ng/mL) VitE+BPS-D $^{\&\&}p<0.01$ vs. VitE-D.

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3.4 Seminiferous tubules morphological analyses

The Ctrl group showed the highest values for the tubular and epithelial area (μm^2) across all phases of the seminiferous epithelium cycle (I-VII, VIII-IX, IX-XIV) (Table 2). In the Ctrl-D group, there were notable reductions in these parameters across all phases as compared with the Ctrl group, although the differences were not significant (Table 2). The BPS-D group showed reduced tubular and epithelial area in phases VIII-IX compared with the Ctrl group ($*p < 0.05$) (Table 2). The tubular area of IX-XIV phases in the VitE +BPS-D group group was smaller than that of the VitE-D ($p \leq 0.05$), but larger than the BPS-D and Ctrl-D groups

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(Table 2). Therefore, Vit-E seems to protect seminiferous tubules, particularly in the presence of harmful substances.

Table 2. Quantitative analysis of seminiferous tubular and epithelial area (μm^2) across phases of the seminiferous epithelium cycle (I-VII, VIII-IX, IX-XIV) in experimental groups.

Groups	**I-VII**		**VIII-IX**		**IX-XIV**	
	Tubular (μm^2)	Epithelial (μm^2)	Tubular (μm^2)	Epithelial (μm^2)	Tubular (μm^2)	Epithelial (μm^2)
Ctrl	46637 ± 2861	36591 ± 3092	44273 ± 2329	34586 ± 2486	39585 ± 3213	30513 ± 2554
Ctrl-D	35182 ± 3914	29561 ± 3676	34134 ± 3849	27158 ± 3519	31553 ± 3974	24780 ± 2949
VitE-D	42132 ± 2665	34705 ± 1501	41644 ± 4610	33618 ± 3444	37940 ± 3487	29205 ± 1836
BPS-D	35732 ± 4289	28237 ± 3097	34703 ± 2558*	25999 ± 1405*	30799 ± 3437	24268 ± 2660
VitE+BPS-D	41443 ± 1854	34751 ± 2739	41055 ± 2267	31788 ± 3024	33884 ± 722.8*#&$	26836 ± 1515

VitE + BPS-D compared to the Ctrl-D group (#$p \leq 0.05$); the VitE-D group (&$p \leq 0.05$); and versus the BPS-D group ($$p \leq 0.05$).

Regarding histopathological evaluation, our study revealed that the seminiferous tubules of the Ctrl group displayed a well-organized structure, with germ cells at various stages of spermatogenesis, including spermatogonia, spermatocytes, and spermatids, supported by a Sertoli cell layer and intact basement membrane with minimal testicular damage (Figure 5 and Table 3). At the same time, the Ctrl-D group exhibited disorganized germ cells, thinning of the seminiferous epithelium, pyknosis, tubules without spermatids, moderate epithelial

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vacuolization, and occasional multinucleated cells, with some loss of spermatids (Figure 5 and Table 3).

Disorganization persisted in the seminiferous tubules of the VitE-D group. However, their arrangement improved. The tubules partially maintained testicular structure, thereby reducing damage to spermatogenesis, although moderate epithelial vacuolization and cellular scaling remained (Figure 5 and Table 3). In contrast, the seminiferous tubules of the BPS-D group were severely disorganized, and reductions in germ cell populations, disrupted Sertoli cell structure, clear epithelial vacuolization, and multinucleated cells were observed, indicating the damaging effects of BPS on spermatogenesis (Figure 5 and Table 3). In the VitE + BPS-D group, the seminiferous tubules were most severely damaged across all parameters, suggesting that VitE fails to mitigate and may even exacerbate the adverse effects of BPS (Figure 5 and Table 3).

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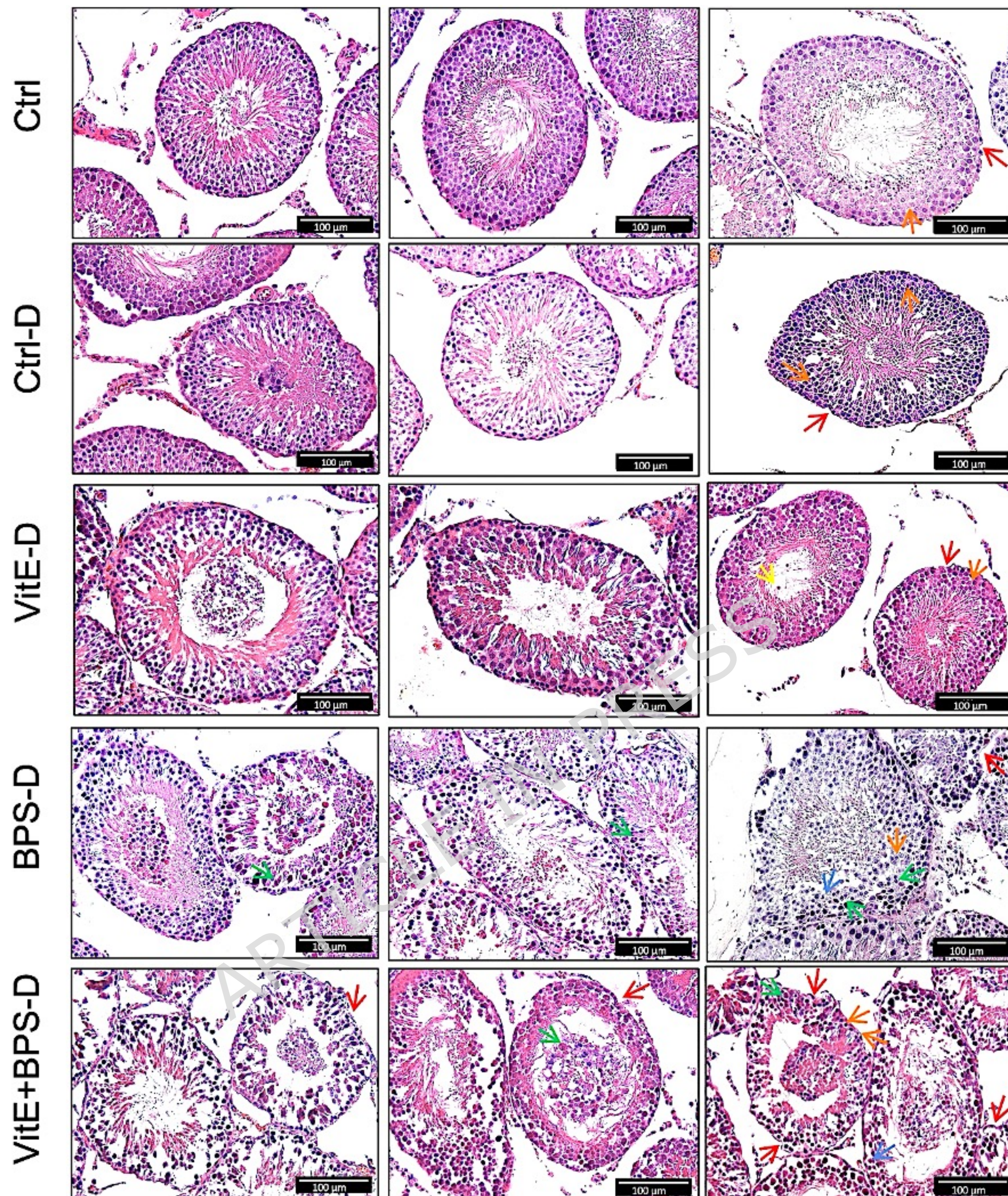

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Figure 5. Histological evaluation of seminiferous tubules stained with hematoxylin-eosin in different experimental groups. Representative images for each group (Ctrl, Ctrl-D, VitE-D, BPS-D, VitE+BPS-D) are shown. The parameters for evaluating the histopathological damage index were folding of the basal lamina (red arrow); epithelial vacuolization (orange arrow); multinucleated cells (green arrow); pyknosis (blue arrow); tubules without spermatids; tubules without spermatocytes; and tubules without

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spermatogonia. The arrow indicates the corresponding histopathological alteration. Scale bar: 100 μm.

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Table 3. Histopathological assessment of testicular damage induced by BPS and/or VitE in Wistar rats.

Group	ID	Folding of the basal lamina	Epithelial vacuolization	Multinucleated cells	Pyknosis	Tubules without spermatids	Tubules without spermatocytes	Tubules without spermatogonia
Ctrl	1	+	+	-	-	-	-	-
	2	+	+	+	-	-	-	-
	3	+	+	-	-	-	-	-
	4	+	+	-	-	-	-	-
	5	+	+	-	-	-	-	-
	6	+	+	-	-	-	-	-
Ctrl-D	1	+	++	+	-	-	-	-
	2	+	+	-	+	-	-	-
	3	+	+	+	+	+	-	-
	4	+	+	+	+	-	-	-
	5	+	+	+	+	-	-	-
VitE-D	1	+	+	-	-	-	-	-
	2	+	+	+	-	-	-	-
	3	+	+	+	+	-	-	-
	4	+	++	+	+	-	-	-
	5	+	++	+	+	-	-	-
BPS-D	1	++	++	++	+	+	-	-
	2	++	+++	+	+	+	-	-
	3	+++	++	++	+	-	-	-
	4	++	++	++	+	+	-	-
	5	++	++	++	+	+	-	-
VitE + BPS-D	1	+++	+++	++	++	-	-	-
	2	+++	+++	++	++	+	-	-
	3	+++	+++	++	++	+	-	-
	4	+++	+++	++	+++	++	-	-
	5	+++	+++	++	+++	++	-	-

ID: animal identification; -: no damage; +: scarce damage; ++: moderate damage; +++: severe damage.

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3.6 Johnsen scoring of the seminiferous tubules

The Johnsen score of the seminiferous tubules in all treatment groups was assessed as an index of healthy spermatogenesis (Figure 6). The Ctrl-D, BPS-D, and VitE+BPS-D groups exhibited significantly lower Johnsen scores than the Ctrl group. No significant differences were observed between the Ctrl and VitE groups. VitE did not reduce the damage to the seminiferous tubules when co-administered with BPS, as this group received the lowest score among the five treatments (Figure 6).

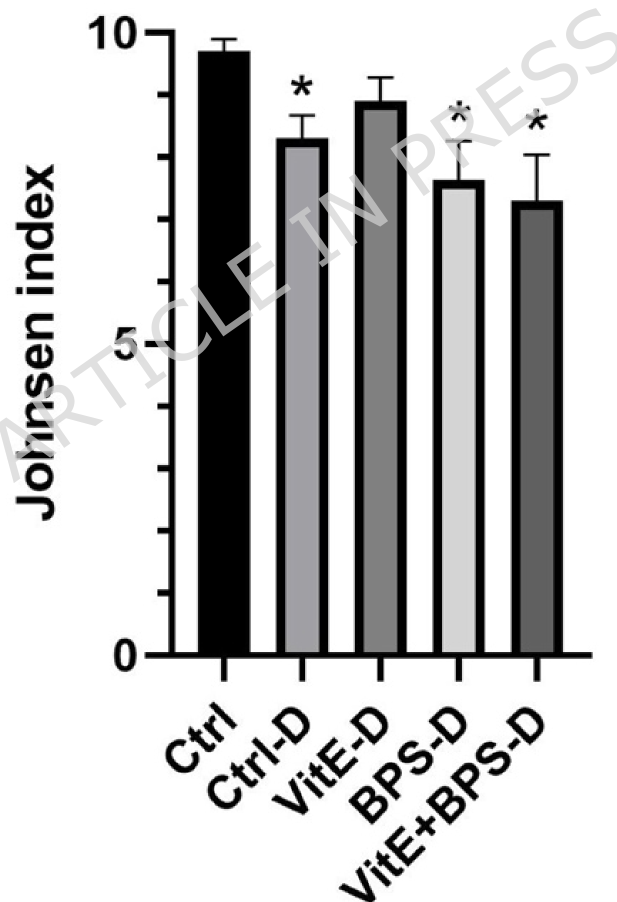

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Figure 6. Mean Johnsen score of testis seminiferous tubules of rats among various treatments. Ctrl-D, BPS-D, VitE+BPS-D vs. Ctrl group (*$p<0.05$). Values are presented as mean ± standard error of the mean (SEM).

3.5 Blood-testis barrier integrity assay

An *in vivo* functional assay was used to assess the presence of an effective BTB that blocked the entry of a fluorescence marker to the apical compartment following its administration. The semi-quantitative data obtained from such an assay (D_{Signal}/D_{Radius}) were used for statistical comparison and analysis as described in Materials and Methods. In the Ctrl and Ctrl-D groups, the fluorescence signal did not enter the apical compartment (Figure 7A). It exhibited the lowest D_{Signal}/D_{Radius} values, indicating the presence of an effective BTB. In contrast, in the VitE-D, BPS-D, and VitE+BPS groups, the fluorescence signal penetrated the apical compartment in some seminiferous tubules (Figure 7A), indicating the rupture of the BTB since it was no longer capable of blocking the passage of biotin across the immunological barrier. BTB permeability was evaluated, and all three groups showed higher D_{Signal}/D_{Radius} values (Figure 7B) than the Ctrl and Ctrl-D groups. Both the VitE-D and VitE+BPS-D groups significantly differed from the Ctrl-D group,

while the VitE-D was also significantly different from the Ctrl group. Our results show the

damaging impact of BPS and VitE on the BTB integrity (Figure 7).

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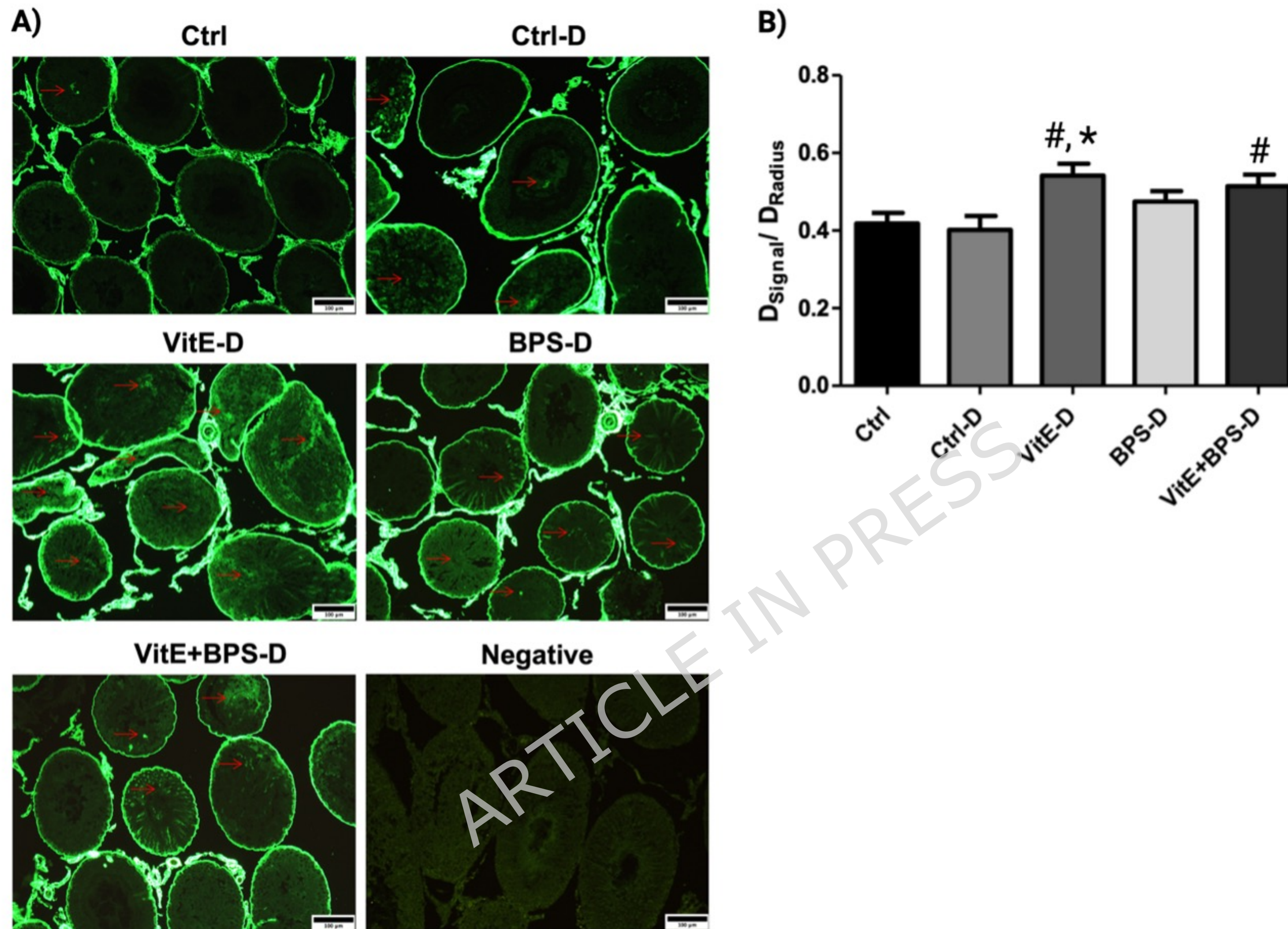

Figure 7. A) Biotin signal (green) in seminiferous tubules. The negative control shows no biotin signal. The red arrow points to the biotin tracer penetrating the apical zone of the seminiferous tubules. **B)** Quantification of the biotin tracer penetration in seminiferous tubules. VitE-D vs. Ctrl group (*$p<0.05$); VitE-D and VitE+BPS-D vs. Ctrl-D group $^{\#}p<0.05$. Values are shown as mean ± standard error of the mean (SEM). Scale bar = 100 μm.

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4. Discussion

DM comprises a group of metabolic disorders marked by persistent hyperglycemia due to defects in insulin secretion, insulin action, or both. [48]. To date, numerous countries are facing this health issue—a global "epidemic" of diabetes that is spreading rapidly worldwide. [49]. The disorder has been commonly classified as type 1 diabetes (TD1), type 2 diabetes (TD2), or gestational or monogenic diabetes [50]. According to the International Diabetes Federation Atlas, about 415 million people worldwide had DM in 2015. That total is projected to increase to 642 million by 2040 [51].

The results in this article show that diabetes increased the number of apoptotic cells/tubules, with a significant increase observed in all diabetic groups (Ctrl-D, VitE-D, BPS-D, VitE+BPS-D) compared with the Ctrl group. Furthermore, BPS administration in the BPS-D and VitE+BPS-D groups led to an additional increase in the number of apoptotic cells/tubules compared with VitE alone in diabetic rats. Moreover, the growth of apoptotic cells in BPS-treated groups is evident compared with diabetic controls, although no significant differences were observed. An increase in sample size may be required to detect statistical differences because, given the small group sizes, statistical power is limited, and precision of estimates is reduced; therefore, non-significant findings should be interpreted cautiously.

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These results are consistent with previous findings. A 2021 study [52] in adult male C57BL/6 mice found that exposure to BPS for 28 consecutive days induced apoptosis of spermatogenic cells in a dose-dependent manner, observing a significant increase in the 200 mg/kg bw group compared to the control group. Another study [53] on CD-1 mice exposed to BPS from gestational day 11 until birth showed a significantly ($p < 0.01$) higher number of TUNEL-positive germ cells at all doses tested (0.5, 20, and 50 µg/kg day), indicating that BPS induced apoptosis in the neonatal testis. Another study [54] carried out on adult male Parkes (P) strain mice treated with BPS (150 mg/kg bw) for 28 days showed a significant ($p < 0.001$) increase in the number of TUNEL-positive testicular cells. In 2021, Kumar *et al.* assessed the effects of 75 mg/kg bw of BPS in adult male golden hamsters and performed Western blot analysis for caspase 3, a well-known apoptosis biomarker. This study [55] found that BPS treatment enhanced caspase-3 expression in the testis and, consequently, testicular germ cell apoptosis.

In 2022, a study [56] evaluated the reproductive effects in adult male rats exposed to low doses of BPS (0.216 ng/g bw/day and 21.6 ng/g bw/day) via breast milk from post-natal day 0 to 15. In this study, TUNEL assays were performed on blastocysts flushed after mating with exposed males and revealed increased γH2AX in blastomeres in the 21.6 ng/g bw/day BPS group, indicating DNA damage and apoptosis. According to our results, VitE does not decrease the number of apoptotic cells/tubules

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when administered with BPS. These findings indicate that BPS is not a safe substitute for BPA, as it causes markedly high levels of testicular cell apoptosis—which may result in abnormal spermatogenesis—and that co-supplementation with VitE fails to mitigate its harmful effects.

Bisphenols have pleiotropic effects; it has been established that they induce apoptosis in testicular cells through various signaling pathways, including oxidative damage. Oxidative damage to cellular components, including lipids, proteins, and DNA, can trigger the intrinsic apoptotic pathway [57]. Additionally, bisphenols bind to estrogen receptors in testicular cells, altering the transcription and translation of genes involved in cell proliferation and survival [58]. This disruption can result in hormonal imbalances and trigger apoptosis by activating various signaling pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and G protein-coupled receptor (GPR30), or by increasing global DNA methylation levels in testicular cells, which are associated with apoptosis and impaired cell function [58]. Bisphenols can induce endoplasmic reticulum (ER) stress by disrupting protein folding and processing [59]. If ER stress is severe, it can lead to apoptosis, increasing the production of reactive oxygen species (ROS) within testicular cells [60,61].

Studies in rats have shown that oxidative stress caused by BPA and BPS impairs the enzymatic antioxidant defense system in the testes. In rats exposed to BPA, VitE was found to restore enzymatic antioxidant

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defenses by increasing the levels of SOD and CAT in the testes. However, the potential of VitE to restore antioxidant defenses disrupted by BPS has not yet been investigated [62].Additionally, oxidative stress induced by BPA can be further aggravated by coexisting conditions such as diabetes [63]. Furthermore, it has been shown that VitE can act as a pro-oxidant and promote additional DNA damage, as demonstrated by Vivarelli et al. (2019) [64].

An excessive level of free radicals and oxidative stress has been associated with sperm damage (abnormalities in sperm morphology, concentration, and motility), reduced testosterone production, and male infertility [61]. Although antioxidant enzymes are highly effective, cells still experience oxidative damage under normal physiological conditions. [65]. The testes possess several antioxidant enzymes and radical scavengers to prevent oxidative stress from affecting normal spermatogenic and steroidogenic functions [66]. The antioxidant enzymes existing in the testis comprise SOD, CAT, GPx, GST, and GR, and the antioxidant factors are zinc and vitamins C (ascorbic acid) and E (α-tocopherol) [67,66].

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Our results show that GR increases significantly in the VitE+BPS-D groups compared with the VitE groups. GPx activity was significantly reduced in the BPS-D and VitE-D groups, suggesting that VitE and BPS exposure significantly decreases GPx. SOD activity significantly decreased in the VitE-D, the BPS-D, and the VitE+BPS-D groups.

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Similarly, CAT activity was significantly lower in the VitE-D and Vit-E+BPS-D groups. Therefore, VitE did not mitigate the detrimental effects of BPS on SOD and CAT antioxidant activities.. Dai *et al*. [52] similarly found that BPS at a dose of 200 mg/kg significantly ($p < 0.05$) reduced SOD and GPx activities in the testes of mice compared to the control group. However, in their study, GST activity also declined, which contrasts with our results. Another study [53] supports that BPS (0.5 and 20 µg/kg/day) significantly reduced the mRNA expression of oxidative-stress-related genes Gpx4, Sod2, and Cat and increased GR in the testis. Moreover, Sahu *et al.* (2023) and Kumar *et al.* (2021) both confirmed that BPS (150 mg/kg bw and 75 mg/kg bw, respectively) significantly decreased SOD and CAT activities in the testis of rats treated for 28 days [54,55]. Additionally, Kose *et al.* (2019) also evidenced that BPS (50–600 µM for 24 hours) reduced GPx, SOD, and CAT activity in RWPE-1 epithelial cells and significantly increased GR activity [20].

Consequently, exposure to BPS altered antioxidant activity in a diabetic rat model: decreased levels of GPx (and general peroxidase), SOD, and CAT, consistent with reduced antioxidant activity, and increased levels of GR. Under physiological conditions, antioxidant enzymes increase their activity in response to oxidative stress to scavenge toxic oxygen metabolites and free radicals [68]. SOD is one of the principal and most effective intracellular antioxidative enzyme systems [69,70]. There are three isoforms of SOD in mammals, all of which catalyze the dismutation

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of superoxide ions into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is then reduced to water and oxygen by CAT and GPx [68].

The antioxidant effects of VitE have been studied in rats, yielding inconsistent findings.. For example, Golestani *et al*. (2006) assessed the effects of different doses of vitamin E (100, 300, and 600 mg/kg body weight), administered twice weekly for six weeks, on erythrocyte SOD activity and plasma total antioxidant capacity in rats. The results showed that while a 100 mg/kg dose of VitE increased SOD activity after six weeks, higher doses (300 and 600 mg/kg body weight) led to a decrease in SOD activity after the fourth and sixth weeks of treatment, thereby reducing overall antioxidant activity. [69]. VitE (α-tocopherol) is a liposoluble vitamin that rapidly accumulates in the liver and adipose tissue and is commonly known for its antioxidant properties. However, several other studies have proposed VitE as a pro-oxidant. VitE needs co-existing antioxidants, such as vitamin C and coenzyme Q10, for its regeneration. VitE's antioxidant mechanism was explained by Traber et al. (2020) as follows: "During lipid peroxidation, the hydroxyl group of the VitE reacts with the peroxyl radical to form the corresponding lipid hydroperoxide and the chromanoxyl radical […] The free radical reaction is halted because the chromanoxyl radical of VitE then reacts with vitamin C (or other hydrogen donors), thereby oxidizing the latter and regenerating vitamin E in its reduced form" [71]. Since vitamin C and

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ubiquinol decrease the activity of α-tocopheroxyl radical to α-tocopherol [72,73], they act as co-antioxidants, and their presence is necessary for VitE's optimum free-radical scavenging activity. When co-antioxidants are exhausted at high doses of VitE and/or during prolonged VitE treatment, VitE acts as a pro-oxidant, as reported by Golestani et al. (2006) [69].

As noted in the antioxidant results, DM did not impair testicular redox balance. Indeed, in some cases (e.g., SOD activity), DM appears to enhance enzymatic function, although there were no significant differences compared with the Ctrl group. Although TUNEL staining clearly shows apoptosis induced by DM. In diabetic testes, sustained ROS pressure can induce adaptive up-regulation of enzymatic defenses without entirely preventing cell loss; antioxidant induction and apoptosis are not mutually exclusive but may co-occur when detoxification capacity is exceeded [74,75].

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Our experimental data showed that BPS exposure diminished sperm concentration, and VitE treatment did not mitigate this effect when BPS is present. A lower sperm concentration is an indicator of abnormal sperm quality and is correlated with male infertility [76]. Diverse studies have examined BPS's influence on sperm concentration and production, consistently reporting its harmful effects on these parameters. For example, lower sperm concentration was observed in mice [53,54,52], rats [77,67], hamsters [55], and zebrafish [78] in different life stages

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following the administration of various doses of BPS. Additionally, a cross-sectional study involving 158 men from couples undergoing fertility treatment found that BPS was present in 76% of all urine samples. Higher urinary BPS levels were linked to poorer sperm parameters, with the strongest associations observed in overweight and obese men [79]. Consequently, there is enough evidence to support that BPS exposure diminishes sperm concentration and motility, which further leads to infertility. Sperm is particularly vulnerable to oxidative stress due to its high content of unsaturated fatty acids; excessive ROS can cause cellular damage, lipid peroxidation, and DNA fragmentation [80]. GPx uses selenocysteine as the active site to catalyze the reduction of hydrogen peroxide or organic hydroperoxides to water or corresponding alcohols [81]. GPx, which was significantly reduced in diabetic animals treated with BPS in our study, is known to be the enzyme responsible for protecting sperm DNA from oxidative damage [82].

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Although testosterone levels in the VitE group did not differ significantly from those in the Ctrl-D and BPS-D groups, they tended to be higher. It is possible that with a longer treatment duration, the difference could become significant, as reported in other studies where seven months of VitE administration stimulated testosterone production [83]. There were substantial differences in testosterone levels between the VitE+BPS-D and the VitE groups ($p<0.01$). It is likely that, in combination with BPS, a negative feedback mechanism was triggered, resulting in a

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significantly lower testosterone level.. In addition, testosterone levels in the control groups (Ctrl and Ctrl-D) were similar to other reports, in which intact and castrated males presented very low levels, ranging from 1.6 to 4.2 pg/mL. This suggests that VitE treatment plays an essential role in the response to testosterone.

Other studies have documented alterations in testosterone concentration due to BPS exposure. Molangiri *et al.* (2022) reported that a dose of 0.4 μg/kg/day from gestational day 4 to day 21 increased serum testosterone levels in male rats [84]. However, Naderi *et al.* (2014) [78], Kumar *et al.* (2021) [55], and Ullah *et al.* (2021) [85] all report that BPS administration decreased serum testosterone levels in different animal models and doses. Serum testosterone levels were not different between the experimental groups and the control vehicle following low BPS doses (0.001-50 μg/kg bw/day) [53,86]. It suffices to say that BPS exposure produces changes in the testosterone concentration, which is related to impaired spermatogenesis [87] and is further discussed in this section.

Even to mitigate random variation, all blood samples were collected within a fixed morning window, under identical handling and processing conditions. We recognize that a limitation of our study is that only measured testosterone was collected at one terminal time point; in the future, it is necessary to incorporate serial sampling across multiple time windows.

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The histopathological microscopic examination of the testes of Wistar rats showed standard tissue architecture in the Ctrl group, with sequentially arranged germ cells at different stages of spermatogenesis, a lumen containing spermatozoa, and a small number of basal lamina folds and epithelial vacuolization. However, diabetic rats showed alterations in testicular tissue architecture: sloughing of germ cells into the lumen, an increase in the interstitial space, and epithelial vacuolization; the seminiferous tubulalso showed a reduction in germ cell and spermatozoa numbers. Animals in the VitE group showed improved histomorphology of seminiferous tubules. This could be explained by the fact that the administration of VitE activates spermatogenesis and the development of smooth-surfaced ER in Leydig cells, suggesting the enhancement of steroidogenesis [88,83]. The damage was increased in diabetic rats treated with BPS, with marked interruptions at different stages of spermatogenesis, larger vacuoles and intercellular spaces, and a significant increase in multinucleated cells and pyknosis, indicating higher rates of DNA damage and apoptosis. Coadministration of BPS and VitE further augmented morphological and histopathological alterations. Although VitE showed antioxidant effects in previous studies, in this context, it failed to mitigate the negative impact of BPS, possibly due to the severity of BPS-induced oxidative stress in combination with diabetes and the probable pro-oxidant effect of VitE. Moreover, Johnsen’s score was lower in the Ctrl-D, BPS-D, and BPS-D+VitE groups compared to the

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Ctrl group, reflecting hypospermatogenesis. Likewise, administering VitE did not alleviate the damaging effects of BPS and diabetes.

Other studies corroborate that BPS is detrimental to the male reproductive system since it instigates histological abnormalities and spermatogenesis impairment. A study [52] conducted on male C57BL/6 mice treated with 2–200 mg/kg/day of BPS for 28 days reported irregular arrangements of spermatogenic cells, increased dispersion of spermatocytes, reduced epithelial height, and swollen, vacuolated mitochondria at all doses. Additionally, autophagic vacuoles and condensed, marginated chromatin were observed at doses of 20–200 mg/kg.. Another study [54] on adult mice treated with 150 mg/kg/d for 28 days found marked presence of vacuoles, reduced germ cell number, germ cell loosening, lumen without sperm, a significant ($p < 0.001$) decrease in height of the germinal epithelium, seminiferous tubule diameter, and area in the BPS only treated group as compared to the control.

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Ullah *et. al*. (2021) assessed the effects of chronic exposure to BPS (0.5, 5, and 50 µg/L in drinking water for 48 weeks) on weaning male Sprague-Dawley rats (22 days old). In groups exposed to 5 µg/L and 50 µg/L, they observed a significant reduction in testicular epithelial height, disrupted spermatogenesis, empty seminiferous tubule lumens, and caput epididymis [85]. Řimnáčová *et. al.* (2020) evaluated the harmful effects of very low doses (0.001, 1.0, or 100 µg/kg bw/day) of BPS on adult ICR

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male mice and concluded that, compared to vehicle, exposure to 100 μg/kg/day of BPS leads to significant testicular damage, evidenced by vacuolization of germ layer cells, enlarged multi-nuclear cells, presence of atypical residual bodies, and fewer mature spermatozoa in the germ layer [86].

Histological damage induced by low doses of BPS was corroborated in another study in adult male rats [67]. The group treated with 50 μg/L of BPS in drinking water for 10 weeks showed alterations in the testicular histoarchitecture (not present in the control group) and lower numbers of spermatogonia, spermatocytes I, spermatocytes II, and spermatids. In line with our results, Johnsen's score was significantly lower in the BPS group than in the control group. Therefore, there is sufficient evidence that BPS, even at low doses, induces alterations in testicular histoarchitecture and impaired spermatogenesis.

Bisphenols can cross BTB by reducing the expression of several junction proteins, directly damaging the structures and functions of germ and Sertoli cells [89,77]. Also, bisphenols have been shown to impair Leydig cell function, thereby reducing testosterone biosynthesis. These results suggest that bisphenols can inhibit spermatogenesis[90,91]. A study [92] explains that BPS impairs the blood-testis barrier and the integrity of the apical ectoplasmic specialization by altering actin-binding protein expression, leading to cytoskeletal actin disorganization and diminishing junctional protein expression.

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This may be attributed to the imbalance between mTORC1 and mTORC2. An *in silico* docking study [67] described a possible mechanistic pathway by which BPS may be responsible for decreased steroidogenesis and spermatogenesis: BPS binds to the steroidogenic acute regulatory (StAR) protein, thereby affecting cholesterol transport into mitochondria. Likewise, BPS exposure affects gonadotropin-releasing hormone (GnRH) transcript expression in the hypothalamus [93]. This is significant since GnRH drives steroidogenesis, germ cell progression, and acquisition of spermatozoa functions [94]. BPS exposure also affected steroidogenic gene expression in H295R adrenal carcinoma cells [95].

Results demonstrate that although BPS is used as an alternative to BPA in some countries, its exposure can lead to similar toxic effects, including but not limited to decreased sperm concentration, altered hormone levels, changes in antioxidant enzymes, and oxidative damage, which can lead to infertility and the exacerbation of other chronic conditions such as diabetes.

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6. Conclusions

Exposure to BPS negatively affects male reproductive health by inducing oxidative stress, cellular apoptosis, and structural damage to testicular tissue. While VitE has been considered a protective antioxidant, in this study, it failed to counteract the detrimental effects of BPS. It even exacerbated the damage, likely due to its potential pro-oxidant activity in

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the absence of co-antioxidants. These findings emphasize the need for careful evaluation of antioxidant use under specific conditions and the development of integrated therapeutic strategies to mitigate the effects of endocrine disruptors such as BPS, especially in individuals with comorbidities like diabetes. Further research is crucial to fully understand these processes and develop strategies to mitigate the adverse effects of bisphenol exposure.

Competing interests

Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Declarations

Ethics approval and consent to participate

Not Applicable

Clinical trial number

Not applicable

Consent for publication

Not Applicable

Availability of data and material

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Competing interests

Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Authors' contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas. That is revising or critically reviewing the article; giving final approval of the version to be published; agreeing on the journal to which the article has been submitted; and, confirming to be accountable for all aspects of the work.

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