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Chronic unpredictable stress disturbs the blood–testis barrier affecting sperm parameters in mice

BIOGRAPHY

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KEY MESSAGE

Chronic stress disrupted the blood–testis barrier by altering the expression levels of tight junction proteins zonula occludens-1 and claudin-11 in mice. Sperm parameters were affected negatively. The results demonstrate the molecular effects of stress in male infertility and can help create prevention and treatment strategies to minimize adverse effects.

ABSTRACT

Research question: Does chronic stress affect the key proteins and sperm parameters of the blood–testis barrier (BTB)?

Design: C57Bl/6 mice were divided into two groups: a non-treated control group and a chronic unpredictable stress (CUS) applied group. The stress status of the animals was confirmed with behavioural tests. Histopathologic evaluation was conducted by haematoxylin and eosin staining and electron microscope. Malondialdehyde, corticosterone and testosterone levels were evaluated in peripheral blood. Expression levels of BTB proteins, namely zonula occludens-1 (ZO-1), claudin-11 (CLDN11) and clathrin in Sertoli cells, were assessed by Western blotting and immunofluorescence techniques. Sperm samples were collected from cauda epididymis, and sperm parameters analysed.

Results: The stress model was confirmed by behavioural tests. Histopathological evaluation of the testes demonstrated a mild degeneration in seminiferous tubules. Malondialdehyde ($P = 0.008$) and corticosterone levels increased ($P = 0.004$) and testosterone levels decreased ($P = 0.005$) in the CUS group. Electron microscopic evaluation confirmed the damage in BTB integrity in the CUS group. Western blot analysis showed that ZO-1 and CLDN11 levels were significantly decreased, although clathrin levels were unchanged. Although sperm concentration and total motility rate were not significantly different between the groups, progressive motility ($P = 0.03$), normal sperm morphology ($P = 0.04$), chromatin integrity (toluidine blue) ($P = 0.002$) and the acrosomal reaction rate ($P = 0.002$) were significantly decreased, and acrosomal abnormality rate was dramatically increased ($P = 0.04$) in the CUS group.

Conclusions: In mice, CUS disrupted BTB integrity and impaired sperm parameters. A decrease in ZO-1 and CLDN11 expression levels may be proposed as the causative factor.

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KEYWORDS
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INTRODUCTION

Stress is described as the 'epidemic of the 21st century' by the World Health Organization (*Fink, 2017*). Hans Selye defined stress as a 'non-specific response of the body to any demand for change' in the 1920s (*Selye, 1936*). Since then, researchers have investigated the effects of acute and chronic stress on emotional, behavioural and physiological changes. Because acute stress keeps the body alert against external threats, it makes the animal (or the individual) ready to fight. Although acute stress is known not to cause severe pathological conditions, chronic stress is reported to lead to psychological and physiological traumatic effects (*Cannon, 1927*).*Katz et al. (1982)* reported that rats exposed to various stress factors, e.g. shocking, cold swim, food, water deprivation, heat and shaker stress and reversal of daily cycle, displayed a lower desire to drink and they exhibited anhedonic behaviours (*Katz, 1982*). Manipulations were made in the protocols to moderate stressors for ethical reasons and take daily stressors into account (*Willner et al., 1987*). Also, the term 'unpredictable' was added to chronic stress models, in which the stressors were applied randomly to break the resistance of the animal (*Willner, 2017*). Recently, many animal models have been developed to investigate the effects of chronic stress on the nervous system (*Qiao et al., 2016*; *Cameron and Schoenfeld, 2018*), the cardiovascular system (*Golbidi et al., 2015*), the urinary system (*Samarghandian et al., 2016*; *Samarghandian et al., 2017*) and the reproductive system (*Divyashree and Yajurvedi, 2018*; *Yazir et al., 2018*). It has been suggested that stress affects organ systems in the human body, including the reproductive system, causing infertility (*Nargund, 2015*).

Infertility is defined as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse, a multifactorial condition affecting 8–12% of couples, especially in developing countries (*Ombelet et al., 2008*). Male-induced infertility constitutes up to around 50% of all infertility cases (*Vander Borght and Wyns, 2018*). The incidence of increasing stress and the number of couples experiencing infertility problems in recent years have led researchers to conduct studies in the field of stress-induced infertility (*Bräuner et al., 2020*).

Spermatogenesis occurs in seminiferous tubules lined by seminiferous epithelium with Sertoli and spermatogenic cell populations. In the seminiferous tubules, the Sertoli cell junctional complex divides the seminiferous epithelium into basal and adluminal compartments, and this junctional complex is known as the blood–testis barrier (BTB) (*Jiang et al., 2014*). The BTB is thought to be one of the tightest barrier structures in the human body (*Siu and Cheng, 2004*; *2008*), which contains four types of junctional connections, including tight junctions, ectoplasmic specializations, desmosomes and gap junctions (*Mruk and Cheng, 2004*). The tight junction proteins belong to the integral membrane protein family and are classified as occludins, claudins and cell adhesion molecules (*Cereijido and Anderson, 2001*). Claudins (claudin 3,5,11) and occludins contribute to preserving the integrity of the BTB structure (*Chung and Cheng, 2001*).

Chronic stress is reported to be associated with sperm parameters and testosterone levels, along with anomalies in testicular histopathology (*Hirano et al., 2015*; *Sakr et al., 2015*; *Zhang et al., 2019*; *Xiao B, 2019*). Although the decrease in sperm count may be related to the induction of extrinsic and intrinsic pathways of apoptosis in spermatogonia (*Juarez-Rojas et al., 2015*), glucocorticoid receptor-mediated apoptosis or inhibition of testosterone in Leydig cells (*Yazawa et al., 2000*), further evidence is needed to clarify the underlying mechanism of sperm count decline. The BTB structure and the effects of chronic stress on the male reproductive system is presented in FIGURE 1.

The aim of present study was to examine the effect of chronic unpredictable stress (CUS) on the expression levels of proteins that contribute to the architecture of the BTB and on sperm parameters.

MATERIALS AND METHODS

Ethical statement

The present study was conducted in accordance with the ethical standards of the Declaration of Helsinki and national and international guidelines. It was approved by the Ethics Committee of Istanbul Medipol University (38828770-E.42875, 17 November 2017).

Animals and experimental design

Male C57BL/6 mice (aged 8 weeks, weighing 20–25 g) were used in the present study. The test animals were obtained from the animal care facility at Istanbul Medipol University. The mice were fed *ad libitum* and individually caged in a controlled environment at 21°C, with 60% humidity and a 12-h light–dark cycle. The mice were allowed a 1-week adaptation to their new surroundings before the experiment. A total number of 24 mice were divided into two groups. Control group animals (n = 10) were handled once a week but otherwise allowed free access to commercial pellet food, water *ad libitum* and left undisturbed. The mice in the stress group (n = 14) were exposed to 7 weeks of CUS because, in mice, spermatogenesis requires 48 days, and the stress protocol cannot be shorter than the spermatogenesis process. The general experimental design is presented in FIGURE 2.

Chronic unpredictable stress paradigm and behavioural assessment

The CUS protocol was carried out as described previously by *Monteiro et al. (2015)*, with some minor modifications. For CUS, seven different stressors were applied randomly. The stressors used were as follows: 4-h restraint (mice were placed in 50-ml plastic Falcon tubes with openings to enable airflow), 1-h shaking (mice were placed in a plastic cage and put on a shaker at 150 revolutions per min) 8-h tilted cage (mice were placed in a plastic cage tilted in a 45° angle), 12- h inverted light cycle (light was off during the daytime and on during night time), overnight illumination (the light left on during the night), for 10-min, hot air stream (mice were exposed to a hair-dryer from a 30-cm distance) and 8-h soiled cage (200 ml water in 100 g sawdust bedding) (TABLE 1).

Behavioural experiments were conducted in a quiet testing room, and mice were transported to the testing room for 1 h before habituation. For behavioural assessment, open field, elevated-o-maze (EOM), and tail suspension tests (TST) were applied three times in that order. The first test was carried out just before the CUS protocol was applied. The second was applied in the fourth week and the last at the end of the seventh

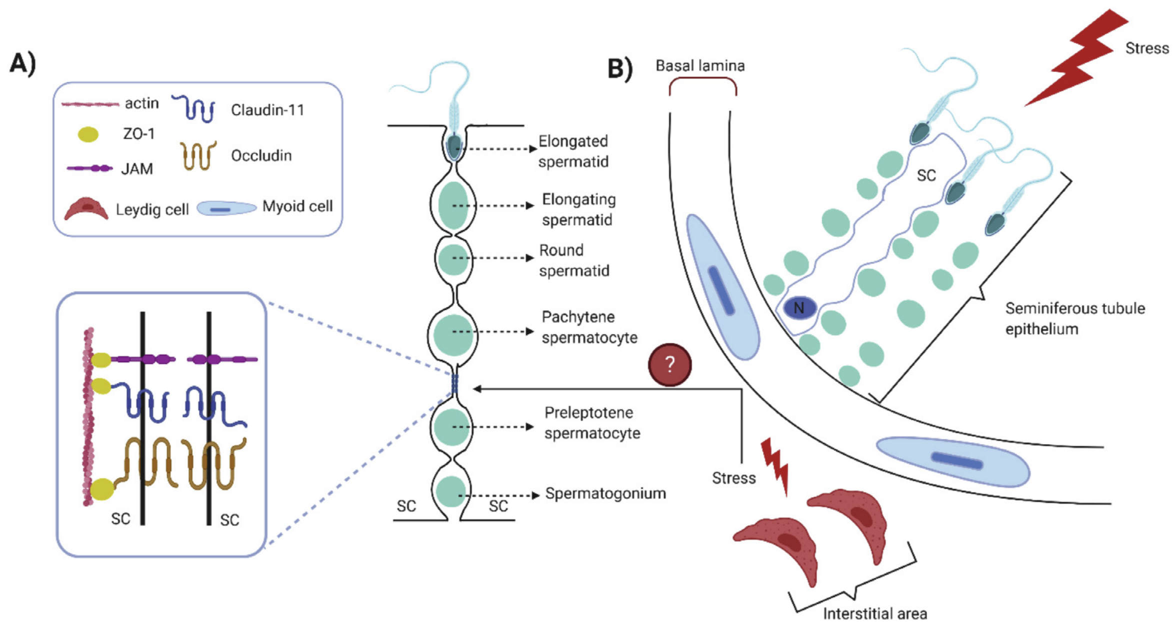

FIGURE 1 The structure of the blood–testis barrier (BTB) and the effects of stress on sperm parameters and Leydig cells. (A) A cross-section of the seminiferous epithelium in mammalian seminiferous tubule in testes. The BTB establishes between two adjacent Sertoli cells (SC) and anatomically divides the seminiferous epithelium into the basal and adluminal compartments. The BTB consists of tight junctions, basal ectoplasmic specializations, desmosome and gap junction. The focus here is on tight junctions, which are composed of occludins, claudins and cell adhesion molecules. Claudin-11 and zonula occludens-1 (ZO-1) proteins are some of the most important in BTB structure: (B) the seminiferous tubule of mammalian testis. The seminiferous epithelium consists of germ cells and SCs. Stress affects both sperm parameters, e.g. concentration, morphology and count, and Leydig cell activity. Testosterone secretion from Leydig cells decreases under chronic stress conditions. The question mark denotes that the mechanism of how chronic stress affects the expression level of proteins in the BTB is still not understood.

week. Exploratory and rearing activities were measured by an open field test, and anxious behaviour was assessed using the EOM as described by *Kilic et al. (2014)*. Briefly, each mouse was released on the central zone of the open field chamber and monitored by the electronic imaging system (ANY-maze, Stoelting Co., USA) for 10 min. The total travelled distance and rearing activity was recorded to assess the exploratory activity. The EOM has a runway with an outer diameter of 46 cm, placed 40 cm above the floor. It has two dark areas surrounded by 16-cm high inner and outer walls and two open areas. Each mouse was released on the light zone (open area) of the EOM and monitored by the electronic imaging system (ANY-maze, Stoelting Co., USA) for 10 min. The time spent in the light zone was recorded to assess anxious behaviour. Depressive-like behaviour was assessed by TST, as described by *Chatterjee et al. (2012)*. Each mouse was then individually suspended in the metal bar in the text box, which is 60 cm above the platform, and the tip of the tail was attached with an adhesive tape and observed by a researcher for 6 min. The first minute was considered as an adaptation period and not included in the statistical analysis. Duration of immobility was recorded. Graph Pad Prism 6 (GraphPad Software Inc., CA, USA) software for statistical analysis was used for data analysis.

Light microscopic examination of spermatogenesis

The right testes of the animals were fixed in Bouin's solution and embedded in paraffin. A total of 4-µm-thick tissue sections were obtained using a microtome (HM 340E; Thermo Scientific, Waltham, MA, USA). Sections were stained with haematoxylin–eosin according to the manufacturer's instructions (Empire Genomics, BPK 4088-2, NY, USA) for general morphologic evaluation and Johnsen's score, which is used for categorization of spermatogenesis (*Johnsen, 1970*). For Johnsen's scoring, every section was assessed in 10 similar fields of view under a Nikon Eclipse light microscope (Nikon® Instruments Inc., USA) using 200 X magnification. For each section, 50 seminiferous tubules were scored. A grade from 1 to 10 was assigned to each tubule cross-section according to the following criteria: 10, complete spermatogenesis; 9, disorganized germinal epithelium and decreased number of mature spermatid; 8, only few spermatozoa present; 7, no spermatozoa but many spermatids present; 6, no spermatozoa and very few spermatids present; 5, no spermatozoa or spermatids but many spermatocytes present; 4, only a few spermatocytes; 3, spermatogonia are the only germ cells present; 2, no germ cells but Sertoli cells are present; and 1, no cells in the tubular section.

Malondialdehyde assay

Malondialdehyde (MDA) was determined spectrophotometrically by evaluating the absorbance of the pink colour formed as a result of the reaction between the lipid peroxidation product MDA and thiobarbituric acid (*Ledwozyw et al., 1986*). Two tubes were prepared as sample and blind tube. A total of 50 µl of serum and 0.25 ml of 1.22 M trichloroacetic acid were added into the sample tube and kept at room temperature for 15 min. A total of 0.15 ml of 47mM thiobarbituric acid was added and incubated in a water bath for 30 min. At the end of the incubation, 0.40 ml of n-butanol was added. The sample mixture was centrifuged at 1400 g for 10 min and 0.2 ml of butanol phase was put into 96-well plate wells. A total of

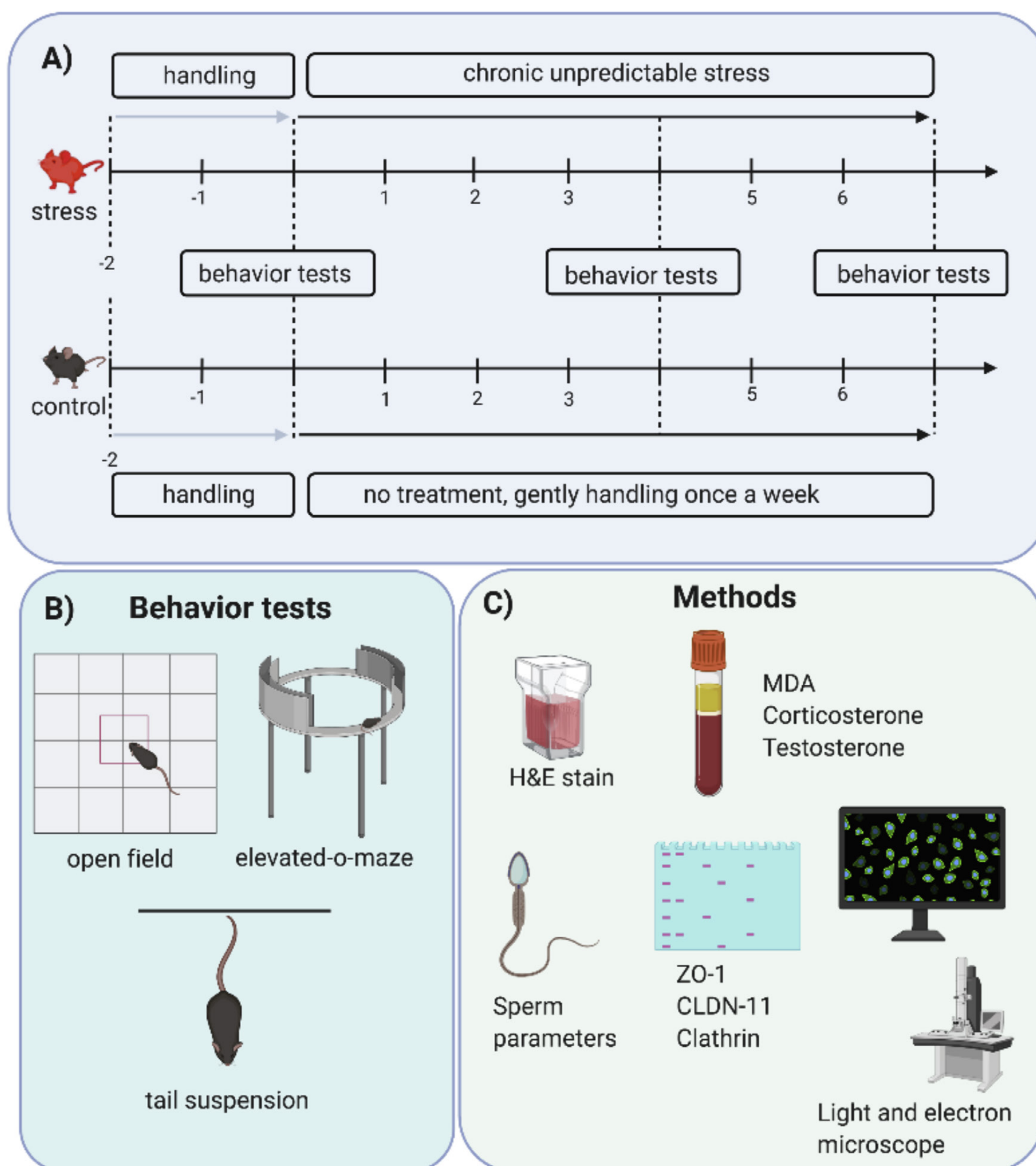

FIGURE 2 Study design. (A) Schedule for the experimental procedures (numbers refer to weeks); (B) behaviour tests to measure the stress levels of mice. An open field test was used to determine the exploratory and rearing activities. Elevated-o-maze and tail suspension tests were used to measure the anxious behaviour and depressive-like behaviour, respectively; (C) methods used for experiments. Haematoxylin and eosin staining was applied to observe general morphology and Johnsen's scoring. In peripheral blood, malondialdehyde (MDA), corticosterone and testosterone levels were measured. Sperm parameters were analysed. Western blotting was conducted to detect the protein expression levels of blood–testis barrier (BTB) proteins (zonula occludens-1 [ZO-1] and claudin-11 [CLDN-11]) and clathrin expression levels in Sertoli cells. The BTB structure was imaged with a confocal and electron microscope.

0.2 ml of n-butanol was used as a blank. Samples and the blank were measured at 532 nm. BioTek Synergy HTX multimode reader (BioTek Instruments, Winooski, VT, USA) was used to measure MDA levels. The extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used, and MDA levels are given in μmol/l.

Corticosterone and testosterone assay

Electrochemiluminescence (ECL) technology was used to measure corticosterone and testosterone levels in blood serum with the use of a cobas e 411 autoanalyzer (Roche -Diagnostics, Mannheim, Germany) (Mathew et al., 2019). Assay sensitivities were 1.5–2.2% and 2.8–3.2% for corticosterone and testosterone, respectively.

Assessment of sperm function parameters

After scrotal incision, epididymides were removed and placed in pre-warmed (37°C) HEPES buffered G-Gamete

TABLE 1 CHRONIC UNPREDICTABLE STRESS PROCEDURE

Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1	Restraint	Restraint	Shaking	Damp sawdust	Hot drier	Restraint	Tilted cage
2	Restraint	Shaking	Damp sawdust	Hot drier	Restraint	Damp sawdust	Overnight illumination
3	Hot drier	Damp sawdust	Restraint	Hot drier	Damp sawdust	Inverted light	Shaking
4	Damp sawdust	Restraint	Hot drier	Shaking	Restraint	Tilted cage	Hot drier
5	Restraint	Tilted cage	Hot drier	Restraint	Overnight illumination	Hot drier	Inverted light
6	Hot drier	Tilted cage	Restraint	Inverted light	Restraint	Damp sawdust	Hot drier
7	Restraint	Damp sawdust	Restraint	Shaking	Shaking	Restraint	Overnight illumination

Solution (Vitrolife, Göteborg, Sweden) and cut into pieces mechanically by a syringe to allow dispersion of spermatozoa. Sperm concentration, total motility and progressive motility were evaluated 10 min later by a Macler Counting Chamber (Sefi Medical Instruments LTD, Haifa, Israel). One hundred squares of each sample were counted. The results were given as million/ml.

For sperm morphology and abnormal acrosome morphology analyses, 10 µl of sperm pellet was smeared on a positively charged slide and allowed to dry. Slides were stained with Diff-Quick (Abris+, Moscow, Russia), viewed under a Nikon Eclipse light microscope (Nikon® Instruments Inc., Melville, NY, USA) at 100 x magnification with immersion oil and scored by counting at least 100 spermatozoa. Abnormal spermatozoa (head and tail defects and cytoplasmic droplet formation in neck region) were evaluated according to the criteria described by Adamkovicova et al. (2016). Briefly, a total of 100 spermatozoa from each mouse were examined and individually scored as normal or abnormal forms, according to the strict sperm morphology criteria classified into head and tail defects. The percentages of normal and abnormal shaped spermatozoa and acrosomes were calculated, and the results were given as normal sperm morphology and abnormal acrosome morphology per cent.

Sperm chromatin integrity (toluidine blue) was evaluated using toluidine blue staining. Slides were fixed with 96% alcohol: acetone (1:1) and hydrolyzed with 0.1 N hydrogen chloride and stained with toluidine blue for 5 min at room temperature. They were then viewed under a Nikon Eclipse light microscope (Nikon® Instruments Inc., USA) at 100 x magnification, with at least 100 sperm cells counted and scored. Pale blue sperm nucleus refers to normal chromatin integrity (toluidine blue), and the dark blue nucleus refers to abnormal chromatin integrity (toluidine blue) (Pourmasumi et al., 2019). Pale and dark blue sperm nuclei were classified, and the results were given as normal chromatin integrity rate (%).

To evaluate the acrosome reaction status, sperm suspension was incubated with 10 µM calcium ionophore A23187 (Sigma-Aldrich, USA) to trigger the acrosome status of spermatozoa for 1 h at 37°C in 5% CO_2 (Fan et al., 2015). Then, to observe the spermatozoa acrosome status, the suspension was smeared on positively charged slides, stained with FITC-PNA (Sigma-Aldrich, USA), and viewed under a confocal microscope (LSM 780, Carl Zeiss, Jena, Germany) with 40 X oil objective. The percentage of acrosome-reacted cells was calculated by scoring 100 spermatozoa on every slide. Number of acrosome-intact spermatozoa (displays intensively green fluorescence), perforated acrosomes (displays disrupted fluorescence) and acrosome-reacted spermatozoa (displays no fluorescence) were counted. The results were given as per cent acrosome reacted spermatozoa.

Protein extraction and Western blotting

Testes tissue samples were harvested from the scrotum. Tissues that belonged to the same group were pooled and homogenized by radioimmunoprecipitation assay buffer lysis (sc-24948, Santa Cruz Biotechnology, Dallas TX, USA) with a protease inhibitor cocktail and then centrifuged at 10,000 g at 4°C for 15 min, after which supernatant was collected. Protein content was measured by Qubit™ 3.0 Fluorometer (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). Equal amounts of protein (30 µg) were loaded on 8% SDS–PAGE gel for electrophoresis (1 h, 200 V), then transferred to a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer System (7 min, 2.5A) (Bio-Rad, Life Sciences Research, Hercules, CA, USA). Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS-T) (containing 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated overnight with rabbit polyclonal anti-zonula occludens-1 (ZO-1) (1:1000; 61–73000, Thermo Fisher Scientific, Waltham, MA, USA), rabbit polyclonal anti- claudin-11 (CLDN11) (1:1000; 36–4500, Thermo Fisher Scientific, Waltham, MA, USA), and mouse monoclonal anti-clathrin (1:500; MA1-065, Thermo Fisher Scientific, Waltham, MA, USA). Each antibody was diluted in the blocking solution (5% non-fat milk in TBST). The following day, membranes were washed with TBS-T three times. Membranes were then incubated with peroxidase-labelled anti-rabbit (1:1000; sc-2004; Santa Cruz Biotechnology) and anti-mouse (1:1000; sc-2005) (Santa Cruz Biotechnology, Dallas, TX, USA). After washing with TBS-T, membranes were incubated with the enhanced chemiluminescence detection solution (1705060) (BioRad Life Sciences Research, Hercules, CA, USA) for 5 min at room temperature. The signals were detected by the ChemiDoc MP imaging system (1708280) (BioRad Life Sciences Research, Hercules, CA, USA). Protein loading was controlled with a rabbit polyclonal anti-beta-actin antibody (1:1000; sc-47778) (Santa Cruz Biotechnology, Dallas, Tx, USA). Three independent blots were quantified and normalized with beta-actin by densitometry using Image J software (US National Institutes of Health, Bethesda, MD).

Immunofluorescence

For each animal, 7 µm-thickness sections were cut with a cryostat (CM1950) (Leica, Wetzlar, Germany) and collected into positively charged slides. After fixation in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 20 min at room temperature, sections were rinsed three times in PBS for 5 min. Sections were then blocked with a blocking agent containing 10% normal goat serum, 3% bovine serum albumin, 0.1% sodium azide and 1% Tween-20 for 1 h at room temperature. Without washing, slides were incubated with the primary antibodies anti-ZO-1 (rabbit, 1:100; 61–7300) (Thermo Fisher Scientific, Waltham, MA, USA), anti-CLDN11 (rabbit, 1:100; 36–4500) (Thermo Fisher Scientific, Waltham, MA, USA), and anti-clathrin Heavy Chain (mouse, 1:200; MA1-065) (Thermo Fisher Scientific, Waltham, MA, USA) for 20 h at 4°C except for anti-ZO-1, which was incubated at 37°C. The following day, slides were rinsed with PBS and incubated with the secondary antibodies goat anti-rabbit immunoglobulin G (IgG) H&L-Alexa Fluor 488 (1:400; ab 150077) (Abcam, Cambridge, UK) and goat anti-mouse IgG H&L-Alexa Fluor 568 (1:400; ab 175473) (Abcam, Cambridge, UK) for 1 h at room temperature for nuclear staining, sections were incubated with DAPI for 5 min. Negative controls also imaged without using primary antibody (data not shown). Sections were analysed under a confocal microscope (LSM 780) (Carl Zeiss, Oberkochen, Germany).

Transmission electron microscope

For transmission electron microscope analysis, testes tissues were prepared as described by *Hua et al. (2015)*. For each animal, 1-mm^3 testes tissues were fixed with the solution containing 1.25% glutaraldehyde, 2.5% paraformaldehyde, 2.2 mg calcium chloride and 0.08M cacodylate buffer (pH 7.4) warmed to 37°C. Tissues were fixed in this solution for 20 min at room temperature. Subsequently, tissues were stained before embedding and dehydrated in a graded alcohol series. Tissues were then embedded in the Epoxy Embedding Medium kit (45359, Sigma-Aldrich, USA). Semi-thin sections (1 µm) of the sample were cut with Leica EM UC7 (Leica Microsystems, Vienna, Austria) and stained with toluidine blue to detect the region of interest. Ultra-thin sections (70 nm) were analysed with STEM (Gemini SEM 500) (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Graph Pad Prism 6 (GraphPad Software Inc., CA, USA) software was used for all statistical analyses. Data were expressed as mean ± standard error mean. Student's t-test was used to assess differences in measured variables between the two groups. The Mann–Whitney U-test was used for sperm parameters to evaluate the differences between the groups. The results were considered statistically significant at $P < 0.05$.

RESULTS

Analysis of behaviour tests

As the main index for evaluating exploratory activity, anxiety and depressive-like behaviours, open field, EOM and TST tests of each group were measured at week 0 and 7 weeks after CUS procedures, respectively. At the end of the CUS procedure, the open field test showed that total distance and rearing activity decreased in the CUS group at week 7, compared with the CUS group at week 0 ($^*P = 0.03$, $^{**}P = 0.008$) (FIGURE 3A and FIGURE 3B). Also, a significant decrease occurred between the control and CUS group at week 7 of the stress protocol, in total distance and rearing activity ($^§P = 0.03$, $^{§§§}P < 0.001$). The EOM and TST analyses revealed that the CUS procedure decreased the time spent in the light zone (s) ($^{**}P = 0.009$) (FIGURE 3C) and increased

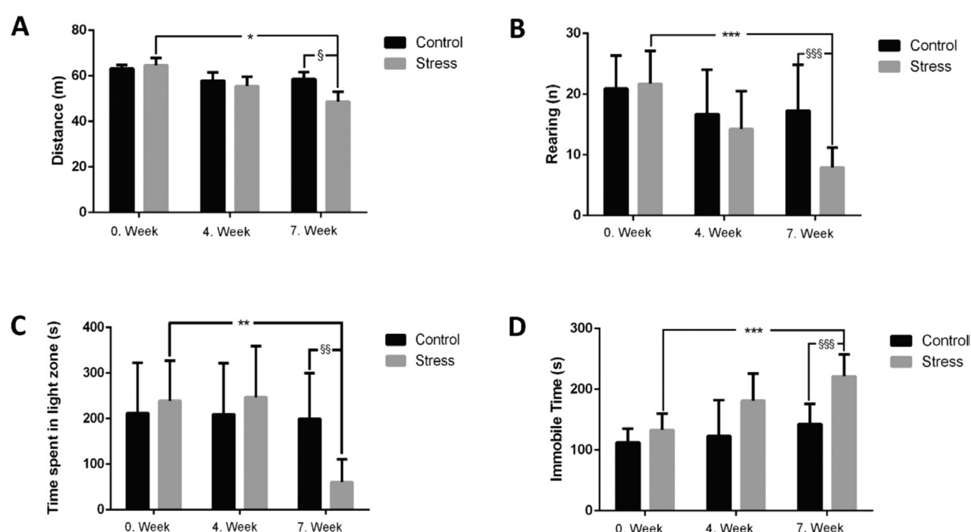

FIGURE 3 Effects of chronic unpredictable stress (CUS) on behaviour tests. (A) Effects of CUS on exploratory activity; and (B) rearing in open field. In the CUS group, CUS decreased total distance travelled (*) and rearing (***) activity when week 0 and week 7 were compared, respectively. At week 7, total distance travelled (§) and rearing activity (§§§) decreased significantly, when control and CUS groups were compared. In the CUS group, CUS decreased the time spent in the light zone at week 7 (**), compared with the (C) CUS group in elevated-o-maze. When the control and CUS groups were compared at week 7, a significant difference was found between the control and CUS groups (§§). The immobile time decreased at week 7 in the CUS group (***) compared with the CUS group at week 0 in (D) tail suspension tests. At week 7, the immobile time increased in the CUS group compared with the control group (§§§). No significant difference was seen in the control group when week 0 and week 7 of the experiment in each test were compared. The results are expressed as mean ± standard error mean (A: $^*P = 0.03$; $^§P = 0.03$, B: $^{**}P = 0.008$; $^{§§§}P < 0.001$, C: $^{**}P = 0.009$; $^{§§}P = 0.004$, D: $^{***}P < 0.001$; $^{§§§}P < 0.001$).

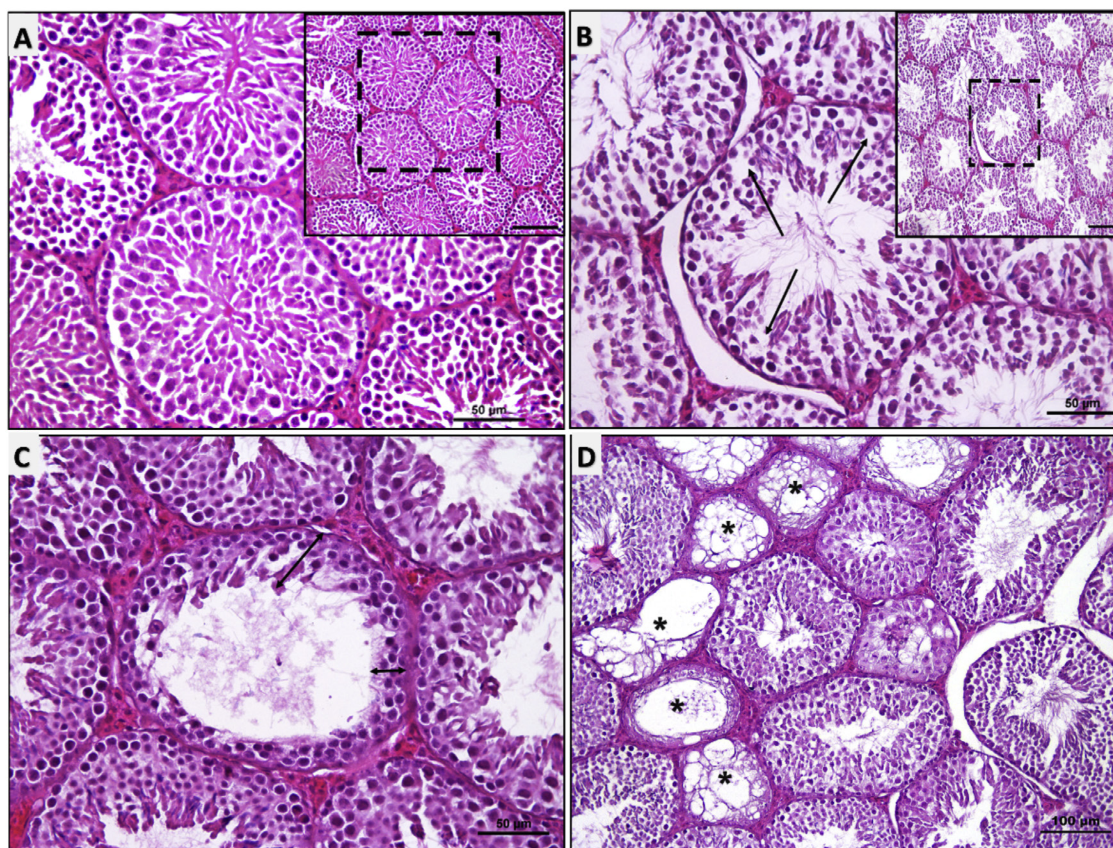

FIGURE 4 Representative micrographs of testicular tissue sections stained with haematoxylin and eosin. (A) Control group mice showed normal seminiferous tubule morphology. In the chronic unpredictable stress group (B–D), seminiferous tubule epithelium showed separations (↖) (B), seminiferous tubule epithelium thickness decreased (↕) (C), and atrophic tubules were observed locally (*) (D) (A, B, C: 40 X; D, insert A, B: 20 X. Scale bar A, B: 100 μm.

the immobile time (s) (***$P < 0.001$) (FIGURE 3D) at week 7 compared with week 0 of the procedure, respectively. Also, when the time spent in the light zone was compared between control and CUS group at week 7, a significant decrease occurred in the CUS group (§§$P = 0.004$). When the control and CUS group were compared at week 7, immobile time increased significantly (§§§$P < 0.001$). No statistical difference was seen in the control group at weeks 0 and 7 for each test.

Histopathological analysis of testes

Johnsen's testicular score was significantly lower in the CUS group than the control group at week 7 (5.42 ± 0.03 versus 6.36 ± 0.2, *$P = 0.03$, respectively). Although testes of the control group displayed normal histological findings (FIGURE 4A), some spermatogenic cells were found to be dismembered from the seminiferous epithelium in the CUS group (FIGURE 4B). Also, the seminiferous epithelium was thinner in the CUS group compared with the control group (FIGURE 4C), and atrophic tubules were observed in some regions of the section (FIGURE 4D).

Serum malondialdehyde assay and hormone levels

In the CUS group, the mean MDA level (μmol/l) was significantly higher than the control group (2.19 ± 0.13 versus 1.17 ± 0.11, **$P = 0.008$), whereas mean plasma testosterone levels (ng/ml) were significantly lower (1.17 ± 0.18 versus 3.56 ± 0.75, **$P = 0.005$) and mean corticosterone (μg/dl) level was higher at the seventh week (0.64 ± 0.08 versus 0.29 ± 0.03, **$P = 0.004$) (TABLE 2).

Spermatozoa parameters

Sperm parameters were analysed, including sperm concentration, progressive motility, total motility, morphology, chromatin structure analysis, acrosomal index and the acrosomal reaction of the CUS and control groups to evaluate the quality of spermatozoa collected from the epididymis (TABLE 3). No significant difference was found in sperm

TABLE 2 MALONDIALDEHYDE, TESTOSTERONE AND CORTICOSTERONE LEVELS OF MICE IN THE CONTROL GROUP AND CHRONIC UNPREDICTABLE STRESS GROUP

	Control (n = 10)	CUS (n = 14)
Malondialdehyde, μmol/l	1.17 ± 0.11	2.19 ± 0.13[a]
Testosterone, ng/ml	3.56 ± 0.75	1.17 ± 0.18[b]
Corticosterone, μg/dl	0.29 ± 0.03	0.64 ± 0.08[c]

[a] $P = 0.008$.
[b] $P = 0.005$.
[c] $P = 0.004$).CUS, chronic unpredictable stress.

TABLE 3 COMPARISON OF SEMEN PARAMETERS BETWEEN THE CONTROL AND CHRONIC UNPREDICTABLE STRESS GROUPS

Sperm parameters	Control (*n* = 10)	CUS (*n* = 14)	*P*- value
Sperm concentration, million/ml	52.5 ± 5.33	56.6 ± 7.03	0.08
Total motility, %	67.2 ± 6.06	67.2 ± 5.30	0.07
Progressive motility, %	16.3 ± 2.98	9.3 ± 1.34	0.03
Normal morphology, %	48.8 ± 2.88	37.7 ± 3.99	0.04
Chromatin integrity, %	60.2 ± 5.10	24.4 ± 4.17	0.002
Acrosome reaction, %	44.5 ± 2.35	19.1 ± 2.02	0.002
Abnormal acrosome, %	16.7 ± 2.27	40.8 ± 6.44	0.04

Values are mean ± standard error mean unless otherwise stated.

concentration (control: 52.5 ± 5.33 million/ml; CUS: 56.6 ± 7.03 million/ml, *P* = 0.08) and total sperm motility (control: 67.2 ± 6.06 [%]; CUS: 67.2 ± 5.30 [%], *P* = 0.07). A statistically significant decrease was found in the progressive motility parameter in the CUS group (control: 16.3 ± 2.98 [%]; CUS: 9.3 ± 1.34 [%], *P* = 0.03) The normal morphology rate was 48.8 ± 2.88 [%] and 37.7 ± 3.99 [%] in the control and CUS groups, respectively (*P* = 0.04). In the CUS group, the number of cells having normal chromatin integrity decreased. The pale and dark stained sperm heads were indicated with an arrow in the control and CUS group, respectively (FIGURE 5A). Although the rate was 60.2 ± 5.10 (%) in the control group, this ratio decreased to 24.4 ± 4.17 (%) in the CUS group (*P* = 0.002).

The rate of acrosomal reaction, which is determined by the presence of acrosomal disruption, was significantly lower in the CUS group, compared with the control group (control: 44.5 ± 2.35 [%]; CUS: 19.1 ± 2.02 [%], *P* = 0.002) (FIGURE 5B). Testicular cryosections were used as positive control of fluorescein isothiocyanate conjugated–peanut agglutinin, which is demonstrated by the presence of green fluorescence in the acrosomal regions of round spermatids and sperm cells in the adluminal compartment of the seminiferous tubules (Supplementary Figure 1). A statistically significant increase was observed in the number of spermatozoa with abnormal acrosomes in the CUS group (control: 16.7 ± 2.27 [%]; CUS: 40.8 ± 6.44 [%], *P* = 0.04).

An interesting finding of the recent study is the presence of agglutination in six out of 14 samples observed in the CUS group (Supplementary Figure 2 and Supplementary video 1). No agglutination, however, was detected in any of the 10 samples in the control group.

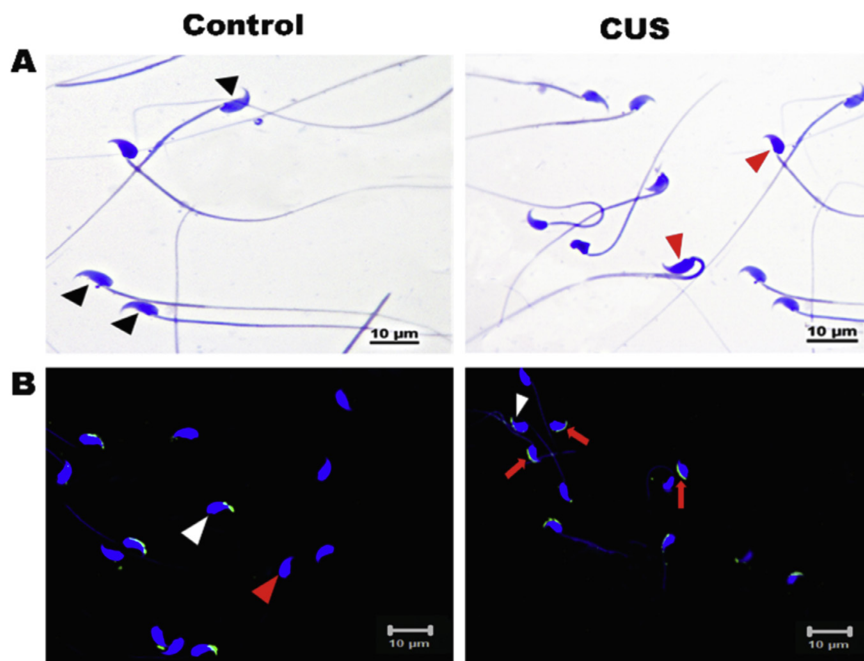

FIGURE 5 Sperm chromatin integrity and acrosome reaction status. (A) Sperm smears stained with toluidine blue. Chromatin integrity (toluidine blue) in control and chronic unpredictable stress (CUS) groups. In the control group, pale stained sperm heads are indicated with a black arrowhead. In the CUS group, dark stained sperm heads are indicated with a red arrowhead; (B) fluorescein isothiocyanate conjugated–peanut agglutinin staining for acrosome reaction. In the control group, acrosome reacted (▲) and perforated acrosome (Δ) spermatozoa. In the CUS group acrosome-intact sperms (red arrow) (A: 100 X, B: 40 X).

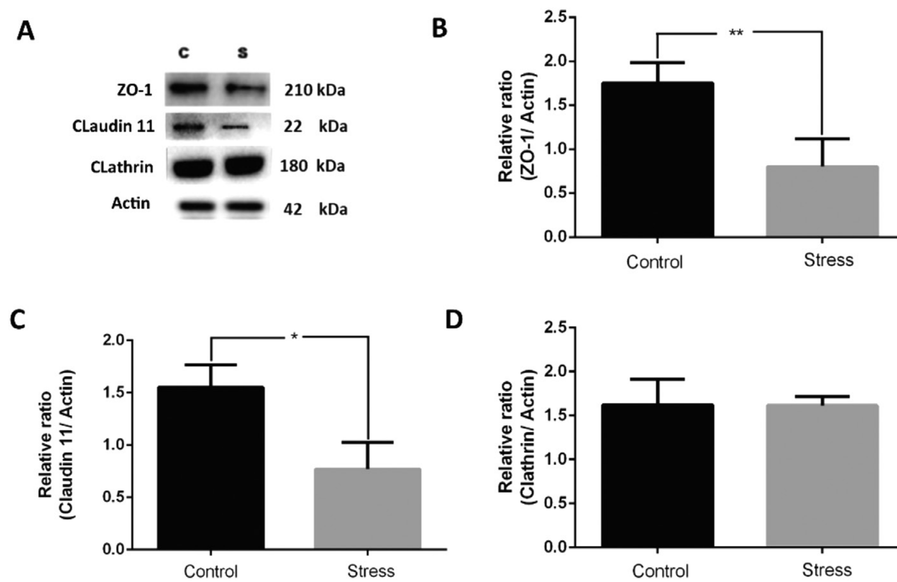

FIGURE 6 Representative immuno-Western blot analysis of zonula occludens-1 (ZO-1), claudin-11 and clathrin expression in the testes of the control and chronic unpredictable stress (CUS) groups. (A) Representative blots are also shown. Relative ratios were quantified and normalized relative to beta-actin against (B) ZO-1, (C) claudin-11 and (D) clathrin. Data are presented as mean ± standard error mean (three independent blots per group). Beta-actin was used as a loading control.

Zonula occludens-1, claudin-11 and clathrin expression

Expression of ZO-1 and CLDN11 decreased in the CUS group (***P* = 0.003, **P* = 0.01, respectively) (FIGURE 6), although no significant difference was observed in the testicular clathrin protein expression (*P* = 0.9).

Immunofluorescence analysis

The basal compartment of the seminiferous tubules was continuous in the control group compared with the CUS group. Clathrin expression was found to be localized in the cytoplasm of Sertoli cells in both groups (data not shown). Fluorescence intensity of ZO-1 and CLDN11 expressions were lower the basal compartment, and BTB integrity was impaired in the CUS group (FIGURE 7).

Electron microscopic examination of testicular tissue

Although the seminiferous tubule morphology was normal in the control group, seminiferous tubule epithelium was observed to be thinner in the CUS group, and the tight junction regions were observed to be disrupted, which contains CLDN11 and ZO-1 proteins (FIGURE 8 and Supplementary Figure 3).

DISCUSSION

Environmental factors, sedentary lifestyles and unbalanced and unhealthy nutrition have increased the rate of couples who have had infertility problems over the past 50 years (Ahmad et al., 2008). In addition to genetic and physiological factors, environmental factors play an important role in this dramatic increase in infertility problems (Younglai et al., 2005). Stress is considered one of the most significant environmental factors affecting this decline in fertility status (Sheiner et al., 2003).

In the present study, the effects of chronic stress on sperm parameters and BTB were examined in detail, especially at BTB protein expression level. We applied CUS randomly for 7 weeks, and confirmed that the CUS model was established by behavioural experiments (total distance travelled, rearing activity, the period spent in the lightened area, the period spent immobile) (Monteiro et al., 2015).

It has been shown in several studies that stress reduces the number of spermatozoa, causes disorders in testicular morphology and causes a decrease in testosterone levels (Retana-Marquez et al., 2014; Sakr et al., 2015). Similarly, in the present study, a significant decrease occurred in testosterone levels in the CUS group. According to the Johnsen scoring analysis, a decrease in the number of germ cells in the seminiferous tubule epithelium was observed in the CUS group. Moreover, vacuolization, atrophic and degenerative tubules were observed in the seminiferous tubule epithelium in the CUS group, unlike the control group.

Chronic stress is also associated with increased oxidative stress (Belviranlı and Gökbel, 2006). It is suggested that free oxygen radicals disrupt cell membranes with lipid peroxidation (Niki, 2008). One of the markers of the lipid peroxidation level in an in-vivo environment is MDA, which is shown to be increased in stressed animals (Fahim et al., 2019; Fu et al., 2019; Shehu et al., 2019). In the present study, MDA levels were also significantly increased in the CUS group compared with the control group in week 7. Excessive serum corticosterone levels indicate that mice experienced psychological stress. Consistent with previous studies, serum corticosterone levels increased in the CUS group compared with the control group (Guo et al., 2017; Xiong et al., 2019).

The relationship between stress and male infertility has been examined in a limited number of clinical studies, with conflicting results. Hjollund et al. (2004) reported no effect of psychologic stress on semen quality. In contrast, other investigators have reported that a stressful lifestyle is associated with reduced semen quality (Gollenberg et al., 2010; Niederberger, 2015; Nordkap et al., 2016). Several animal

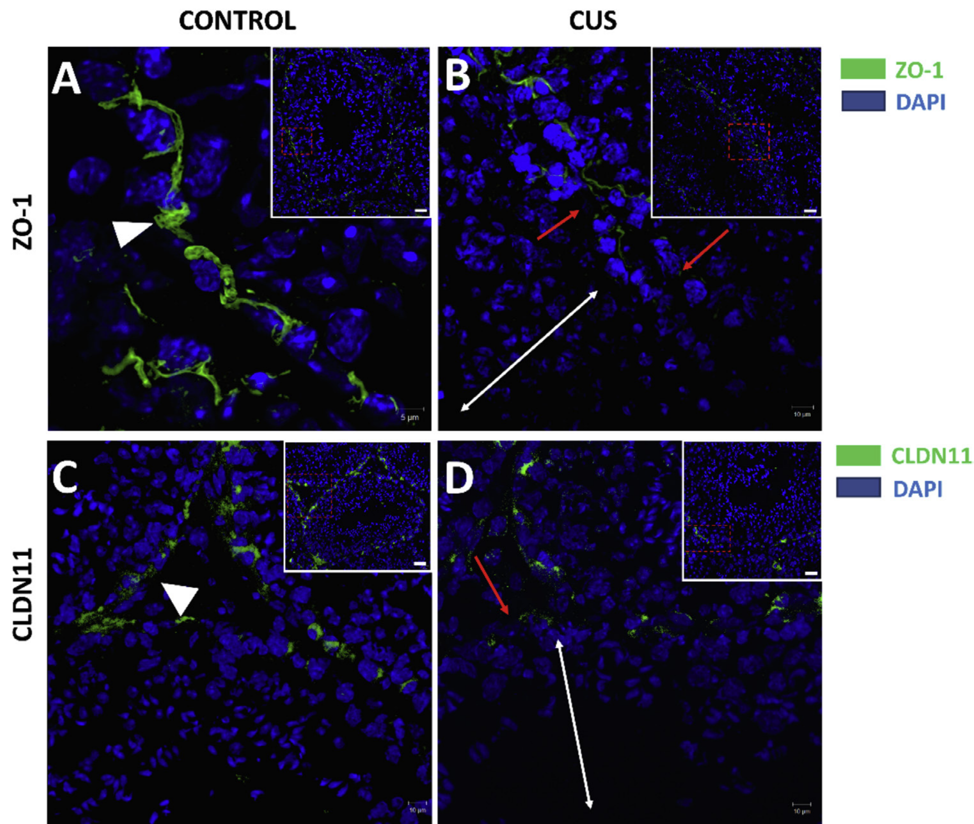

FIGURE 7 Representative confocal microscopic images of testes from each group. Cryosections immunostained for zonula occludens-1 (ZO-1) and claudin-11 (CLDN11) (green) and nuclei by DAPI (blue). Expression of ZO-1 and CLDN11 in the control group showed continuity indicated by a white arrowhead (A and B, respectively). In the chronic unpredictable stress (CUS) group, ZO-1 and CLDN11 expression decreased, and the disruption of continuity is indicated by a red arrow; thinning in the seminiferous epithelium is indicated by a double-sided arrow (↕) (C and D, respectively). All images were taken with 40 X oil objectives; inserted images were taken with 20 X objectives (scale bar: 20 μm) (image A also contains digital zooming).

studies have also suggested that CUS is associated with decreased sperm concentration (Arun et al., 2018; Zou et al., 2019); however, we did not observe such a significant decline in sperm concentration collected from epididymides in the CUS group, possibly because different animals were used as models. Similarly, no difference was found in total motility between the groups, whereas the progressive motility rate was significantly decreased in the CUS group. These results are in agreement with previously published studies showing that CUS reduces the rate of progressive motility (Juarez-Rojas et al., 2015; Sakr et al., 2015). The decrease in testosterone levels may be the reason for the decrease in total sperm motility as total sperm motility is shown to be affected by androgen levels (Robaire et al., 2006). Moreover, the reduction of progressive motility could result from the overproduction of epididymal reactive oxygen species and lipid peroxidation, caused by increased corticosterone levels (Dhanabalan et al., 2011). Another reason for progressive motility may be that lipid peroxidation causes a defect in the middle portion of the spermatozoon (Rao et al., 1989), and changing the sperm plasma membrane (Agarwal et al., 2014) can eventually impair sperm progressive motility.

In a study of infertile individuals, an increase in the number of spermatozoa with abnormal morphology was observed (especially with cytoplasmic droplets or head abnormalities) in patients exposed to stress in the previous 3 months (Auger et al., 2001; Arun et al., 2018). In another study, stressful life events can were shown to cause an increase in abnormal sperm count (Janevic et al., 2014). Similarly, in the present study, we observed a decrease in the number of spermatozoa with normal morphology in the CUS group. This abnormality in sperm morphology may be a consequence of a disrupted BTB structure, which may occur because of decreased ZO-1 and CLDN11 expressions observed in the basal compartment.

We observed that the number of spermatozoa that undergo acrosomal reaction decreased and abnormal acrosome morphology rate increased in the CUS group compared with the control group. In a chronic stress study conducted in rats, immobility stress was applied for 42 days, and acrosomal reaction analysis was carried out by staining with Coomassie blue. As a result of this analysis, an increase was observed in the stress group's acrosome reacted cell number. This was suggested to be a result of stress affecting the expression of Golgi-associated PDZ and coiled-coil motif-containing, which among proteins involved in acrosome functionality (Arun et al., 2016). In another study, however, increased MDA levels were shown to cause abnormal acrosome morphology and decrease acrosome reaction (El-Taieb et al., 2015).

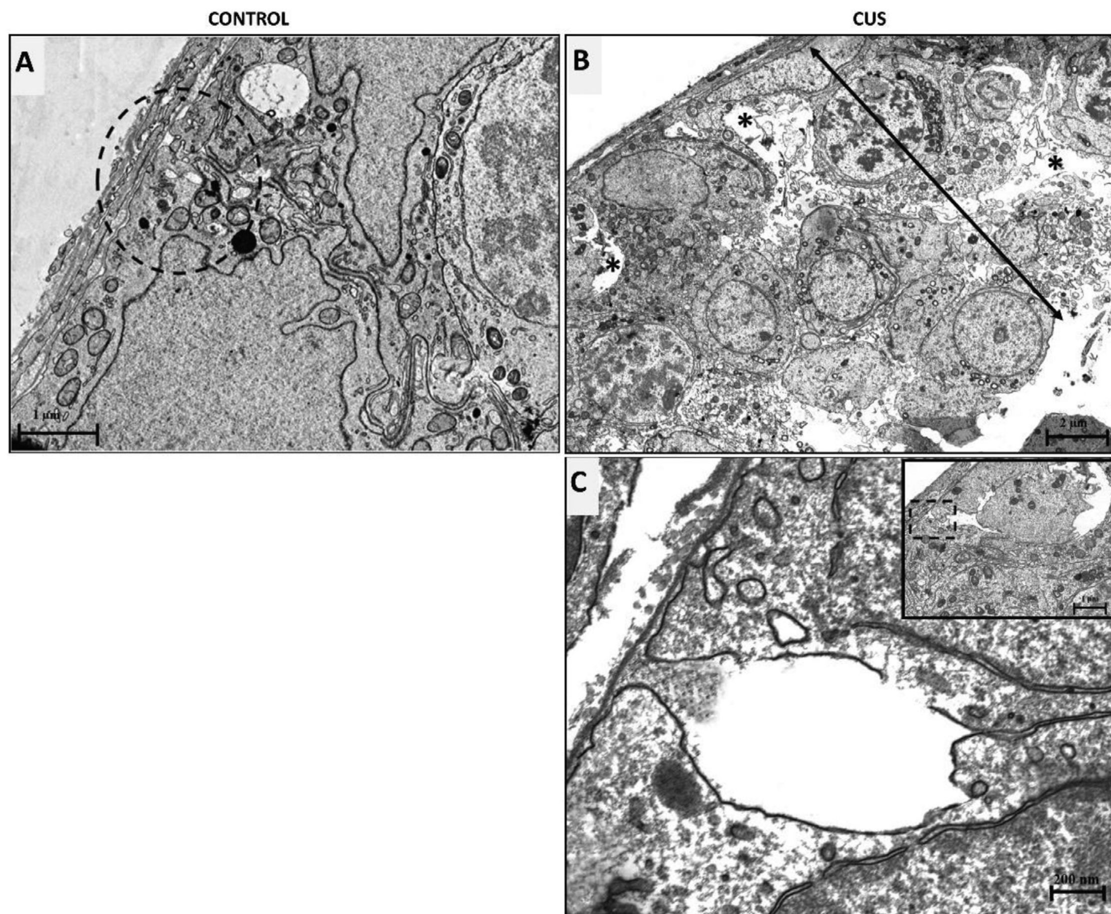

FIGURE 8 Electron microscopic examination of testes. (A) In the control group, no openings were observed in the basal compartment in the area indicated by dashed lines; (B) openings in the seminiferous tubular epithelium in the chronic unpredictable stress (CUS) group (*) and spills in the germ cell line ( ); (C) disruption in the blood–testis barrier structure in the CUS group. (A: 14.57 kx; B: 5.13 kx; C: 51.02 kx; inserted area: 21.52 kx).

Another parameter analysed in the present study is chromatin integrity (toluidine blue). To the best of our knowledge, we demonstrated for the first time that chromatin integrity (toluidine blue) of sperm cells was decreased under stress conditions in the CUS group, which may be explained by a result of increased oxidative stress or problems in repairing mechanisms.

Interestingly, we observed agglutination in the CUS group, which is an indirect indicator of immunologic infertility. Intense agglutination was observed in 42.9% of the samples in the CUS group, unlike the control group samples (Cui et al., 2015). This may be explained by data showing that anti-sperm antibodies (ASA) attack sperm cells when BTB is disrupted, causing agglutination. It is possible that immune cells pass freely into the seminiferous tubules, and ASAs are formed as a result of the damage of BTB. Although the results require confirmation at the immunoglobulin M and IgG levels, the situation could provide evidence for the disruption of BTB. Further studies should be conducted to establish the mechanism underlying immunologic infertility cases.

In the present study, our in-vivo data show that levels of protein of the tight junction molecules, ZO-1 and CLDN11, significantly decrease after the CUS procedure. The results are consistent with a previous study indicating that, after scrotal heat exposure, tight junction proteins (ZO-1 and claudin-3) mRNA and protein levels decreased significantly (Cai et al., 2011). In contrast, the CLDN-11 level increased after heat exposure. The difference between the results may be a result of the difference in the protocol applied.

To the best of our knowledge, by means of clathrin expression, this is the first study examining the level of clathrin in Sertoli cells of unpredictable chronic stress model.

Chronic stress is reported to cause thinning in the seminiferous tubule basement membrane and apoptosis in Leydig cells (Hou et al., 2014). When we observed ultrastructural changes in the seminiferous tubules with a transmission electron microscope, we observed significant disruptions (openings) at the basal compartment of the seminiferous tubules in the CUS group. The decrease of tight junction protein levels can be explained by these observations. We also observed that the seminiferous epithelium was thinner in the CUS group.

In conclusion, our results show that CUS disrupts BTB integrity and impairs sperm parameters in a mouse model. A decrease in ZO-1 and CLDN11 expression levels may be proposed as the causative factor. The present study has two limitations. The first is that

we could not use isotype control for primary antibodies, which were used for immunofluorescence analysis. The second is that, during sperm motility analysis, we observed agglutination in the stress group and took a video, but we did not take an image from the control group. As mentioned in the results section under subsection 'Sperm parameters', we did not observe agglutination in the control group. Further studies are needed to investigate the effect of chronic stress on other BTB proteins located in the BTB architecture and to analyse its effect on male infertility.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.12.007.

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