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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)

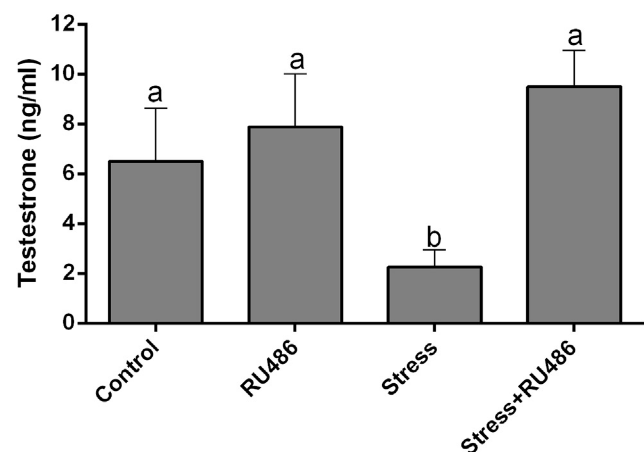

Figure 1. The mean and standard error of serum testosterone concentration after chronic restraint stress-induced rats ($n = 6$).
[a,b] Different superscript letters indicate significant differences between groups. RU486, glucocorticoid receptor antagonist.

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