Age-Dependent Effects of Chronic Unpredictable Stress on Male Reproductive Organs in Rats

Beheshteh Abouhamzeh[1], Kianoosh Gholami[2], Behnoush Sabayan[3], Mohammad Reza Ghasemzadeh[4], Parvin Zareian[5], Esmaeil Samizadeh[6]

[1]Department of Anatomical Sciences, School of Medicine, AJA University of Medical Sciences, Tehran, Iran

[2]Anesthesiology Resident, Department of Anesthesiology, AJA University of Medical Science, Tehran, Iran

[3]Ph.D. Candidate of Clinical Psychology, Department of Clinical Psychology, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

[4]Assistant Professor of Psychiatry, Department of Psychiatry, AJA University of Medical Sciences, Tehran, Iran

[5]Department of Physiology, School of Medicine, AJA University of Medical Sciences, Tehran, Iran

[6]Pathology department of Imam Reza hospital, AJA University of Medical Sciences, Tehran, Iran

Background: Stress occurs in response to external harmful factors and can have detrimental effects on organ functions.

Objective: This study investigated the age-dependent effects of chronic unpredictable stress (CUS) on serum testosterone levels, testicular promyelocytic leukemia zinc finger (PLZF) expression, and histopathological changes in young and old male rats.

Methods: Thirty-two male Wistar rats were randomly assigned into four groups (young control, young stress, old control, and old stress; 8 rats in each group). Rats in the stress groups were subjected to one unpredictable stressor daily for eight consecutive weeks. Serum testosterone was measured using the Enzyme-Linked Immunosorbent Assay (ELISA), PLZF-positive spermatogonial cells were evaluated by immunohistochemistry (IHC), and testicular tissue was assessed using hematoxylin and eosin (H&E) staining. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc testing.

Results: CUS significantly reduced serum testosterone levels in both stress groups in comparison to control groups ($p < 0.001$). Also, the proportion of PLZF-positive spermatogonial cells significantly decreased in the stress groups ($p < 0.001$). Histological results demonstrated reduced spermatogonia population in both stress groups while sloughing of seminiferous tubules was observed only in the old stress group.

Conclusion: CUS impairs endocrine and histological markers of the male reproductive system, with different responses based on age. These findings suggest that CUS may contribute to male infertility through changes in testosterone production, spermatogonial maintenance, and testicular histology.

Keywords: Chronic Unpredictable Stress, Testicular Tissue, Testosterone, Rats

Category: Original Research

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Correspondence to:

Kianoosh Gholami

Anesthesiology Resident, Department of Anesthesiology, AJA University of Medical Science, Tehran, Iran

kianooshg@outlook.com

Introduction

Chronic stress is a common problem in the world that occurs in response to long and distressing situations.[1] It constitutes a generalized systemic reaction to persistent internal and external stressors and may cause psychological and biological disturbances by affecting the endocrine system, immune responses, physiological processes, and behaviors.[2,3]

Some factors influencing male infertility include genetic, hormonal, environmental, lifestyle, and psychological factors.[4] Experimental studies have demonstrated that prolonged stress exposure like CUS can cause reduced testosterone concentration, seminiferous tubule degeneration, increase germ-cell apoptosis, impair spermatogenesis, and decreased sexual behavior.[5-7] CUS is one of the validated experimental models of prolonged psychological stress, and it is commonly employed to induce anxiety or depression in rodents, which reproduce many neuroendocrine and physiological changes observed in humans.[8] Compared with a single stressor, CUS is based on several forms happening in an unpredictable order, which reduces habituation and more accurately reflects chronic stress experienced in daily life.[9]

Persistent activation of the hypothalamic–pituitary–adrenal (HPA) axis, by CUS, affects testosterone secretion by suppressing Gonadotropin-Releasing Hormone (GnRH), Luteinizing Hormone (LH), and Follicle-Stimulating Hormone (FSH) secretion from the anterior pituitary gland.[10] Additionally, Corticotropin-Releasing Hormone (CRH) inhibits gonadotropin production as a result of chronic stress exposure that is also controlled by the hypothalamus itself.[11] Consequently, chronic psychological stress is frequently associated with reduced testosterone levels and impaired male reproductive function.[12]

Moreover, it is not established whether stress causes different reproductive system damage based on age or whether changes in PLZF expression align with age-related histopathological changes.[13] As a result, the interaction between aging and CUS on spermatogonial maintenance remains insufficiently characterized.[14]

PLZF is a transcription factor for spermatogenesis and is essential for stem cell maintenance and self-renewal activities.[15] The involvement of PLZF in chromosomal translocation with the RARαα gene is well known.[11] Decreased PLZF expression is associated with decreased spermatogonial stem cells, impaired spermatogenesis, and male infertility.[16] Therefore, PLZF appears to be a valuable molecular marker for detecting early stress-induced changes in spermatogonial stem cells.[15]

Although chronic stress is known to decrease male reproductive function it remains unclear whether stress-induced reproductive damage differs with age or whether alterations in PLZF expression parallel age-related histopathological change.[7,13] Therefore, the interaction between aging and CUS in regulating endocrine function, spermatogonial stem cell maintenance, and testicular histology remains insufficiently characterized.[7,14,17] Understanding these age-dependent effects may improve our knowledge of stress-related infertility throughout the reproductive lifespan.[17]

Accordingly, the present study evaluated the effects of CUS on blood testosterone levels, PLZF expression, and histological changes on male reproductive organs in young and old rats.

Method

Animal and Experimental design

The experiment was performed on 32 Wistar rats obtained from the Razi Institute. The rats were divided into two age groups: young rats (8 weeks old, weighing 160 ± 30 gr) and old rats (52 weeks old, weighing 400 ± 50 gr). Eight-week-old rats are considered young adults with optimal reproductive capability, while 52-week-old rats are past their reproductive peak, demonstrating natural age-related changes in testicular function and hormone levels. This division allows for the evaluation of stress effects across the reproductive span. Each age group was divided into two groups: control and stress, resulting in four groups:

1. Young Control group
2. Young Stress group
3. Old Control group
4. Old Stress group

Each group consisted of 8 rats. We selected 8 animals per group based on CUS studies that demonstrated statistically significant differences with comparable sample sizes. To prevent bias, rats were assigned to groups randomly using a computer-generated randomization sequence before starting the CUS protocol. We conducted the experiment over 8 weeks.

The adaptation progress

Rats underwent 7-day acclimatization under standardized conditions. Throughout this period, they had unrestricted access to food and water, were housed at 24 °C under a 12-hour light/12-hour dark cycle, and were observed each day for changes in body weight, overall behavior, and obvious stress indicators such as excessive grooming or decreased activity, confirming sufficient acclimatization before random assignment.

CUS Induction

After the stabilization period, we used the conventional method of CUS to induce stress, which is one of the methods that creates a similar model to depression in humans for rats.[18-20] Each day between 8:30 a.m. and 12:30 p.m. for eight weeks, the rats were randomly assigned to experience a distinct stressor, with only one stressor administered per day. Control groups did not undergo any stress procedures. Table 1 details the specific stressors and days of the experiment used in this study.

Evaluation Indicators, Procedures, and Anesthesia

After the last experiment, rats were anesthetized using a combination of ketamine (75–100 mg/kg) and Xylazine (5–10 mg/kg) administered via intraperitoneal injection 10 minutes after the last stressor. The following evaluations were performed:

1. Examination of structural changes in the testis using H&E staining.
2. IHC analysis for PLZF in testicular tissue.
3. ELISA assay for plasma testosterone concentration.

Table 1. Schedule of chronic unpredictable stressors administered during the eight-week experimental period

Week	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
1	-	-	PET	BSTC	CiR	BAC	8 GSC
2	MS	darkness	ISC	ISC	ISC	FS	WD
3	FL	restrainer	temperature of 45 °	FD	-	8 GSC	PET
4	MS	CiR	darkness	ISC	ISC	ISC	BAC
5	WD	restrainer	FS	temperature of 45 °	FD	FL	8 GSC
6	ISC	ISC	ISC	darkness	-	PET	CiR
7	BAC	MS	FS	restrainer	8 GSC	FD	temperature of 45 °
8	darkness	MS	ISC	ISC	ISC	-	FL
9	PET	CiR	Day of Sacrifice	-	-	-	-

24 hours food deprivation (FD), 24 hours water deprivation (WD), Bend the animal cage at a 45-degree angle for 24 hours (BAC), 1 pinch at 1 cm end of the tail (PET) 4 hours in the restrainer (restrainer), 5 minutes at a temperature of 45 ° C (temperature of 45 °), 5 minutes forced swimming in 4 ° C water (FS), 24-hour exposure of the animal to light (FL), Darkness 3 hours a day (darkness), Isolation in a small cage (three days) (ISC), Gather the animals in a small cage for 12 hours (GSC), Moisten sawdust under the animal for 24 hours (MS), Change under animal instead of rigid for 24 hours (CiR), The base stress of a tall column that was placed on a platform for 20 minutes at a height of 100 cm from the ground (BSTC).

Surgical Process and Sampling

Following the anesthesia, bilateral orchiectomy was performed through a midline scrotal incision.and the testicular tissue biopsies were obtained for fixation and tissue passage.

Investigation of testicular tissue changes in H&E staining

Testicular tissue samples were rinsed with phosphate-buffered saline (PBS) and then preserved in a 10% formaldehyde solution. Several ethanol concentrations were used for the specimens' dehydration. At the next step, we used xylene for clarification. To make tissue incisions, we impregnated the samples with paraffin. Then we made 3 µm-thick incisions on the slides using a rotary microtome. After preparation, the slides were stained with H&E and subsequently examined under a light microscope.

Measurement of serum testosterone

The blood samples were obtained from the rats after anesthesia and the surface plasma contents were extracted. Serum testosterone concentrations were measured using the ELISA kit (Ideal Tashkhis Atieh, Iran; Cat. No. TST96).

IHC analysis for PLZF

The tissues were cut to a thickness of 2µm and stored on a positively charged slide at 37 °C for 24 hours. The specimens were then degreased and dehydrated using xylene, ethanol, and PBS. The Rabbit anti-PLZF primary antibody (Abcam, United Kingdom; Cat. No. ab305064; dilution 1:200) was diluted in a suitable buffer and applied to the sections, then incubated at 4 °C for four days. After removing the primary PLZF antibody, the FITC-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc; Cat. No. sc2357; dilution 1:500) was added and incubated at the same dilution for an hour. Tissues were examined under a fluorescence microscope following propidium iodide staining of the nuclei.[21] PLZF immunostaining specifically was used to identify the population of PLZF-positive undifferentiated spermatogonia (spermatogonial stem cell - SSC). This analysis therefore focuses on the SSC niche rather than differentiated germ-cell population.

Image preparation and analysis

Images were captured using an advanced color Moticam 2300 camera (Motic Inc, China) at a resolution of 1,352×1,016 pixels under standard lighting conditions at 200x magnification. PLZF-positive cells located along the basal membrane of seminiferous tubules were counted using ImageJ v1.47 software. Representative images from each experimental group were captured under identical microscopic conditions at ×200 magnification. The selected images illustrate the typical IHC staining pattern for each group and were used only for qualitative presentation rather than quantitative evaluation. PLZF-positive spermatogonial cells were expressed as a percentage of total spermatogonial population within each analyzed field.

Statistical method

Statistical analyses were conducted using SPSS version 20. Data were analyzed via two-way ANOVA followed by Bonferroni post hoc tests where appropriate, with statistical significance defined as $p < 0.05$. All results are presented as mean ± standard deviation (SD).

Ethical Approval

All experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (2007) and complied with institutional guidelines approved by the Ethics Committee of Aja University of Medical Sciences (approval ID: IR.AJAUMS.REC.1397.021).

Large-language Method Assistance

An open-weight large language model was used solely for grammar checking and revising phrasing in specific sections. No results, data analyses, statistical calculations, or scientific interpretations were generated or changed by the model. All LLM-generated text was carefully checked, verified, and edited by the authors to ensure academic accuracy.

Result

Table 2 summarizes plasma testosterone concentrations in all groups. Plasma testosterone concentrations were significantly reduced in both stress groups compared to their specific control groups ($p < 0.001$). The reduction in testosterone concentration was greater in young stressed rats than in old stressed rats.

Table 3 presents the percentage of PLZF-positive cells identified by IHC. The percentage of PLZF-positive spermatogonial cells was significantly lower in both stress groups than in their specific control groups ($p < 0.001$). While stress reduced PLZF expression in both

Table 2. The changes in plasma testosterone in the designated groups after the experiment

Plasma Testosterone	Control (ng/mL)	Stress (ng/mL)	p-value
Young	302.5±21.0	197.0±31.7	p< 0.001
Old	236.1±34.5	194.9±35.2	

*Values are presented as mean ± SD.

Table 3. The percentage of PLZF-expressing cells in the designated groups after the experiment

Tissue PLZF	Control	Stress	p-value
Young	88.5±2.8	71.7±6.6	P= 0.000
Old	43.7±2.8	26.5±1.2	

stress groups, the young stress group showed a higher decrease in PLZF-positive cells in comparison with the old stress group.

Figure 1 presents representative immunohistochemical staining for PLZF in each experimental group. PLZF-positive spermatogonial cells were primarily detected along the basal layer of the seminiferous tubules. Histological observations were consistent with the quantitative data, demonstrating a reduction in PLZF-positive cells following CUS, with the lowest immunoreactivity observed in the old stress group.

H&E staining sections demonstrated a decrease in spermatogonia in both stress groups compared to the controls (Figure 2). Only the old stress group exhibited sloughing of the seminiferous epithelium.

Discussion

In the current study, CUS significantly decreased serum testosterone levels in both young and old rats. This finding supports the previous reports indicating that chronic stress disrupts testicular steroidogenesis by activating the HPA axis and inhibiting the hypothalamic–

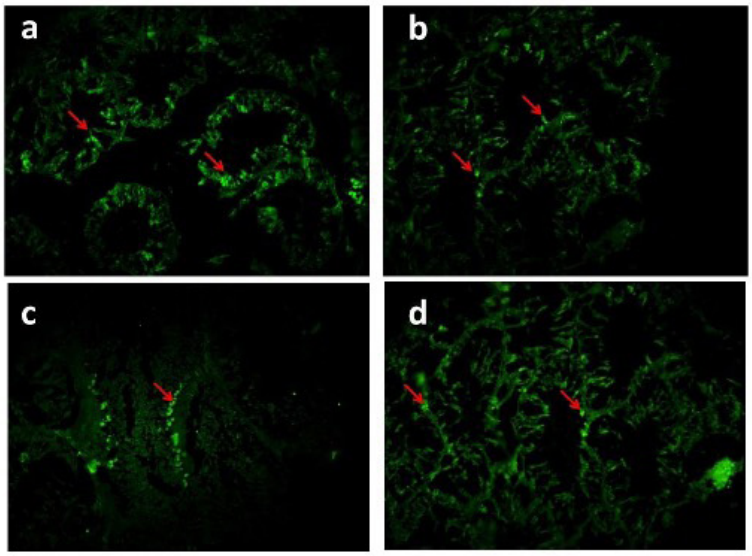

Figure 1: Representative IHC staining for PLZF in testicular tissue

Representative IHC staining for PLZF in testicular tissue from (a) young control, (b) old control, (c) young stress, and (d) old stress rats (original magnification ×200). PLZF-positive spermatogonial cells (red arrows) are located along the basal membrane of the seminiferous tubules. Representative images show reduced PLZF immunoreactivity in both stress groups compared with their respective controls, with the greatest reduction observed in the old stress group. These images are representative of each experimental group; quantitative analysis of all animals is presented in Table 3.

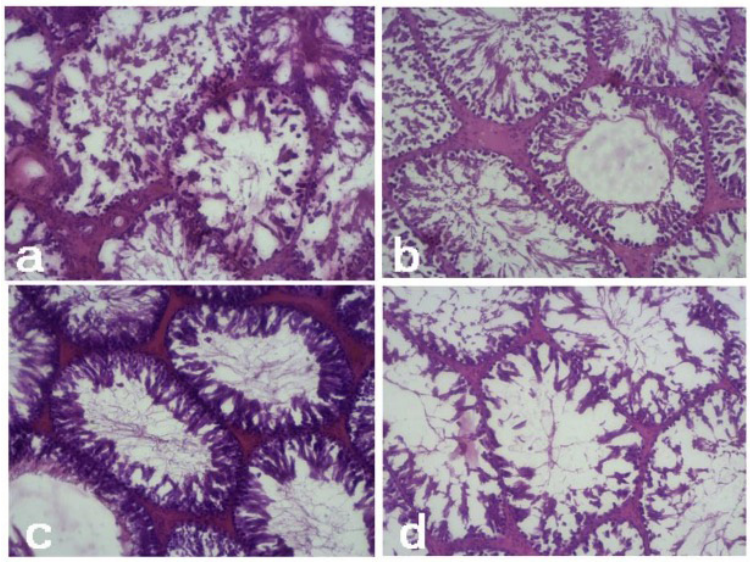

Figure 2: Representative H&E sections of testicular tissue

Representative H&E sections of testicular tissue from (A) young control, (B) old control, (C) young stress, and (D) old stress groups following eight weeks of CUS. Reduced spermatogonial cell population was observed in both stress groups, whereas seminiferous epithelial sloughing was observed only in the old stress group.

pituitary–gonadal (HPG) axis function.[22,23] Elevated glucocorticoid concentrations suppress GnRH secretion, thereby reducing LH-mediated stimulation of Leydig cells and limiting testosterone synthesis.[24,25]

The inhibitory effect of glucocorticoids on testosterone production is mediated through multiple mechanisms. Experimental studies have shown that glucocorticoids downregulate the expression of key steroidogenic proteins and enzymes, including steroidogenic acute regulatory protein (StAR), CYP11A1, HSD3B, CYP17A1, and HSD17B, enzymes that play essential roles in testosterone synthesis.[26] In addition, chronic glucocorticoid exposure may reduce Leydig-cell number through apoptosis and impair cellular proliferation, resulting in a decline in testosterone synthesis.[27]

Previous studies have shown that chronic stress impairs reproductive function through activation of the HPA axis. Increased CRH suppresses GnRH secretion and reduces pituitary LH release. In addition, the expression of CRH receptors in Leydig cells provides evidence for a direct inhibitory effect of stress signaling on testosterone synthesis. These mechanisms suggest that the reduction in testosterone is the result of endocrine dysfunction involving the HPA–HPG axis[24].

Following our study, the young stressed group had significantly lower plasma testosterone levels than older stressed group. Furthermore, the relative reduction in testosterone following CUS was greater in young rats than the older rats. This may reflect the lower baseline testosterone levels associated with reproductive aging, which limit the additional decline under stress. This explanation is supported by studies demonstrating progressive impairment of Leydig-cell function and androgen synthesis with advancing age.[25]

Stress exposure significantly reduced the PLZF expression in testicular tissue in both old and young rats, with a more significant reduction in the young group. As a key transcription factor that regulates the self-renewal and maintenance of the undifferentiated state of SSCs, PLZF plays a central role in normal spermatogenesis. Therefore, reduced expression may impair long-term spermatogenic capacity by disrupting the SSC niche.[15] These findings support the previous studies linking altered PLZF signaling to spermatogonial stem cell depletion and impaired fertility.

Importantly, the decrease in PLZF-positive cells (Figure 1) closely aligned the histological finding of reduced spermatogonial population. This concordance between molecular and histological findings suggests that chronic stress disrupts SSC maintenance and germ-cell survival, rather than causing isolated alterations in protein expression.

The testes produce sperm from spermatogonial cells during mitosis and meiosis division. This process demands the use of suitable environments. Any disruption in this procedure can result in inappropriate sperm production and infertility.[25]

Structural degeneration of the seminiferous tubules is a recognized feature of impaired spermatogenesis. The greater structural damage observed in old rats may result from diminished regenerative capacity, increased oxidative stress, and reduced tolerance to chronic endocrine disturbances.[25] Our study indicated a decrease in the spermatogonia population in both young and old groups. Also, in the old stress group, the seminiferous tubules showed sloughing. Male fertility relies on sufficient sperm production in the seminiferous epithelium.

One of the main strengths of this study is the combined evaluation of endocrine, molecular, and histopathological outcomes in young and old rats. Although the negative effects of CUS on male reproduction are well established, comparative studies examining young and old rats using PLZF as an indicator of SSC maintenance remain limited. These findings offer new insight into age-dependent responses to CUS and reproductive aging.

Conclusion

The findings of this study show that CUS affects the male reproductive system differently depending on age. Stressed young rats experienced a much greater drop in testosterone levels compared to older stressed rats. Similarly, PLZF-positive cells in testicular tissue decreased more sharply in young rats. Histological examination also showed decreased spermatogonial population in both groups, even though the seminiferous tubule damage was only detected in the older stressed rats. Our results suggest that young males may be more influenced by the negative effects of CUS, including declined testosterone levels and reduced spermatogenesis.

Acknowledgement

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Conflict of Interest

There are no conflicts of interest between the authors of this article in terms of funding, research support, consultation fees, patents, or financial interests. Additionally, there are no conflicts of interest, such as unpaid membership in a government or non-governmental organization.

Source of Funding

This study did not receive any external funding.

Ethical Considerations

The authors of this study have adhered strictly to ethical issues (such as plagiarism and data fabrication, as well as double publication). Ethics approval was also received from Aja University of Medical Sciences and the ethics committee (IR.AJAUMS.REC.1397.021).

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Supplementary Files

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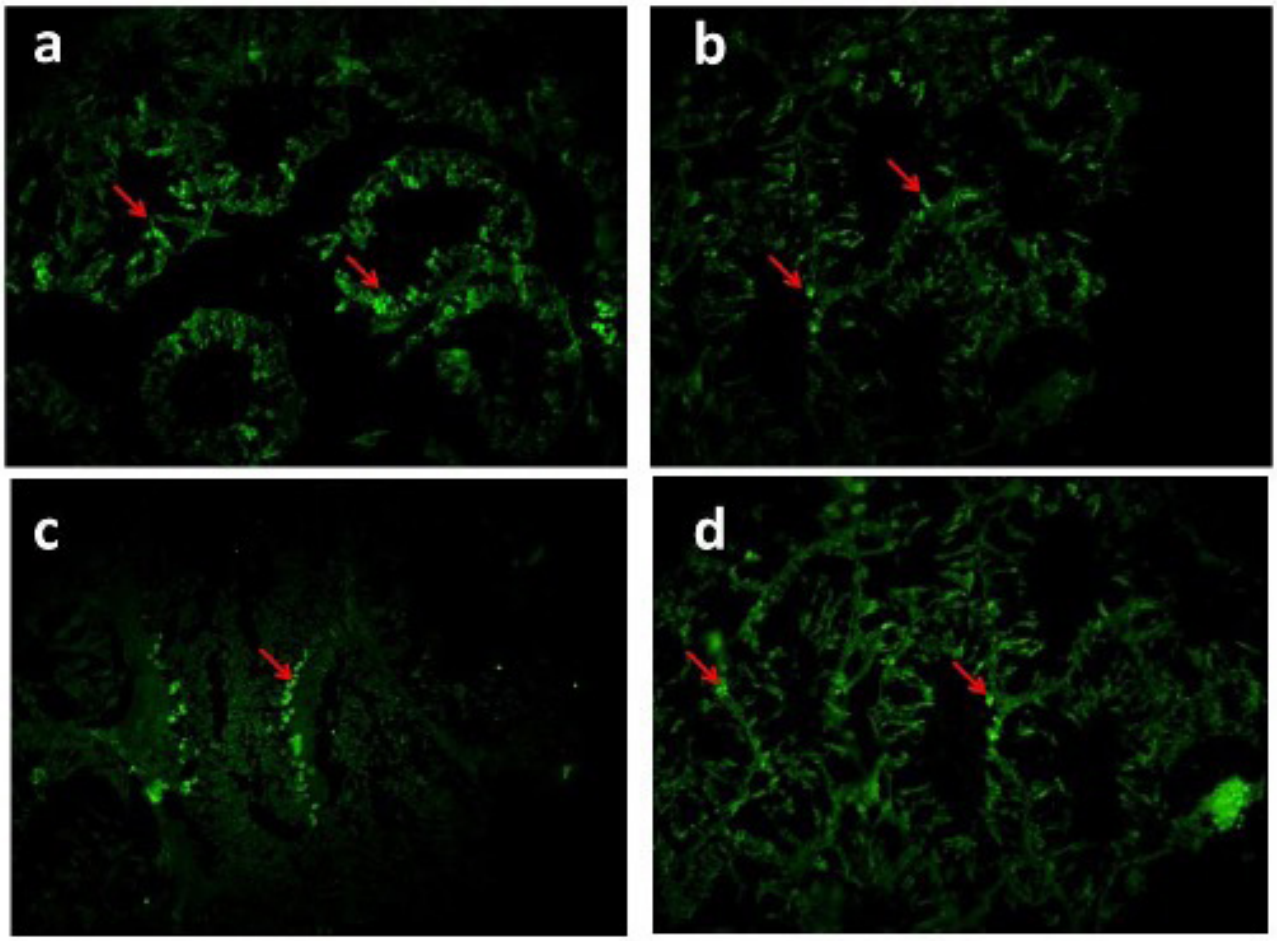
Appendix 1. Representative IHC staining for PLZF in testicular tissue



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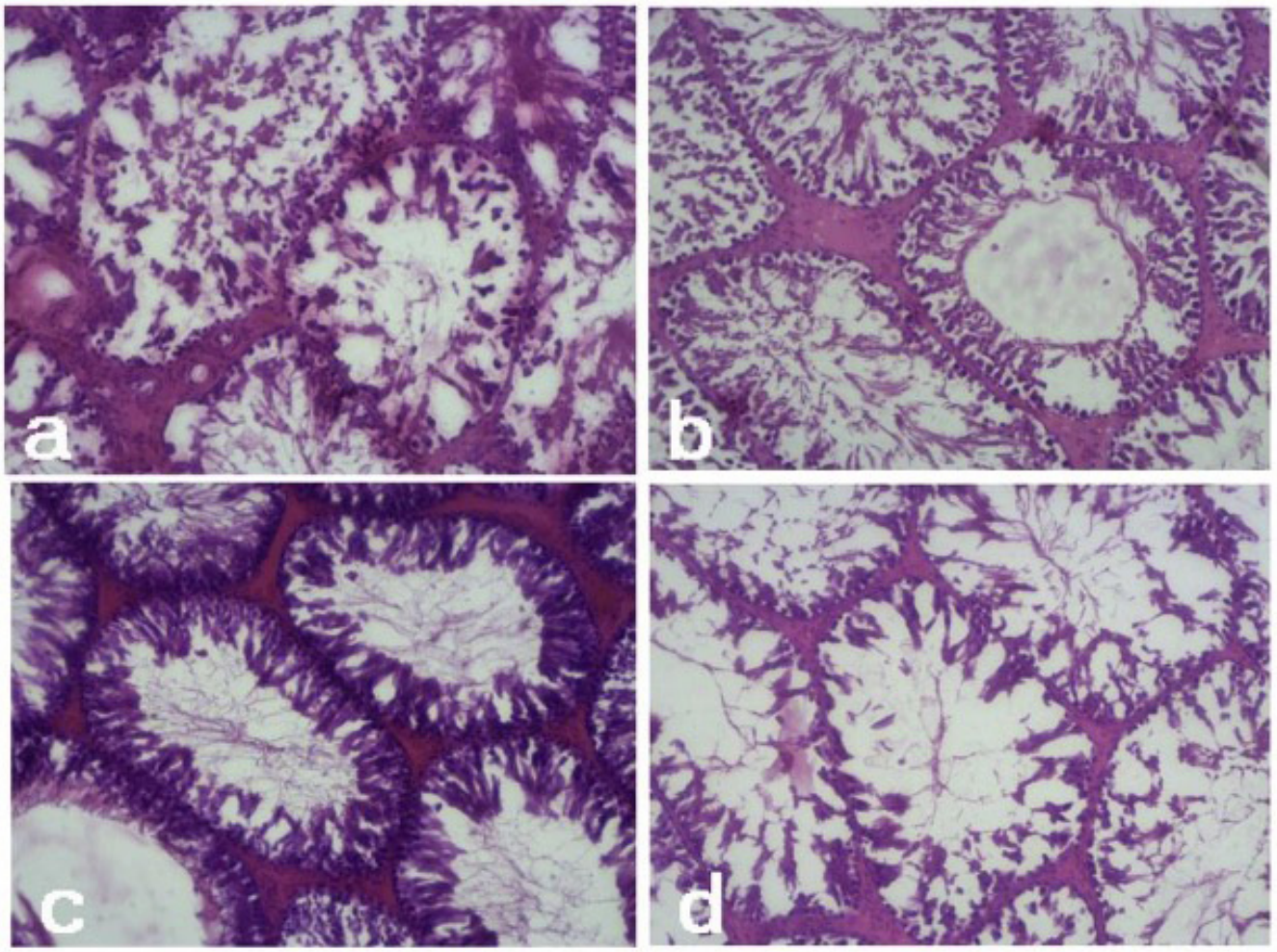
Appendix 2. Representative H&E sections of testicular tissue



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5	WD	restrainer	FS	temperature of 45 °	FD	FL	8 GSC
6	ISC	ISC	ISC	darkness	-	PET	CiR
7	BAC	MS	FS	restrainer	8 GSC	FD	temperature of 45 °
8	darkness	MS	ISC	ISC	ISC	-	FL
9	PET	CiR	Day of Sacrifice	-	-	-	-

24 hours food deprivation (FD), 24 hours water deprivation (WD), Bend the animal cage at a 45-degree angle for 24 hours (BAC), 1 pinch at 1 cm end of the tail (PET) 4 hours in the restrainer (restrainer), 5 minutes at a temperature of 45 ° C (temperature of 45 °), 5 minutes forced swimming in 4 ° C water (FS), 24-hour exposure of the animal to light (FL), Darkness 3 hours a day (darkness), Isolation in a small cage (three days) (ISC), Gather the animals in a small cage for 12 hours (GSC), Moisten sawdust under the animal for 24 hours (MS), Change under animal instead of rigid for 24 hours (CiR), The base stress of a tall column that was placed on a platform for 20 minutes at a height of 100 cm from the ground (BSTC).

Appendix 4. The changes in plasma testosterone in the designated groups after the experiment

Plasma Testosterone	Control (ng/mL)	Stress (ng/mL)	p-value
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*Values are presented as mean ± SD.

Appendix 5. The percentage of PLZF-expressing cells in the designated groups after the experiment

Tissue PLZF	Control	Stress	p-value
Young	88.5±2.8	71.7±6.6	P= 0.000
Old	43.7±2.8	26.5±1.2	