Indian Journal of Experimental Biology
Vol. 61, June 2023, pp. 393-402
DOI: 10.56042/ijeb.v61i06.1929

Effect of chronic physiological stress on rat oocyte reserve: Role of IGF-1, AMH and Bcl-2

Deniz Ercetin[1]*, Melike Sapmaz-Metin[1] & Ruhan Deniz Topuz[2]

[1]Department of Histology and Embryology; [2]Department of Medical Pharmacology, Faculty of Medicine, Trakya University, Edirne, Turkey

Received 06 November 2022; *revised 02 March 2023*

Ovarian activity has a complex physiology and is related to oocyte quality in women. This study investigates the effects of ovarian activity with chronic stress (CS) on behavioural parameters, estrous cycles, and ovarian follicular development in rats. Here, we examined the ovarian microenvironment against exposure to stress and to elucidate the stress-related ovarian molecular mechanisms. Twenty female Sprague-Dawley rats were divided into the control and the CS groups. The estrous cycle phases were detected by vaginal smear. Rats in the CS group were immobilized 1 h/day for 8 weeks. At the end of the experiment, all animals were subjected to behavioral tests in their metestrus phase and sacrificed on the other day (diestrus phase). The ovaries were harvested for histological analysis, blood samples were taken to measure cortisol levels. The immunoreactivities of ovarian IGF-1, AMH and Bcl-2 proteins were evaluated by immunohistochemistry. Here, we have studied CS-induced prolonged estrous cycles. We showed a lower number of developing follicles but the higher number of atretic follicles in the CS group's ovaries. The dominant structure of ovarian histology was large interstitial glands. CS caused decreases in ovarian Bcl-2, IGF-1 and AMH immunoreactivities which have roles in follicular development. Also, anxiety was detected in CS-exposed animals. Our results showed that chronic restrainer stress can be a serious endocrine disrupter by reducing ovarian paracrine factors.

Keywords: Anxiety, Chronic stress, Estrous cycle, Follicular development, Ovary

Stress leads to deviation from homeostasis in a living being and affects the functions of the central nervous system, the gastrointestinal system, the cardiovascular system, the neuroendocrine system, and the reproductive system[1]. The psychological and physiological reactions emerging after exposure to stress vary depending on gender. Epidemiological data indicate that women are more develop depression than men. Fluctuations in sex hormones are closely associated with stress and depression[2]. The ovarian hormone levels related to menstrual cycle phases also affect post-stress cortisol reactivity[3]. The high level of circulating estradiol may provide individual endurance against chronic stress in females. Chronic stress decreased *in vivo* maturation of oocytes, the viability and increased the percentage of abnormal oocytes[4]. An important regulator of the stress response is the endocrine system, which plays a central role in regulating the adjustment of morphology, physiology and behaviour to deal with current conditions[5]. Successful reproduction is the result of numerous interactions that played essential roles in the ovary.

Insulin-like growth factor-1 (IGF-1) is an intra-ovarian growth factor that plays important endocrine/paracrine roles, it affects female fertility and ovarian function by reducing apoptosis in granulosa cells[6]. B-cell lymphoma 2 (Bcl-2) protects the follicle pool from apoptosis but stress decreased the expression of Bcl-2 and the subsequent activation of caspases that leads to follicular atresia[7]. Anti-Müllerian hormone (AMH) is predominantly produced by the granulose cells of preantral and antral follicles. AMH deficiency has been proposed as a marker of ovarian dysfunction; because it disrupts early folliculogenesis and has an inhibitory effect on follicle recruitment and growth[8]. Although growth factors are important for healthy ovarian dynamics, only a limited number of studies are available on the effects of stress on these factors involved in ovarian molecular mechanisms. Therefore, in this study, we examined the effects of chronic immobilization stress on adult female rats and analyzed behavioral parameters, estrous cycle, serum cortisol level and

*Correspondence:
Phpne: +90 545 510 02 21 (Mob.)
E-Mail: denizmuratoglu@yandex.com
ORCIDs: 0001-9713-044X (DE); 0001-9623-4116 (MSM); 0002-1520-3754 (RDT)

ovarian morphology, as well as IGF-1, AMH, and Bcl-2 immunoreactivities.

Materials and Methods

Animals and experimental design

The experiments were conducted by the Regulation of Animal Research Ethics in Turkey. The ethical approval was granted by the Trakya University Animal Research Ethics Committee (Project number 2016/19, Edirne, Turkey). All experiments were conducted between 9:00 a.m. and 11:00 a.m. under standard laboratory conditions (12-h light/ dark cycle with lights; 22±2°C room temperature). Food pellets and tap water were provided *ad libitum*. Female, Sprague Dawley rats aged 8-12 weeks were purchased from Trakya University, Animal Care and Research Unit, Edirne, Turkey.

Twenty Sprague-Dawley female rats with regular estrous cycles were accepted to study. The rats were randomly divided into the control (n=10) and chronic stress (CS) groups (n=10). Vaginal smears were collected from both groups, every morning at 09:00 a.m. for 8 weeks to detect estrous cycle phases. The rats were also weighed every week to define the effects of stress on body weight. Restrainer was used for the CS group (1 h/day for 8 weeks, at room temperature (22±2°C)[9]. Restraint stress was applied to the rats, in-housed individual PVC cylinders inside the cage for 1 h between 10:00 and 11:00 a.m.[10]. At the end of the experiment, all animals were subjected to behavioural tests in the metestrus phase and the other day (diestrus phase) they were sacrificed under xylazine/ketamine anesthesia. Serum samples were collected for cortisol analysis and bilateral ovaries were harvested for histological analysis.

Behavioural tests for anxiety detection

At the end of the 8 weeks, all animals were subjected to behavioral tests in the metestrus phase at 09:00-12:00 a.m. rotarod, open field and elevated plus maze tests were performed, consecutively, on both groups in the Trakya University, Faculty of Medicine, Medical Pharmacology Department. Rotarod test: The rat underwent three consecutive trials with 3 min intervals. Rats that were able to stay for a total of 3 min on this roller were considered to have normal motor activity[11]. Open field and plus maze tests were placed in an isolated room, far from movement and noises. The open field test was performed to measure anxiety. The test apparatus be composed of a black square box with an open top (dimensions of 80×80 cm). The test was carried out in an isolated dark room illuminated with 30 lux light. During the test, the rat was placed in the center of the square box and allowed to move freely. Total time spent in the center was used as a measure of anxiety, where higher values are related to less anxiety. Total distance moved was measured as a general locomotion indicator. All behaviours were monitored for 10 min and calculated by the Ethovision XT (Noldus, Holland)[12]. The elevated plus maze test is another test to evaluate anxiety. The apparatus has a black acrylic surface and include four arms (two open and two closed), (L×W: 110 cm**)** and the height of the test apparatus has 60 cm above the floor. A rat was placed in the central area, facing the open arms of the apparatus. The rat was allowed to move freely for 5 min and recorded by the camera[13]. The number of entries into the open/closed arms and the time spent were calculated[14].

Study of the estrous cycle

To determine restrainer stress-induced alterations in the reproductive cycle, vaginal smears were taken from all rats in the control and CS groups. In this process; the cotton-coated end portions of the sterile rods were moistened with saline, then a swab gently was taken from the vagina and placed on a slide[15]. Hematoxylin-Eosin (H&E) (Sigma Aldrich, Germany) staining was applied to dried smear samples and then examined by light microscope (Olympus BX51, Japan), and rats were assigned to one of four substages: proestrus, estrus, metestrus and diestrus. The cycle length was defined as the number of consecutive days between the estrus smears during the previous and subsequent cycles. The smear data were used to analysis of "The total number of estrous cycles/Total number of experiment days", "The duration of an estrous cycle" and "The diestrus index"[16]. The diestrus index was calculated by dividing the total number of diestrus days by the duration of the experiment and multiplying the result by 100 (total diestrus days/total experiment days × 100)[17].

Serum cortisol assay

Cardiac blood serum samples were obtained from animals and analyzed by Trakya University Health Research and Application Center Hormone Analysis Laboratory. The serum cortisol levels (ug/dL) were measured in the Beckman Coulter DXI600 devise by the CIA method (Beckman Coulter Access Cortisol Kit, Lot: 922008).

Histological studies

Animals were sacrificed at the diestrus phase and ovaries were harvested. Ovarian tissues were fixed in neutral buffered formalin (Sigma Aldrich, Taufkirchen, Germany). The tissue samples were dehydrated by passing through the rising alcohol series, cleared in toluene (Merck Millipore) and embedded in paraffin (Merck Millipore). The sections passing through the equator of the ovary were selected to see all follicles properly. They were stained with H&E and Masson's Trichrome to analyze ovarian histology under the light microscope (Olympus BX 51, Japan). As a sign of stress-induced changes in steroidogenic activity in the ovary, the interstitial gland area was measured by imaging analysis system (version 2.11.5.1, Camera, Argenit, Turkey), on average 1.42 mm^2 surface field/ovary with Masson's trichrome stained sections. Sections were examined under light microscopy at 100X magnification.

H&E staining

The ovarian paraffin sections were twice dewaxed in xylene and rehydrated in descending grades of alcohol, then stained with H&E and ovarian histopathology was considered under a light microscope (Olympus BX 51, Japan)[18,19].

Masson's trichrome staining

Ovarian tissue sections were deparaffinized twice in xylene, then rehydrated through graded ethanols. Masson's trichrome staining was used to perform morphometric measurements for interstitial glands[16].

Immunohistochemistry

The paraffin sections of control and CS groups were incubated overnight at 56°C, and deparaffinization was performed. Then sections were subjected to antigen retrieval using boiled citrate buffer (pH 6.0, Thermo Scientific, AP-9003-500, Calif., USA), and treated in 3% (v/v) hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were blocked with 1% pre-immune rabbit serum (Thermo Scientific, Ultra V Block, TA-125-UB, Calif., USA) for 20 min to block non-specific antibody binding. Later, incubated overnight with primary antibodies; Anti-IGF-1 antibody (LifeSpan LS-C388624) and Anti-Mullerian hormone antibody (Santa Cruz, MIS, sc- 6886) at 1/100 dilution, and Anti-Bcl-2 antibody (LifeSpan LS-B6548) at 1/200 dilution. Followed by the application of HRP biotinylated secondary antibody solution (Thermo Scientific, Streptavidin peroxidase, TS-125-HR, Calif., USA) for 10 min, AEC chromogen (Abcam ab64252) was applied. Sections were counterstained with hematoxylin[20]. IGF-1, AMH, and Bcl-2 immuno-reactivities were assessed using the histological score (H-score), which is obtained by multiplying the number of stained cells by the intensity of staining (low=1, medium=2, high=3)[21]. The sections were observed by light microscopy at 200X.

Statistical analysis

We used the SPSS 20.0 program (License number: 10.240.642) in the Department of Biostatistics and Medical Informatics, School of Medicine, Trakya University. Mann-Whitney U test and Student-t test were used to determine differences between groups and data were recorded as means ± SD. Values for $P \leq 0.05$ was considered significant.

Results

Effect of chronic stress (CS) on body and ovarian tissue weight

Stress affected rat weight gain in the present study. The initial mean body weight of the control group was 220 g and the latest weight was 245 g. Whereas the initial mean body weight of the CS group was 220 g and the latest weight was 223 g. After eight weeks, we showed CS group weighed less than the control group ($P < 0.0001$). The ovarian tissue weights of the CS group (0.09 g) were also significantly lower than the control group (0.012 g) (p=0.013).

Effect of CS on behavioural parameters

In the rotarod test, all subjects could stay on roller cylinders for 3 min and their motor activities were accepted as normal. During the open field test, the anxiety levels of the subjects were evaluated by considering their total time spent in the center and the total distance moved. In the CS group, the total time spent in the center was 11.140 s and the total distance moved was 1487.12 cm. These data were lower than the control group (67.259 s, 1627.29 cm). In the elevated plus maze test, anxious behaviours were evaluated based on the length of time spent in and entering frequency to the closed arms. CS-exposed rats more often entered the closed arms than the control rats (mean times 7.4 *vs*. 5.5). All results suggest an increase in anxiety.

Effect of CS on estrous cycle

The smear data were used to calculate the cycle number during the eight-week experiment, the duration of the cycle and the diestrus indices were shown in Table 1. It was determined that the cycle number observed during the experiment of the CS group was significantly lower than the control group

Table 1 — Analysis of 8-week vaginal smear findings of the groups

	Control group (n=10)	CS group (n=10)	p
The cycle number	11.7	9	<0.0001*
Length of a cycle (day)	5.36±0.56	6.92±1.17	<0.001*
Diestrus index	23.40±5.54	28.70±4.45	0.011*

[*Compared with the control group, *P* <0.05 was considered significant]

(9 *vs*. 11.7, *P* <0.0001). However, CS caused extended cycle duration, the average duration of a cycle was 5.36 days in the control group, whereas it was 6.92 days in the CS group (*P* <0.001). The frequency of the diestrus phase increased in the CS group rats. Thus, a higher diestrus index was determined in the stress group than in the control group (28.7 *vs*. 23.4, p=0.011). The four phases of the estrous cycle exhibited regularly unique cellular properties in the control group. In the proestrus phase, clustered and round epithelial cells were shown (Fig. 1A). The phase having the characteristic of only nuclei-free cornified cells was accepted as the estrus phase (Fig. 1B). The large-volume nucleated epithelial cells and neutrophils were marked in the metestrus phase (Fig. 1C). In the diestrus phase, a few nucleated epithelial cells and neutrophils were observed in the smear samples (Fig. 1D). CS-induced neutrophil infiltration in the proestrus (Fig. 1E), but there were no differences in the estrus phase (Fig. 1F). A significant increase in neutrophil density was shown in the metestrus and diestrus phases of CS group animals (Fig. 1 G and H).

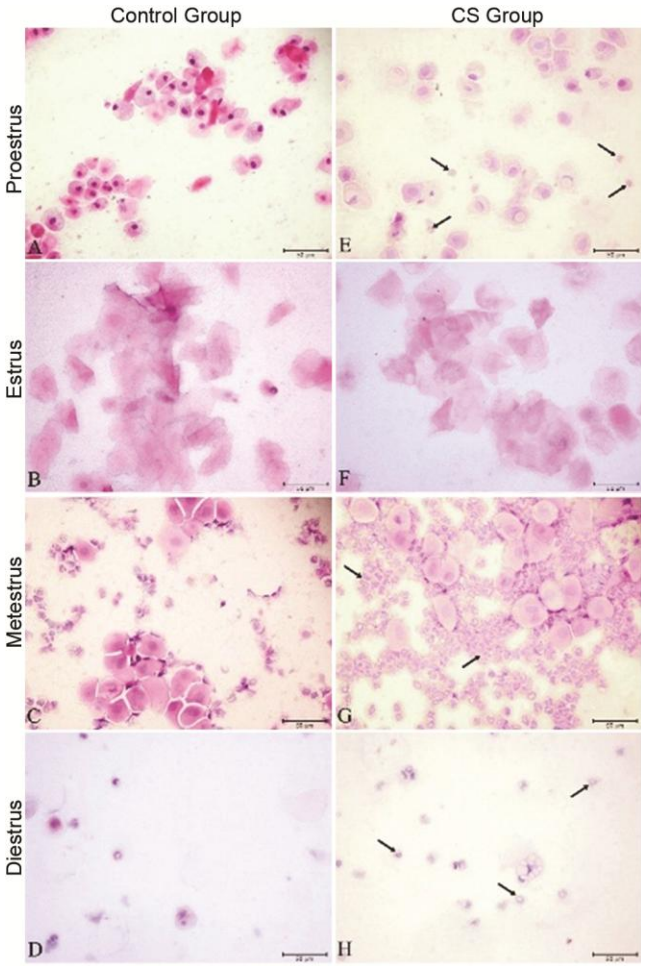

Fig. 1 —Vaginal smear samples taken from (A-D) control; and (E-H) CS groups rat. [400X, H&E. Black arrows = neutrophil]

Serum cortisol analysis

Serum cortisol level was higher (1.17±0.67 ug/dL) in the CS group than that of the control group (0.88±0.18 ug/dL). However, there was no statistical significance (p=0.384).

Effects of CS on ovary histopathology

The dominant structures of the control ovaries in the diestrus phase were the primary and secondary follicles. Numerous healthy follicles at different developmental levels and also frequent mitotic figures among the granulosa cells were identified in the control group ovaries (Fig. 2A and 3A). However, fewer healthy follicles were found in the CS group and the interstitial glands were marked in the ovarian stroma in the CS group (Fig. 2B and 3B). Apoptosis in atretic follicles (Fig. 2C) and oocyte fragmentation (Fig. 2D) were observed in the ovaries of the chronic stress group, 400X. The interstitial glands, which were the structures that remained from the atretic follicles, existed in the ovarian stroma. This condition is a reflection of increased follicular atresia and also androgen secretion caused by chronic stress. Therefore, we measured interstitial fields on the sections stained with Masson's trichrome with an average of 1.46 mm^2 sectional field for each animal. In the CS group ovaries, a larger interstitial field was shown than those in the control group (0.80 mm^2 *vs*. 0.62 mm^2). A statistically significant difference was found between the ovaries of the two groups in terms of the interstitial field/total sectional field (*P* <0.001; Fig. 3C).

Effect of CS on IGF-1, AMH and Bcl-2 immunoreactivities

Severe IGF-1 expression was found in the cytoplasm of granulosa cells and the oocytes of the multilaminar primary and secondary follicles in the control group (Fig. 4A). In the CS group, the low-moderate immunoreactivity of IGF-1 was detected in the healthy follicles (Fig. 4B). We found a statistically significant difference between the two groups for

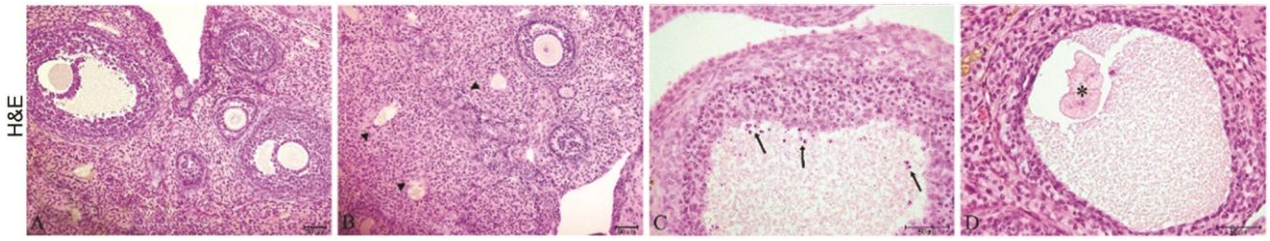

Fig. 2 — Normal ovarian histology in (A) control group 200X; (B) CS group, decreasing number of healthy follicles and an increasing number of hypertrophic interstitial glands (arrowhead) are shown, 200X; and (C & D) Atretic follicles are marked in the CS group ovaries. [Apoptotic bodies (arrow), oocyte fragmentation (*), 400X, H&E]

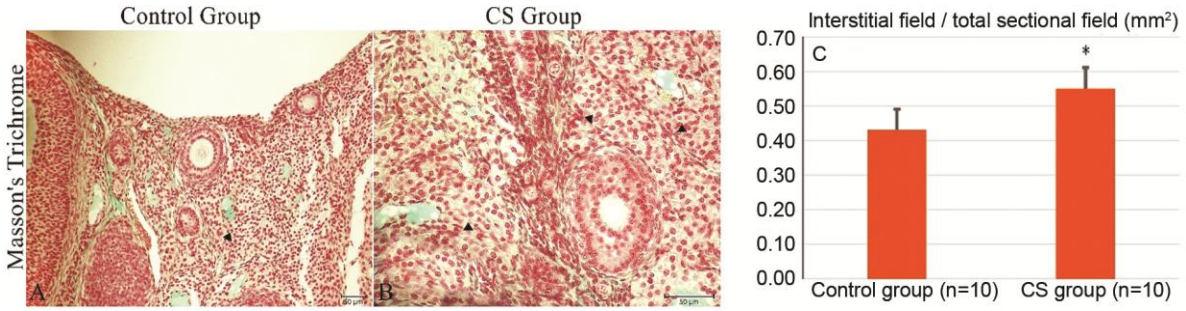

Fig. 3 — Healthy follicles in (A) control group, 200X; (B) Hypertrophic interstitial glands [Arrowhead = interstitial gland, 400X]; and (C) Masson's Trichrome. Interstitial field/total sectional field in the ovaries of two groups. [*P <0.001]

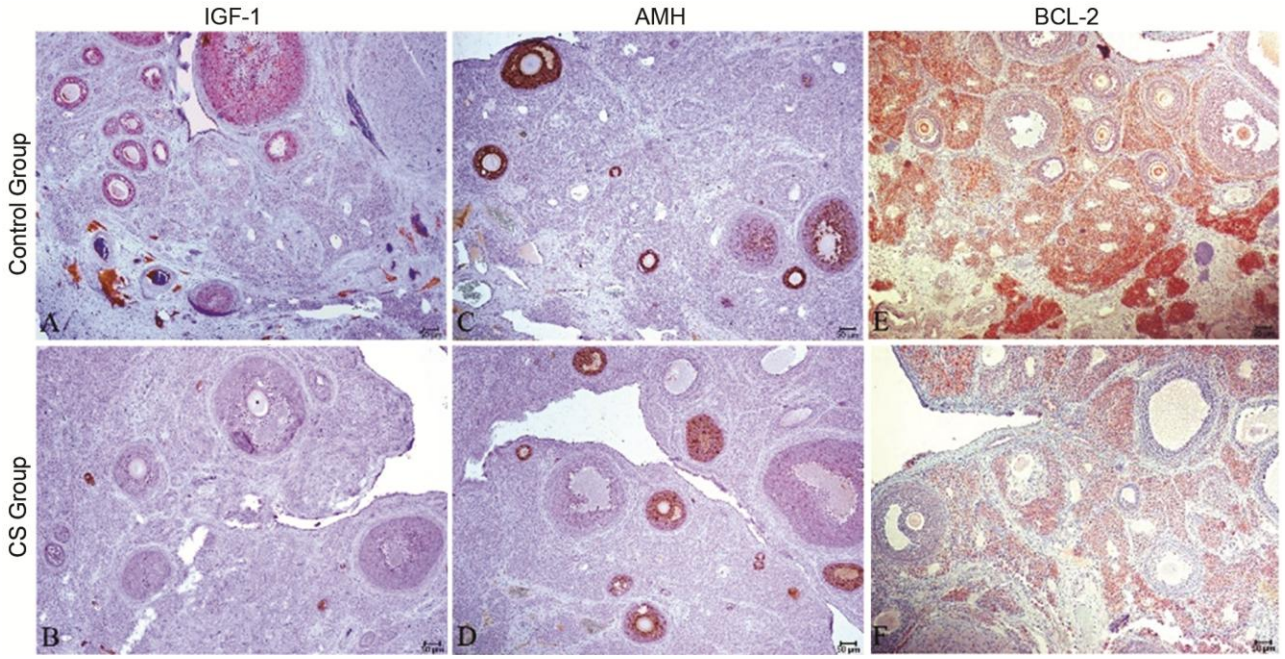

Fig. 4 — (A) Control group showing IGF-1 immunoreactivity as marked in the granulosa cells and oocytes of multilaminar primary and secondary follicles while (B) CS group showing weak to moderate immunoreactivity; (C) control group showing AMH immunoreactivity in all healthy follicles' granulosa cells, and (D) CS group showing less immunoreactivity; and (E) control group showing moderate to strong Bcl-2 immunoreactivity as observed in granulosa cells, oocytes, theca cells, germinal epithelium, corpus luteum and interstitial glands; and (F) CS group showing Bcl-2 immunoreactivity as weak to moderate. [100X, Hematoxylin counterstaining]

IGF-1 immunoreactivity (P <0.0001). The AMH immunoreactivity was observed in all the developing follicles but no staining was detected in the germinal epithelium, corpus luteum, or interstitial glands in the control ovaries (Fig. 4C). The ovaries exposed to chronic stress exhibited fewer AMH-positive follicles when compared with the control ovaries (Fig. 4D). For AMH immunoreactivity, there was a statistically

Table 2 — Immunohistochemical staining H-score values of the groups

	Control group (n=10)	CS group (n=10)	*P*
IGF-1	215.63±21.3	128.75±13.6	<0.0001
AMH	233.75±13	143.75±22.6	<0.0001
BCL-2	142.50±14.9	106.25±14.1	<0.0001

[*Compared with the control group, *P* <0.0001 was considered significant]

significance between the control and CS group (*P* <0.0001). The Bcl-2 expression was observed widely in the ovarian tissue of the control group; most notably in the follicles, germinal epithelium, corpus luteum and interstitial glands. The Bcl-2 immunoreactivity was severe in the oocytes of multilaminar primary and secondary follicles, while it was observed as moderate in the primordial follicles, granulosa and theca cells of developmental follicles (Fig. 4E). However, the Bcl-2 staining was mostly weak in the germinal epithelium, corpus luteum, and interstitial glands in the CS group (Fig. 4F). When Bcl-2 immunoreactivity was evaluated in the ovarian tissue, there was a statistically significant difference between the two groups (*P* <0.0001). It was identified that the IGF-1, AMH and Bcl-2 H-score values of the CS group were lower when compared to the control group (*P* <0.0001; Table 2).

Discussion

The reproductive potential of females is related to ovarian reserve. The ovarian microenvironment has great importance for the development of mature and functionally competent oocytes from this reserve. In this study, we tested the changes of endocrine and paracrine factors playing roles in follicular differentiation and oocyte growth to explain the chronic physiological stress effects on the ovarian dynamic in female rats. In addition, we investigated the effects of restrainer stress on anxiety, estrous cycle and ovarian histology.

Long-term stress leads to increased sensitivity to depression and anxiety[24]. During stress induction, many alterations are seen in behavioral, biochemical, and reproductive parameters[25]. Therefore, the assessment of these parameters will serve as an important basis to produce anti-stress activities[26].

Ovarian hormones commonly affect the behaviors of females[27]. Hormonal fluctuations are seen in women during the menstrual cycle[28]. As we considered the effects of chronic stress and the stage of the estrous cycles on anxiety, the diestrus stage is more suitable than the other stages because of low estrogenic fluctuations in rats. Furthermore, ovarian morphology is more quiescent in this stage. Therefore, we harvested ovaries in that stage to show clearer effects of CS on anxiety, estrous cycle and ovarian morphology. Ramos-Ortalaza *et al*.[12] reported how ovarian hormones may modulate the development of depression and anxiety. They reported decreased anxiety in the estrus stage compared to proestrus and diestrus. Mid-levels of estrogen (estrus) decreased anxiety instead of peak levels of the gonadal hormones (proestrus)[29]. The elevated estrogen hormone levels have effect likeantianxiolytic and antidepressant[30]. Furthermore, during pregnancy applied maternal stress causes lower offspring birth weight and shorter gestation[31].

Previous studies reported that when the open field test was applied to female mice exposed to chronic restraint stress for 4 weeks, total moving distance and time spent in the center were significantly reduced[32]. Also, chronic restrainer stress caused an increase in the duration of staying in close arms in the elevated plus maze test[26]. Similarly, in our study, the CS-exposed rats stayed on the periphery and failed to act in the open field test, additionally increasing entering frequency to the closed arms in the elevated plus maze test. These findings proved the anxiety in rats induced by 8 weeks of restrainer stress.

The circulating estradiol levels may provide resistance to anxiety disorders in females[33]. On the contrary, some of the studies suggest females are more sensitive to anxiety because of changing hormonal levels[34]. Women have various periods which make them susceptible to depression and anxiety, such as puberty, pregnancy and perimenopause[35]. Stress can cause menstrual disorders and infertility in women[36]. A limited number of studies showed the effects of stress on menstrual cycle disorder. When an inflammatory-like stress attack was applied to non-human primates for five days during the follicular phase, it was observed that the follicular phase extended significantly[37]. Ramos-Ortolaza *et al*.[12] reported irregular cycles due to an extension in the estrus phase duration without entering into proestrus phases. Another study shows that disruption of the menstrual cycle has led to a decrease in pregnancy rates and fetal numbers[38].

The vaginal smear is a valuable parameter, in our study, it reflected the effects of chronic restrainer stress on the female genital tract. We showed a

reduced number of cycles in the CS group because of the extension in the durations of the metestrus and diestrus phases. In addition, an extension of one cycle time, as well as a high diestrus index were observed. Although no neutrophils are seen in a normal proestrus phase, we showed neutrophils in proestrus smears of CS group rats. These results can be valuable data on stress-induced estrous cycle irregularity and may be a sign of an inflammatory response against stress.

The hypothalamus-pituitary-adrenal axis (HPA) is affected under stress conditions and the alterations in the levels of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol can cause infertility[39]. The serum cortisol levels vary due to the type and duration of the stress factor. Also, gender is the other factor, that affects cortisol response[40]. A previous study suggested that chronic cold stress did not alter the serum cortisol levels[41]. It was stated that the cortisol level increased to the highest value on the 1st day of unpredictable and repeated restraint stress, while it did not change on the other days[42]. The other study reported that chronic immobilization stress caused a significant increase in serum corticosterone levels in female rats[43]. Similarly, in our study, restraint stress induced an increase in the serum cortisol level. But, it was a slight increase may be due to the adaptation ability of rats to stress.

The interstitial glands derive from the theca interna of the atretic follicles. Thecal cells are converted into hormone-secreting interstitial cells by the atretic processes. It has been accepted for several years that this tissue is abundant in the estrus and appears to increase during pregnancy due to the elevation of progesterone and interstitial gland cells hyper atrophy[44]. The interstitial gland could be considered as a steroid-producing structure of the ovary exhibiting species specificity and they can synthesize and store steroid hormones. Their prominence may differ from one species to another, as well as with stages of the reproductive cycle[45]. In humans, the interstitial glands produce estrogen, while in rabbits, it is well-developed and secretes progesterone[43]. Atrophy of interstitial glands can be induced by hormonal imbalance[46].The hypertrophic interstitial gland shows the aging ovary. Because it becomes ~~as~~ a highly steroidogenic tissue and includes reactive oxygen species then it effects follicular viability[47]. An increase in the interstitial gland number is closely related to the rise in the atresia in the ovary[48].

After exposure to immobilization stress, functional disorders were reported both in ovulation and oocyte development in female rats[49]. Chronic restraint stress caused a decrease in the number of healthy follicles, while an increase in the number of atretic follicles. For the first time, we measured increased interstitial gland area under the chronic stress. This may be an evidence of CS-induced endocrine disruption leading to time extension in the progestive estrous cycle. There are limited studies on interstitial glands so our findings can contribute to the literature to explain stress-induced ovarian disorders.

IGF-1 is a growth factor that is expressed in the follicular cells and oocytes. IGF-1 acts synergistically with FSH to stimulate oocyte growth, increasing the number of developed oocytes and stimulating activity in the ovary[50]. It was reported that IGF-1 null mice could not ovulate and they have low estradiol levels[51]. Immobilization stress caused a reduction in the ovarian and serum IGF-1 levels in rats. This result was explained by the repression of CRH on the IGF-1 protein expression[52]. In accordance with previous studies, we showed that 8 weeks of restrainer stress caused a decrease in IGF-1 immunoreactivity in the rat ovaries.

AMH is another important paracrine factor in the ovary and also an indicator that preserved ovarian follicular reserve. It has been stated that there is a relationship between the low serum AMH level and stress in infertile women[53]. These results may be a sign of primordial follicle loss and disorders in reproduction in rats exposed to chronic restrainer stress. Although many studies measured blood levels of IGF-1 and AMH to evaluate the ovarian function, considering the ovarian dynamics, demonstrating tissue levels and localizations of these factors may become valuable to explain molecular mechanisms under the stress condition. We suggest that our immunohistochemical findings provide information, for the first time, that CS caused decreases in the levels of IGF-1 and AMH.

Bcl-2 mainly plays a role in the apoptotic process as an anti-apoptotic factor. Studies have shown that, both the mRNA expression and the ovarian tissue level of Bcl-2 decline after chronic immobilization stress exposure[54]. Our study revealed that Bcl-2 immunoreactivity decreased in the ovarian tissues of the CS group in comparison with the control group. The increase in the number of atretic follicles confirmed this result.

Conclusion

Our above findings show that chronic stress decreased the body and ovary weights, created an irregularity in the estrous cycle, and disrupted ovarian dynamic by reducing IGF-1, AMH, and Bcl-2 expressions in rat ovaries. Although anxiety was shown by behavioural tests, a slight increase in cortisol levels may be due to the adaptation of rats to stress conditions depending on their learning abilities. Immunohistochemistry showed the reduction of ovarian growth factors, that is may lead to ovarian failure. For the first time, interstitial gland area measurement, as a valuable parameter, is presented in our present study. The increase in interstitial gland area may be an indicator of stress-induced ovarian aging. In light of all these, our results contribute to future studies on protecting the ovarian microenvironment against stress exposure and understanding stress-related ovarian molecular mechanisms.

Acknowledgment

The authors acknowledge Trakya University Research Center, Edirne, Turkey for the financial support (project no: 2016-79).

Statement of Ethics

All experimental protocols were approved by the Trakya University Ethics Board for Animal Experiments (TUHADYEK-2016/19). All researchers were certified for the care and use of laboratory animals, and all the animals received humane care in strict compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health and Turkish bylaws for laboratory animal use.

Conflict of Interest

Authors declare no competing interests.

References

1 Arun S, Burawat J, Sukhorum W, Sampannang A, Maneenin C & Iamsaard S, Chronic restraint stress induces sperm acrosome reaction and changes in testicular tyrosine phosphorylated proteins in rats. *Int J Reprod Biomed*, 14 (2016) 443.

2 Fernandes EV, Carmona LF, Ferreira MCB, Machado FA, Balarin MRS, Ramos SD, Estanislau C & Venancio EJ, Impact of interaction between chronic variable stress and moderate intensity physical exercise on antibody production in Wistar rats. *Indian J Exp Biol*, 59 (2021) 406.

3 Chung KC, Peisen F, Kogler L, Radke S, Turetsky B, Freiherr J & Derntl B, The Influence of Menstrual Cycle and Androstadienone on Female Stress Reactions: An fMRI Study. *Front Hum Neurosci*, 10 (2016) 44.

4 Casillas F, Betancourt M, Juarez-Rojas L, Ducolomb Y, Lopez A, Avila-Quintero A, Zamora J, Ommati MM & Retana-Marquez S, Chronic Stress Detrimentally Affects *In Vivo* Maturation in Rat Oocytes and Oocyte Viability at All Phases of the Estrous Cycle. *Animals* (Basel), 11 (2021) 9.

5 Toth Z, Ouyang JQ & Lendvai AZ, Exploring the mechanistic link between corticosterone and insulin-like growth factor-1 in a wild passerine bird. *Peer J*, 6 (2018) 5936.

6 Han Y, Wang S, Wang Y & Zeng S, IGF-1 Inhibits Apoptosis of Porcine Primary Granulosa Cell by Targeting Degradation of BimEL. *Int J Mol Sci*, 20 (2019) 21.

7 Zheng Y, Ma L, Liu N, Tang X, Guo S, Zhang B & Jiang Z, Autophagy and Apoptosis of Porcine Ovarian Granulosa Cells During Follicular Development. *Animals* (Basel), 9 (2019) 12.

8 Wu X, Wu H, Sun W & Wang C, Improvement of anti-Mullerian hormone and oxidative stress through regular exercise in Chinese women with polycystic ovary syndrome. *Hormones*, 20 (2020) 339.

9 Crema LM, Diehl LA, Aguiar AP, Almeida L, Fontella FU, Pettenuzzo L, Vendite D & Dalmaz C, Effects of chronic restraint stress and 17-beta-estradiol replacement on oxidative stress in the spinal cord of ovariectomized female rats. *Neurochem Res*, 35 (2010) 1700.

10 Dogan M, Sevgili AM, Kozanoglu I & Pehlivanoglu B, Effect of acute or subchronic stress on T cell response in peripheral blood: Regulatory role of vitamin D. *Indian J Exp Biol*, 61 (2023) 14.

11 Tu D, Gao Y, Yang R, Guan T, Hong JS & Gao HM, The pentose phosphate pathway regulates chronic neuroinflammation and dopaminergic neurodegeneration. *J Neuroinflammation*, 16 (2019) 255.

12 Ramos-Ortolaza DL, Doreste-Mendez RJ, Alvarado-Torres JK & Torres-Reveron A, Ovarian hormones modify anxiety behavior and glucocorticoid receptors after chronic social isolation stress. *Behav Brain Res*, 328 (2017) 115.

13 Knight P, Chellian R, Wilson R, Behnood-Rod A, Panunzio S & Bruijnzeel AW, Sex differences in the elevated plus-maze test and large open field test in adult Wistar rats. *Pharmacol Biochem Behav*, 204 (2021).

14 Domonkos E, Borbelyova V, Csongova M, Bosy M, Kacmarova M, Ostatnikova D, Hodosy J & Celec P, Sex differences and sex hormones in anxiety-like behavior of aging rats. *Horm Behav*, 93 (2017) 159.

15 Priyadarshani A, Effects of opium alkaloid, noscapine in RU486 induced experimental model of polycystic ovary syndrome. *Indian J Biochem Biophys*, 59 (2022) 468.

16 Sapmaz-Metin M, Topcu-Tarladacalisir Y, Kurt-Omurlu I, Karaoz Weller B & Unsal-Atan S, A morphological study of uterine alterations in mice due to exposure to cadmium. *Biotech Histochem*, 92 (2017) 264.

17 Mondal M, Sarkar K, Nath PP & Paul G, Monosodium glutamate suppresses the female reproductive function by impairing the functions of ovary and uterus in rat. *Environ Toxicol*, 33 (2018) 198.

18 Wang S, Lin S, Zhu M, Li C, Chen S, Pu L, Lin J, Cao L & Zhang Y, Acupuncture Reduces Apoptosis of Granulosa Cells in Rats with Premature Ovarian Failure Via Restoring the PI3K/Akt Signaling Pathway. *Int J Mol Sci*, 20(2019) 24.

19 Rana A, Kumar NR & Kaur J, Therapeutic effect of propolis on Staphylococcus aureus induced oxidative stress in spleen of Balb/c mice: A biochemical and histopathological study. *Indian J Exp Biol*, 60 (2022) 346.

20 Kizilay G, Ersoy O, Bozer C, Demirtas S, Cikmaz S & Yilmaz A, Effects of electroacupuncture at ST36 and BL20 on the diabetic rat testis. *Acupunct Med*, (2023) 9645284221146196. doi: 10.1177/09645284221146196.

21 Delen O & Uz YH, Protective effect of pyrrolidine dithiocarbamate against methotrexate-induced testicular damage. *Hum Exp Toxicol*, 40 (2021) 164.

22 Broi MG, Giorgi VSI, Wang F, Keefe DL, Albertini D & Navarro PA,Influence of follicular fluid and cumulus cells on oocyte quality: clinical implications. *J Assist Reprod Genet*, 35 (2018) 735.

23 Zhao M, Wang W, Jiang Z, Zhu Z, Liu D & Pan F, Long-term effect of post-traumatic stress in adolescence on dendrite development and H3K9me2/BDNF expression in male rat hippocampus and prefrontal cortex. *Front Cell Dev Biol*, 8 (2020) 682.

24 Westfall S, Caracci F, Estill M, Frolinger T, Shen L & Pasinetti GM, Chronic Stress-Induced Depression and Anxiety Priming Modulated by Gut-Brain-Axis Immunity. *Front Immunol*, 12 (2021) 670500.

25 Zavala E, Voliotis M, Zerenner T, Tabak J, Walker JJ, Li XF, Terry JR, Lightman SL, O'Byrne K & Tsaneva-Atanasova K, Dynamic Hormone Control of Stress and Fertility. *Front Physiol*, 11 (2020) 598845.

26 Fotsing D, Ngoupaye GT, Ouafo AC, Njapdounke SKJ, Kenneth YA & Ngo Bum E, Effects of Gladiolus dalenii on the Stress-Induced Behavioral, Neurochemical, and Reproductive Changes in Rats. *Front Pharmacol*, 8 (2017) 685.

27 Ghazvini H, Tirgar F, Khodamoradi M, Akbarnejad Z, Rafaiee R, Tamijani SMS, Asadi-Shekaari M, Esmaeilpour K & Sheibani V, Ovarian hormones prevent methamphetamine-induced anxiety-related behaviors and neuronal damage in ovariectomized rats. *Neurosci Lett*, 746 (2021) 135652.

28 Miller CK, Halbing AA, Patisaul HB & Meitzen J, Interactions of the estrous cycle, novelty, and light on female and male rat open field locomotor and anxiety-related behaviors. *Physiol Behav*, 228 (2021) 113203.

29 Sayin A, Derinoz O, Yuksel N, Sahin S & Bolay H, The effects of the estrus cycle and citalopram on anxiety-like behaviors and c-fos expression in rats. *Pharmacol Biochem Behav*, 124 (2014) 180.

30 Kundakovic M & Rocks D, Sex hormone fluctuation and increased female risk for depression and anxiety disorders: From clinical evidence to molecular mechanisms. *Front Neuroendocrinol*, 66 (2022) 101010.

31 Nazzari S, Fearon P, Rice F, Dottori N, Ciceri F, Molteni M & Frigerio A, Beyond the HPA-axis: Exploring maternal prenatal influences on birth outcomes and stress reactivity. *Psychoneuroendocrinology*, 101 (2019) 253.

32 Song AQ, Gao B, Fan JJ, Zhu YJ, Zhou J, Wang YL, Xu LZ & Wu WN,NLRP1 inflammasome contributes to chronic stress-induced depressive-like behaviors in mice. *J Neuroinflammation*, 17 (2020) 178.

33 Luine V, Gomez J, Beck K & Bowman R, Sex differences in chronic stress effects on cognition in rodents. *Pharmacol Biochem Behav*, 152 (2017) 13.

34 Soares CN & Zitek B, Reproductive hormone sensitivity and risk for depression across the female life cycle: a continuum of vulnerability?. *J Psychiatry Neurosci*, 33 (2008) 331.

35 Halbreich U & Kahn LS, Role of estrogen in the aetiology and treatment of mood disorders. *CNS Drugs*, 15 (2001) 797.

36 Han J, Yang D, Liu Z, Tian L, Yan J, Li K, Fang Z, Chen Q, Lin B, Zhang W, Xi Z & Liu X, The damage effect of heat stress and psychological stress combined exposure on uterus in female rats. *Life Sci*, 286 (2021) 120053.

37 Xiao E, Xia-Zhang L & Ferin M, Stress and the menstrual cycle: short- and long-term response to a five-day endotoxin challenge during the luteal phase in the rhesus monkey. *J Clin Endocrinol Metab*, 84 (1999) 623.

38 Retana-Marquez S, Juarez-Rojas L, Avila-Quintero A, Rojas-Maya S, Perera G, Casillas F, Betancourt M & Gomez-Quiroz L, Neuroendocrine disruption is associated to infertility in chronically stressed female rats. *Biol Reprod*, 20 (2020) 474.

39 Phumsatitpong C, Wagenmaker ER & Moenter SM, Neuroendocrine interactions of the stress and reproductive axes. *Front Neuroendocrinol*, 63 (2021) 100928.

40 McLaughlin C, Schutze R, Henley D, Pennell C, Straker L & Smith A, Prenatal and childhood stress exposure and the sex specific response to psychosocial stress in adulthood. *Psychoneuroendocrinology*, 125 (2021) 105.

41 Dorfman M, Arancibia S, Fiedler JL & Lara HE, Chronic intermittent cold stress activates ovarian sympathetic nerves and modifies ovarian follicular development in the rat. *Biol Reprod*, 68 (2003) 2038.

42 Gong S, Miao YL, Jiao GZ, Sun MJ, Li H, Lin J, Luo MJ & Tan JH, Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. *PLoS ONE*, 10 (2015) e0117503.

43 Abdel Hafez SMN, Allam F & Elbassuoni E, Sex differences impact the pancreatic response to chronic immobilization stress in rats. *Cell Stress Chaperones*, 26 (2021) 199.

44 Awad MM, Mohamed RH, Amin YA & Hussein HA, Histological and immunohistochemical investigations of ovarian interstitial glands during non-breeding season in camels (Camelus dromedarius). *Reprod Domest Anim*, 53 (2018) 872.

45 Tsvetkov T, Stanchev V & Takeva T, Comparative study of the ovarian interstitial gland in non-hibernating and hibernating mammals. *Bulg J Agric Sci*, 9 (2003) 565.

46 Gopinath C, Toxicology and pathology of female reproductive tract. *Cell Biol Toxicol*, 29 (2013) 131.

47 Diaz-Hernandez V, Caldelas I, Montano LM & Merchant-Larios H, Morphological rearrangement of the cortical region, in aging ovaries. *Histol Histopathol*, 34 (2019) 775.

48 Miyabayashi K, Tokunaga K, Otake H, Baba T, Shima Y & Morohashi K, Heterogeneity of ovarian theca and interstitial gland cells in mice. *PLoS ONE*, 10 (2015) e0128352.

49 Zhang SY, Wang JZ, Li JJ, Wei DL, Sui HS, Zhang ZH, Zhou P & Tan JH, Maternal restraint stress diminishes the developmental potential of oocytes. *Biol Reprod*, 84 (2011) 672.

50 Devesa J & Caicedo D, The Role of Growth Hormone on Ovarian Functioning and Ovarian Angiogenesis. *Front Endocrinol* (Lausanne), 10 (2019) 450.
51 Li L, Fu YC, Xu JJ, Chen XC, Lin XH & Luo LL, Caloric restriction promotes the reproductive capacity of female rats via modulating the level of insulin-like growth factor-1 (IGF-1). *Gen Comp Endocrinol*, 174 (2011) 232.
52 Liang B, Wei DL, Cheng YN, Yuan HJ, Lin J, Cui XZ, Luo MJ & Tan JH, Restraint stress impairs oocyte developmental potential in mice: role of CRH-induced apoptosis of ovarian cells. *Biol Reprod*, 89 (2013) 64.
53 Dong YZ, Zhou FJ & Sun YP, Psychological stress is related to a decrease of serum anti-mullerian hormone level in infertile women. *Reprod Biol Endocrinol*, 15 (2017) 51.
54 Wang X, Xie Y, Zhang T, Bo S, Bai X, Liu H, Li T, Liu S, Zhou Y & Cong X, Resveratrol reverses chronic restraint stress-induced depression-like behaviour: Involvement of BDNF level, ERK phosphorylation and expression of Bcl-2 and Bax in rats. *Brain Res Bull*, 125 (2016) 134.