



EFFECTS OF ANTIGLUCOCORTICOID PRETREATMENT ON  
TESTES IN CHRONICALLY STRESSED ADULT RATS  
– A HISTOMORPHOMETRIC STUDY

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**Summary**

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Male fertility and reproduction can be affected by stress. The aim of the present study was histomorphometric evaluation of the role of glucocorticoids during chronic restraint stress on spermatogenesis. Twenty four male adult Sprague-Dawley rats were allocated randomly to four equal groups; stress group, mifepristone (RU486)-treated group, stress/RU486 and control groups. In stress group, the rats were restrained 1 h/day for 12 days. In RU486 group, the rats were injected RU486 at a dose of 2.5 mg/kg for 12 days. In stress/RU486 group, the rats were injected RU486 1 h before the stress process for 12 days. The testes of the all groups were removed and on 10 circular transverse sections of tubules stained with hematoxylin-eosin, histomorphometric parameters including cellular (germinal epithelium) diameter and area of the seminiferous tubules, total diameter and cross sectional area, number of seminiferous tubules per unit area, and numerical density of the tubules of the seminiferous tubules were measured. Restraint stress reduced lumen diameter, thickness of germinal epithelium and numerical density of seminiferous tubules ( $P<0.05$ ). This reduction was reversed by subcutaneous injection of the anti-glucocorticoid, RU486 prior to stress session ( $P<0.05$ ). In conclusion, chronic stress acts on testicular tissue via glucocorticoid receptors to suppress spermatogenesis in male rats.

**Key words:** chronic stress, glucocorticoid receptor, histomorphometry, rats, spermatogenesis

## INTRODUCTION

Stressful events can disrupt endocrine signalling and reproduction (Chand & Lovejoy, 2011). The secretion of glucocorticoids is a typical response to stress. Previous studies in rats attempted to correlate stress-induced glucocorticoid secretion with alterations in the structure of the testis and sub-fertility (Yazawa *et al.*, 2000; Mingoti *et al.*, 2003; Swami & Jegannathan Ramanathan, 2007). The mediators of the effects of stress on the hypothalamic-pituitary-gonadal (HPG) axis include glucocorticoids, catecholamines, arginine-vasopressin, corticotropin-releasing factor (CRF), and endogenous opioids. Many of the negative effects of stress on fertility may be attributed to elevated production of stress hormones, especially glucocorticoids, within the circulation and testes. These mediators exert disruptive effects on spermatogenic cells. Hypothalamus neurons express abundant glucocorticoid receptors (Rosenfeld *et al.*, 1988) and dexamethasone as a synthetic glucocorticoid suppresses transcription in immortalised gonadotropin-releasing hormone (GnRH) secreting hypothalamic cell lines (Chandran *et al.*, 1994). In addition, immunohistochemical studies showed a large density of glucocorticoid receptors at the interstitial and basal zone of the testis in rats (Stalker *et al.*, 1989; Biagini *et al.*, 1995).

In a chronically stressed rat model, plasma corticosterone concentration was increased, whereas plasma testosterone was decreased (Yazawa *et al.*, 2000). This association has led to the general argument that glucocorticoids play a profound role in mediating the inhibitory effects of stress on reproduction. In addition, glucocorticoids increase the inhibitory actions of gonadotropin-inhibitory hormone (GnIH), a novel negative regulator of re-

productive axis, on GnRH secretion (Kirby *et al.*, 2009). Even after sexual development, psychophysical stressors exert control over reproduction, causing abnormal estrous cycling and decreased sexual hormone production. Because of the complexity of stress response, how the HPG axis senses external stressors remains to be understood.

Mifepristone (RU486) is a derivative of the progestin norethindrone, and acts as a progesterone receptor antagonist or glucocorticoid receptor antagonist (Cadepond *et al.*, 1997; Munhoz *et al.*, 2006). An advantage of glucocorticoid receptor antagonists is that prolonged antagonism of glucocorticoid receptor may actually result in glucocorticoid receptor up-regulation in hypothalamus and limbic structures. Therefore, it enhances the glucocorticoid receptor negative feedback control of the hypothalamic-pituitary-adrenal (HPA) axis (Wulsin *et al.*, 2010). Moreover, the use of RU486 in individuals with elevated glucocorticoids as a result of chronic stress may be particularly advantageous as preliminary blockade of glucocorticoid receptor can serve as a barrier against the deleterious effects of excess glucocorticoid production (Wulsin *et al.*, 2010). RU486 normalised changes in adult neurogenesis induced after chronic stress in rats (Oomen *et al.*, 2007). RU486 rapidly reversed a chronic corticosterone-induced reduction of adult neurogenesis in rats (Mayer *et al.*, 2006).

Chronic stress was found to suppress expression of GnIH, related to reproductive system (Karami Kheirabad *et al.*, 2015). Systemic administration of RU486 partially reversed the restraint-induced decrease in GnIH expression, confirming that glucocorticoids and its receptor are

involved in steroidogenic suppression (Karami Kheirabad *et al.*, 2015).

The aim of the present study was to determine the effects of chronic exposure to restraint stress on testes of rats and evaluate the use of RU486 as an anti-glucocorticoid agent at testicular level. Using histomorphometric analysis of histological sections at the light microscope level to follow changes in volume, surface area and other structures in three dimensions to be calculated from the two dimensional images of light microscopy.

## MATERIALS AND METHODS

### *Animals*

Twenty four male adult Sprague-Dawley rats weighing 220–240 g were housed in the Laboratory Animal Center, Shiraz University of Medical Sciences, Iran under controlled temperature ( $23\pm 1^\circ\text{C}$ ), 12:12-h light/dark cycle, and  $55\pm 5\%$  relative humidity. They were given standard pellet and water *ad libitum* during the experimental period. The experimental investigation was approved by Ethics committee of Shiraz University of Medical Sciences.

The rats were randomly divided into four equal groups ( $n=6$ ): stress, RU486, stress/RU486, and control groups. Restraint stress was performed daily for 12 days. Briefly, rats were handled before experiments. After that, animals were individually restrained for 1 h through wrapping of their upper and lower limbs by plastic cylinders ( $20.5\times 8\times 6$  cm) with holes for ventilation and their extended tails. The cylinders were just large enough to allow rats of the size used to turn around easily. In RU486 group, the rats were subcutaneously injected RU486 ( $2.5$  mg/kg,  $20$   $\mu\text{L}/\text{rat}$ ; ab120356, Abcam Ltd, Cambridge, UK) for 12 days. In

stress/RU486 group, the rats were subcutaneously injected with the same dose of RU486, 60 min before the stress process for 12 days. The control group was allowed to freely move in the cages and did not receive any stress or drug. All of the experiments were conducted between 8:00 and 12:00 AM.

### *Histomorphometric analysis*

Twenty four hours after the last session of stress, the rats were weighted and anaesthetised by inhalation of ether and euthanised by cervical dislocation. For histological evaluation, the right testes of all groups were dissected out and weighed using a digital weighing scale (precision 0.001 g). The testicular tissues were fixed in fresh 10% buffered formalin solution in room temperature. After that each testis was implanted in paraffin. Ethanol and xylene were used for dehydration step. Samples were embedded in paraffin wax and sectioned at thicknesses of  $5$   $\mu\text{m}$ . Sections were deparaffinised at  $60^\circ\text{C}$  and dehydrated in graded concentrations of xylene and ethanol rehydrated in room temperature and stained with haematoxylin and eosin stain. Finally, sections were viewed and photographed on light microscope (CX21, Olympus, Japan) equipped with an adjusted digital camera (AM423U Eyepiece Camera, Dino-Eye, Taiwan) and histomorphometric indices were measured using Dinocapture 2.0 software (Dino-Eye, Taiwan). Counts used to determine the numerical densities and the volume of seminiferous tubules were made with a  $\times 40$  objective lens and the same eyepiece grid.

Total, lumen and cellular diameters ( $\mu\text{m}$ ), lumen, cellular and cross sectional area ( $\times 10^4$   $\mu\text{m}^2$ ), number of tubules (per  $5\times 5$   $\text{mm}^2$ ) and numerical density were determined randomly in 10 circular trans-

verse sections of different regions of testes (Panahi *et al.*, 2015a).

The mean seminiferous tubule diameter (D) was measured in 10 seminiferous tubules per animal by taking the average of two diameters, D1 and D2 at right angles. Cross-sectional area (Ac) of the seminiferous tubules was determined using the equation  $Ac = \pi(D/2)^2$ , where  $\pi$  is equivalent to 3.142 and D is the mean diameter of seminiferous tubules (Panahi *et al.*, 2015b). The number of seminiferous tubules per unit area (NA) was determined using the unbiased counting frame proposed by Gundersen *et al.*, (1988). Numerical density (Nv) of seminiferous tubules was the number of seminiferous tubules per unit volume and it was using the modified Floderus equation (Panahi *et al.*, 2015a):

$$Nv = NA / (D + T)$$

where NA is the number of seminiferous tubules per unit area, D is the mean diameter of the seminiferous tubule and T is the average thickness of the section ( $\mu\text{m}$ ).

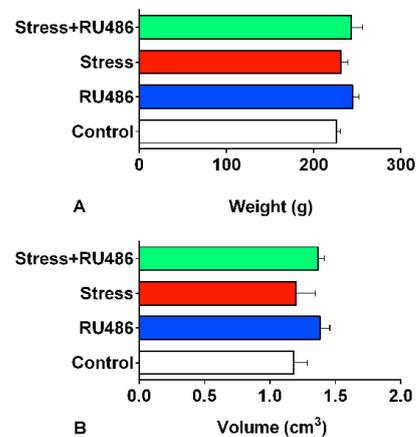
#### Statistical analysis

The data of histomorphometric indices of seminiferous tubules were subjected to Kolmogorov-Smirnov test of normality and analysed by one-way ANOVA and LSD *post hoc* test (SPSS for Windows, version 20, SPSS Inc, Chicago, Illinois). The P-value of less than 0.05 was considered to be statistically significant. Group means and their standard error were reported in the text and graphs (GraphPad Prism version 5.01 for Windows, GraphPad software Inc., San Diego, CA, USA).

## RESULTS

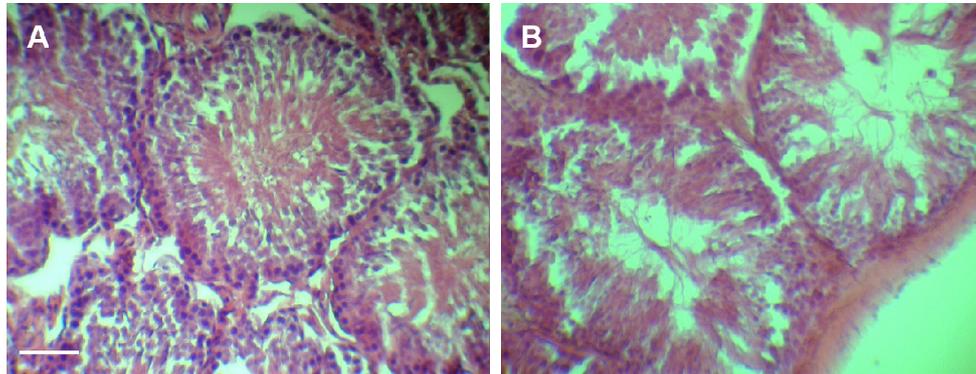
There was no significant difference in body weights (g, Fig. 1A) and volume of

testis ( $\text{cm}^3$ , Fig. 1B) between rats in all groups ( $P > 0.05$ ). Testes in all groups presented tubules with thin basement membrane and tunica propria; as well as normal germinal epithelium showing orderly progression from spermatogonia to spermatocytes with groups of spermatids and mature spermatozoa (Fig. 2A). Sertoli cells (*Epitheliocytus sustentans*) were compressed between the germinal cells and were not easily seen. The interstitium contained normal numbers of Leydig cells (*Endocrinocytus interstitialis*) (Fig. 2B).

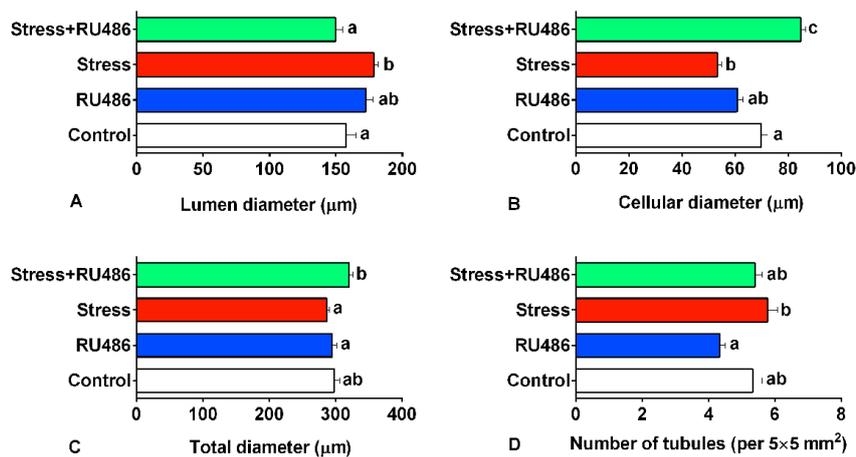


**Fig. 1.** Mean  $\pm$  standard error weight (A) and testis volume (B) in the different groups to evaluate the effects of chronic stress on testes of adult male rats.

In stereological analysis, lumen diameter of the seminiferous tubules in the stress group was higher than both control and stress/RU486 groups ( $P < 0.05$ ; Fig. 3A). Furthermore, luminal area of the seminiferous tubules in RU486 and stress groups was higher vs the stress/RU486 group ( $P < 0.05$ ; Fig. 4A). Moreover, cellular diameter and cellular area in stress/RU486 group were bigger than those of other groups ( $P < 0.05$ ; Figs. 3B and 4B). In addition, cellular diameter and



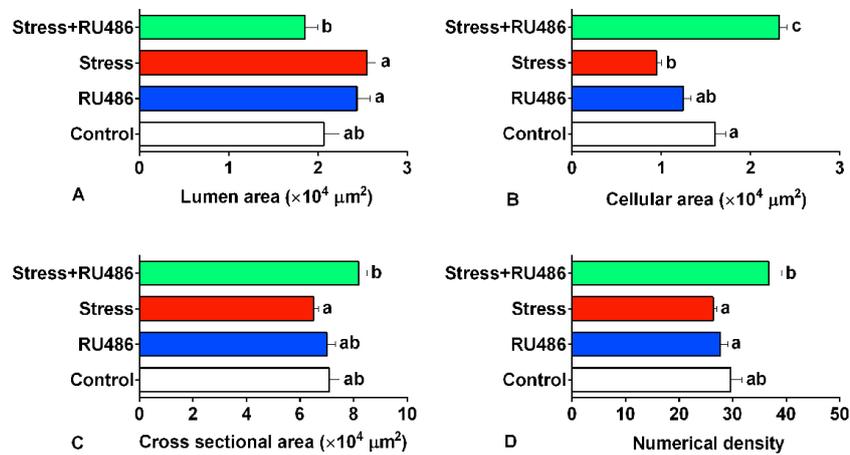
**Fig. 2.** Seminiferous tubules in control rats (A) and rats with chronic stress (B). Seminiferous tubules with thin basement membrane and tunica propria; as well as normal germinal epithelium showing orderly progression from spermatogonia to spermatocytes. H & E, bar=50 µm.



**Fig. 3.** Mean ± standard error of histomorphometric indices of seminiferous tubules in different groups to evaluate the effects of chronic stress on testes of adult male rats. A. Lumen diameter (µm); B. Cellular diameter (µm); C. Total diameters (µm); D. Luminal area ( $\times 10^4 \mu\text{m}^2$ ). <sup>a,b,c</sup> Different letters show significant differences between groups.

cellular area in stress group were inferior to the control group ( $P < 0.05$ ). Altogether, total diameter of the seminiferous tubules in RU486 and stress groups was lower than stress/ RU486 group ( $P < 0.05$ ; Fig. 3C). However, cross sectional area of the seminiferous tubules in stress/RU486 group was larger compared to the stress

group ( $P < 0.05$ ; Fig. 4C). Moreover, the number of seminiferous tubules per unit area of testis in stress group was higher than that in the RU486 group ( $P < 0.05$ ; Fig. 3D) while numerical density of the seminiferous tubules in RU486 and stress groups was lower than the stress/RU486 group ( $P < 0.05$ ; Fig. 4D).



**Fig. 4.** Mean  $\pm$  standard error of histomorphometric indices of seminiferous tubules in different groups to evaluate the effects of chronic stress on testes of adult male rats. A. Cellular area ( $\times 10^4 \mu\text{m}^2$ ); B. Cross sectional area of the tubule ( $\times 10^4 \mu\text{m}^2$ ); C. Number of seminiferous tubules per unit area of testis (per  $5 \times 5 \text{ mm}^2$ ) and D. numerical density of the seminiferous tubules. <sup>a,b,c</sup> Different letters show significant differences between groups.

## DISCUSSION

Twelve consecutive days of restraint stress caused morphometric alterations, i.e. lumen diameter and cellular area were decreased that might be the result of an impairment in the division and conversion of the spermatogonia to the spermatozoa. Consistent with our findings, Retana-Márquez *et al.* (2014) observed that epithelial surface area decreased in testis of male rats stressed for 20 days. In addition, Nirupama *et al.* (2013) reported that following restraint stress, the seminiferous tubules of rats were shrunken and contained less spermatozoa. Moreover, after chronic restraint stress in rat, decrease in both the production of maturing spermatids and concentration of spermatozoa in the cauda epididymidis was observed (Almeida *et al.*, 1998). Following the RU486 treatment of chronic stress in the

present study, thickness of germinal epithelium increased. The chronic restraint stress had a negative action on the epithelium of the seminiferous tubules; it provoked a moderate tubular hypocellularity. There are evidences that stress-induced increases in corticosterone are directly responsible for Leydig cell apoptosis (Bernier *et al.*, 1984; Dong *et al.*, 2004). Localisation of glucocorticoid receptor in the testis and Leydig cells suggests that different stressors directly affect steroidogenesis in the testis (Schultz *et al.*, 1993). Therefore, the effects of chronic stress are most pronounced on the seminiferous epithelium and restraint stress in the rats led to damage to spermatogenesis.

Following chronic restraint stress, body weights and testis volume did not change. Consistent with our results, Almeida *et al.* (1998) reported that no change was observed in the testicular weight following chronic restraint stress. In contrast,

testicular weights decreased on days 20 and 50 in stressed male rats (Retana-Márquez *et al.*, 2014). Present observations of impaired spermatogenesis with chronic stress suggested that spermatogenesis in rats might have resulted from the alteration in the seminiferous tubules milieu due to testosterone deficiency. These findings suggest that tubular cells can be a target of stress. The stress-induced suppression of spermatogenesis was blocked by RU486, an antagonist of type II glucocorticoid receptors, implying that elevation in plasma corticosterone mediates the stress-induced reduction of spermatogenesis via action on this receptor. Furthermore, recent work has demonstrated an up-regulation of hypothalamic RFamide-related peptide-3 (RFRP-3) mRNA on chronically restrained rats (Karami Kheirabad *et al.*, 2015). There is evidence that RFRP-3 neurons express functional glucocorticoid receptors by which stress can influence reproductive function (Kirby *et al.*, 2009). In addition, adrenalectomy blocks the increase in RFRP-3 expression (Kirby *et al.*, 2009). Moreover, stress decreases the sensitivity of gonads in male rats to gonadotropins. This may be caused by reducing LH receptor (Orr & Mann, 1990; 1992). It is not well understood whether the excessive glucocorticoid is responsible for decrease in spermatogenesis activity directly, or affects interstitial cells in the testis and thereby contributes to the stress-induced suppression of spermatogenesis. It should be noted that RU486 might act centrally to improve spermatogenesis.

## CONCLUSIONS

The exposure to chronic restraint stress impairs spermatogenesis in rats via glucocorticoid receptors and seminiferous tubules can be a major target of stress. An-

ti-glucocorticoid pretreatment by mifepristone (RU486) reduced the effect of chronic stress on male rat subfertility.

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