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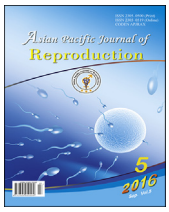

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# Testicular germ cells apoptosis following exposure to chronic stress in rats

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ABSTRACT

**Objective:** To evaluate the effects of chronic stress on testosterone hormone level and germ cells apoptosis in testes and the inhibitory role of glucocorticoid receptor antagonist, RU486.

**Methods:** Adult male Sprague–Dawley rats were randomly assigned to four groups ($n$ = 6): control; stress; RU486; stress/RU486. Animals in RU486 and stress/RU486 subjected to subcutaneous injections of 2.5 mg RU486/kg 1 h before stress session. Rats were submitted to chronic restraint stress (1 h daily for 12 consecutive days) whereas control animals were not subjected to stress. Serum testosterone assay was performed and the occurrence of DNA fragmentation in the testis sections was examined using TUNEL staining.

**Results:** Chronic restraint stress significantly induced a decrease in serum testosterone level with increase in apoptosis in spermatogonia. Systemic administration of RU486 significantly restored serum testosterone levels and attenuates stress-induced apoptosis in spermatogonia.

**Conclusion:** The restraint stress-induced change in serum testosterone levels and seminiferous tubules apoptosis closely associated with the glucocorticoid receptor.

## 1. Introduction

Exposure to psychophysical stressors has adverse effects on reproductive system. Testis performs steroidogenesis and spermatogenesis that are functionally relevant. All species respond to stress by a decrease in the levels of gonadal steroids [1,2]. Psychophysical stress experienced by adolescents can shift the onset of puberty [3]. Chronic environmental stressors are associated with low plasma testosterone in mammals [4]. Testicular germ cell apoptosis has been demonstrated in response to high temperature stress and testosterone reduction [5].

The neuroendocrine mechanisms responsible for testosterone reduction under stressful situations are not clear. A variety of factors could be involved including increase glucocorticoids, catecholamine, or decreased gonadotropins. There is evidence that stress hormones and testosterone are work against each other. Glucocorticoids appear to exert much of their effects through a member of the nuclear steroid receptor superfamily, glucocorticoid receptor, that functions as a ligand dependent transcription factor to regulate the expression of glucocorticoid receptor target genes either positively or negatively [6]. In mammals, the interstitial cells of Leydig which produce steroids, notably testosterone and dihydrotestosterone, contain glucocorticoid receptors [7]. In the presence of luteinizing hormone (LH), Leydig cells produce and secrete testosterone for spermatogenesis through cytochromes P450 enzymes [8].

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Systemic injection of exogenous glucocorticoids causes apoptosis of testicular germ cell [9]. Administration of adrenocorticotropine hormone has been shown to reduce testosterone level [10]. Sensitivity of Leydig cells to gonadotropins decrease by stress [11]. Moreover, cortisol induces reduction in plasma testosterone level, presumably by inhibiting testicular 17α-hydroxylase or 17, 20-lyase activity [12]. Although an inhibitory role of acute stress on testosterone level is well established [13,14], the effect of prolonged stress on testicular steroidogenesis has not been fully examined.

The restraint stress has been used in modeling physical and psychological stress in animal studies [15]. The current study intended to assess the role of glucocorticoids during chronic restraint stress on spermatogenesis. For this purpose, we have examined the effect of glucocorticoid receptor antagonist, RU486, on apoptosis using the terminal deoxynucleotidyl transferase mediated dUDP nick end labeling (TUNEL) method in parallel with serum testosterone concentration changes.

## 2. Material and methods

### 2.1. Animals

Adult male Sprague–Dawley rats bred in Laboratory Animal Center, Shiraz University of Medical Sciences, Iran and raised under controlled environmental conditions (temperature (23 ± 1) °C; 12 h light/12 h dark) with food and water *ad libitum* were used for experiments.

The rats were randomly divided into four groups ($n$ = 6); stress, RU486, stress/RU486, and control groups: control group consisted of unstressed animals; stress group were exposed to restraint (1 h for 12 consecutive days); RU486 group, the rats were injected subcutaneously with RU486 (2.5 mg/kg, 20 μL/rat; ab120356, Abcam Ltd, Cambridge, UK) 60 min before restraint stress was applied; stress/RU486 group, the rats were injected subcutaneously with the same dose of RU486, 60 min before the stress session. Restraint stress was performed daily for 12 d. Briefly, rats were handled before experiments. After that, animals were individually restraint for 1 h through wrapping of their upper and lower limbs by plastic cylinders (20.5 cm × 8 cm × 6 cm) which 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. All experiments were conducted during the light phase between 8 and 12 a.m. The experimental investigation was approved by Shiraz University of Medical Sciences ethics committee. Twenty four hours after the last session of stress, the animals were killed by cervical dislocation and trunk blood was collected. Serum samples were stored at −70 °C until assayed for testosterone by testosterone kit (ELISA, Diagnostics Biochem Canada Inc., Ontario, Canada) and the kit sensitivity was 0.022 ng/mL.

### 2.2. TUNEL assay

Twenty four hours after the last session of stress, the rats were weighted and anaesthetized by inhalation of ether and euthanized by cervical dislocation. Testes were removed and fixed in 4% (v:v) paraformaldehyde for 16 h at 4 °C, then an additional 8 h at 25 °C, prior to dehydration and paraffin embedding. Six-micron-thick testis sections were cut and mounted on Supersoft Plus slides (Fisher Scientific, Pittsburgh, PA). The specimens were subjected to TUNEL staining using a TACS 2 TdT-DAB in situ Apoptosis Detection Kit (Catalogue No. 4810-30-K; Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's protocol. The TUNEL assay was carried out on paraffin-embedded sections of testis. Briefly, sections were deparaffinized and dehydrated in graded concentrations of xylene and ethanol. The sections were digested with 20 μg/mL proteinase K for 15 min at room temperature. The sections were then washed and incubated with the TUNEL reaction mixture (enzyme solution and labeling solution) for 60 min at 37 °C in a humidified atmosphere. Negative controls were processed according to the same protocol, except for the incubation with TdT. TUNEL-positive cell detection was based on dark labeling as intense as, or more intense than, that of apoptotic cells observed in the positive control slide labeled simultaneously. All labeled sections were viewed at 400× magnification with an Olympus BX50 microscope, Tokyo, Japan.

TUNEL analyses were expressed by two indices. First, a histogram of the number of total spermatogonia or spermatocytes per an individual seminiferous tubule was established. Secondly, the number of TUNEL-positive cells in at least five cross sections of the tubules with apoptotic cells was counted. Then the percentages of the number of TUNEL-positive cells were calculated by dividing positive cells to total spermatogonia or spermatocytes.

### 2.3. Statistical analysis

Data of serum testosterone concentration and total and percentage of TUNEL-positive cells were analyzed by one-way analysis of variance (ANOVA) followed by LSD post hoc test (SPSS for Windows, version 20, SPSS Inc., Chicago, Illinois). All data are expressed as means and standard errors of means. Differences were regarded as significant at $P \leq 0.05$.

## 3. Results

Restraint stress (1 h/d for 12 consecutive days) significantly decreased serum testosterone levels ($P < 0.05$), and subcutaneous (sc) administration of RU486 (2.5 mg/kg in 20 μL per rat)

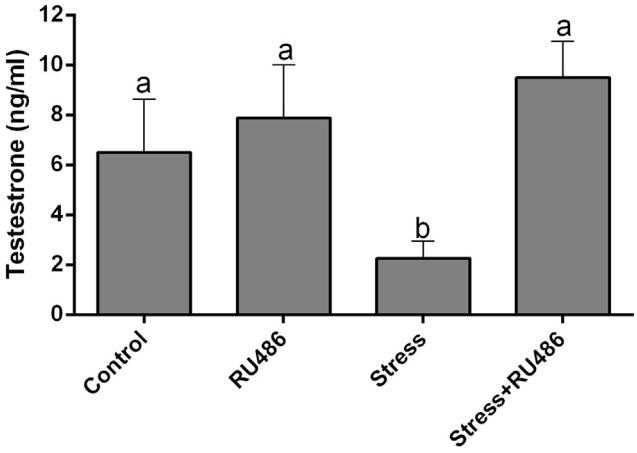


**Figure 1.** The mean and standard error of serum testosterone concentration after chronic restraint stress-induced rats ($n$ = 6).
[a,b] Different superscript letters indicate significant differences between groups. RU486, glucocorticoid receptor antagonist.

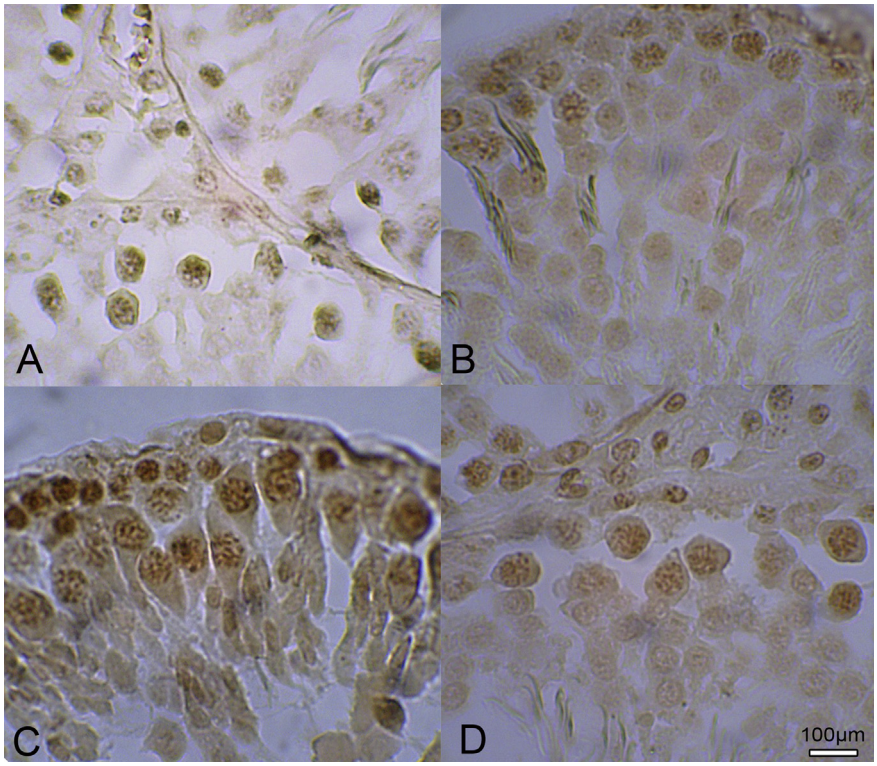


**Figure 2.** TUNEL detection of apoptotic germ cells in testes.
A, TUNEL-positive germ cells are rare in control testes (A), RU486, glucocorticoid receptor antagonist (B) and chronic stress pretreated with RU486 (D). C, high TUNEL-positive spermatogonia and spermatocytes in testes of rats which exposed to chronic stress.

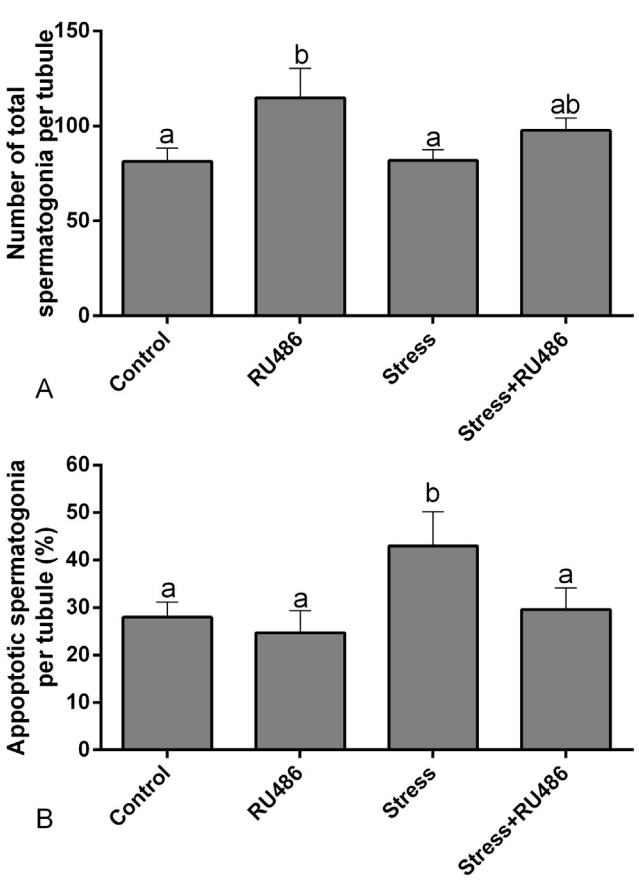


**Figure 3.** The mean and standard error of percentage of TUNEL-positive spermatogonia per seminiferous tubules cross sections after chronic restraint stress-induced rats ($n$ = 6).
[a,b] Different superscript letters indicate significant differences between groups. RU486, glucocorticoid receptor antagonist.

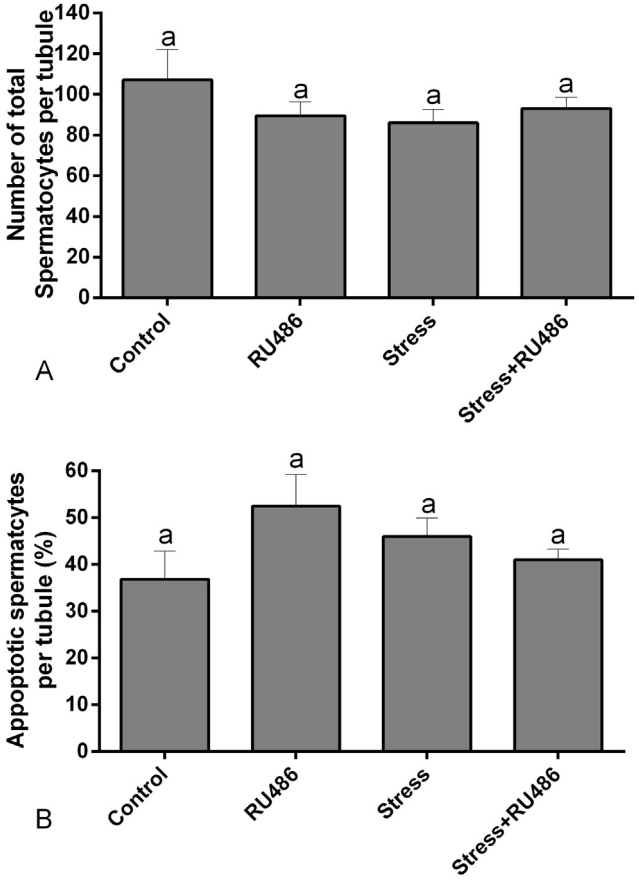


**Figure 4.** The mean and standard error of percentage of TUNEL-positive spermatocytes per seminiferous tubules cross sections after chronic restraint stress-induced rats ($n$ = 6).
[a] Different superscript letters indicate significant differences between groups. RU486, glucocorticoid receptor antagonist.

blunted the inhibitory effects of chronic stress on serum testosterone concentration (Figure 1).

TUNEL analysis of testicular tissues showed that apoptosis was confined to the basal germ cells, indicating suppression of spermatogenesis. The number of TUNEL-positive cells in the testis markedly increased in stress group (Figure 2). TUNEL-positive Leydig cells were detected in the sections prepared from the stressed animals, consistent with the low testosterone concentration in stress group. Apoptotic cells were not detected in spermatids located close to the lumen.

In the stress group, enlarged tubular lumen was seen due to the decrease in the number of germ cells. The numbers of TUNEL-positive spermatogonia per seminiferous tubule of the stress group increased relative to those of the control groups ($P < 0.05$, Figure 3). However, the stress/RU486 group showed a reduction in apoptotic cells when compared with stress group ($P < 0.05$) and to levels equivalent to those observed in control testes. The number of TUNEL-positive spermatocytes per seminiferous tubule was not increased significantly in the stress group, but an increasing trend of apoptotic spermatocyte in this group was observed (Figure 4).

## 4. Discussion

The findings of testicular apoptosis following chronic stress suggest possible role of the androgen changes in germ cell apoptosis. Various types of stress can have adverse effect on fertility and reproductive system. Heat stress causes apoptosis of spermatogonia in mice [16]. Consistent with our findings, exposure of rat to immobilization stress led to increase in testicular germ cell DNA fragmentation [17]. In addition, chronic stress by immobilization causes Leydig cell apoptosis through glucocorticoid receptor while adrenalectomised rats have increased frequencies of apoptosis in Leydig cells [18]. Repeated immobilization stress for 10 d negatively affects testicular steroidogenesis related to cAMP signaling [19]. Glucocorticoid receptor are expressed in various cell types of testis and highly conserved across species [20,21]. Increased glucocorticoids (stress-induced or exogenous administration) exert effects on reproductive system. Glucocorticoids directly inhibit enzymes involve in testosterone biosynthesis and also number of LH receptor in testis [22]. Psychophysical stress causes testicular defects by acting on the testis and decreased enzyme content [19] and testosterone production [23] or whether due to effect on the higher level in hypothalamic–pituitary axis [24], it is something all publications deal with to some extent. Results presented in this study agree with other work in animal investigations [19,25] which showed a significant decrease in serum testosterone level following chronic stress. Results of this study showed that serum testosterone levels were decreased in rats exposed to restraint and increased apoptotic spermatogonia and Leydig cells from these animals. Production of testosterone in the Leydig cells is vital for the spermatogenesis in the seminiferous tubules [26]. Corticosterone suppresses the 3β-hydroxysteroid dehydrogenase type 1 (HSD3B1) and 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3) enzymes mRNA expression in Leydig cells and through that decreased steroidogenic activity of testis [27]. Therefore, in the testis, the enzymes catalyze testosterone synthesis are linked to spermatogenesis and sperm maturation. RU486 pretreatment attenuated the stress-induced apoptosis. Such anti-apoptotic effects of RU486 on the seminiferous tubules appear to closely parallel increases in the serum level of testosterone. The anti-glucocorticoid effect of RU486 is related to masking DNA domain of the glucocorticoid receptor [28], however it appears RU486 to impair nuclear translocation of glucocorticoid receptors maybe due to inefficient release of heat-shock proteins [29].

The present study indicates that administration of RU486 prevent stress induced apoptosis of spermatogonia. Glucocorticoid receptor blockade by intra testicular administration of RU486, prevented the immobilization-induced decline in plasma testosterone levels, so the rapid changes in testosterone suggest a suppression of androgen biosynthesis directly by glucocorticoid through a non-genomic mechanism [30]. Our results are not appropriate to reveal how restraint stress decreased serum testosterone level. Based on other animal studies that serum LH levels are either unchanged or decline during stress [31,32]. This study confirms findings available in literature on the increased in germ cell apoptosis related to glucocorticoid receptor during stress. In view of these glucocorticoids could account for the apoptosis of damaged germ cells, but not spermatocyte, in order to prevent genetic abnormalities to offspring.

In conclusion, our data demonstrate that chronic restraint stress impaired testicular steroidogenesis and causes apoptosis in spermatogonia related to increase in glucocorticoid action on testes.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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