

Protective effect of minocycline on dexamethasone induced testicular germ cell apoptosis in mice

M. ORAZIZADEH, M. HASHEMITABAR, L. KHORSANDI

Department of Anatomical Sciences, Faculty of Medicine, Ahwaz Jundi-Shapour University of Medical Sciences, Ahwaz (Iran)

Abstract. – Background: Apoptosis involves in testicular germ cell loss in animals and humans, and plays an important role in male fertility. Previous studies have reported neuroprotective and antiapoptotic effects of minocycline.

Aim: In this study the protective effect of Minocycline on testicular germ cell apoptosis arising from dexamethasone (Dex) has been evaluated. Dex is a widely used as a glucocorticoid (GC) agent that its apoptotic effect has been shown.

Materials and Methods: Experimental groups of 8 male mice received one of the following treatments daily for 7 days: 100 mg/kg Minocycline, 7 mg/kg Dex and 7 mg/kg Dex +100 mg/kg Minocycline. Control group was treated with 0.5 ml saline given orally for a week. Then the mice were sacrificed, and their testes processed for assessment of germ cell apoptosis (TUNEL method), the quality of spermatogenesis (Johnsen score system) and testicular sperm counts.

Results: Germ cell apoptosis were significantly increased in Dex treated mice compared with control ($P<0.01$). Spermatogenesis and the number of sperms head were significantly reduced in Dex treated mice compared with those of the control group ($P<0.01$). Treatment with Dex and Minocycline resulted in an inhibition effect on germ cell apoptosis and a significant increase in the Johnsen score and the number of head sperm compared with Dex treated mice ($P<0.05$).

Conclusion: The application of Minocycline may serve as a beneficial medication to protect germ cells against apoptosis.

Key Words:

Minocycline, Dexametasone, Germ cells, Apoptosis.

Introduction

Infertility affects an estimated 10% of couples, and in roughly half of these cases the de-

fect can be traced to the male¹. Apoptosis plays an important role in testicular germ cell development, and appears to be the shared responsible mechanism for male infertility caused by testicular exposure to elevated temperature, toxicants, radiation, chemotherapy and hormonal depletion²⁻⁶.

It is well known that glucocorticoids (GCs) induce apoptosis in testis by decreasing the testosterone levels^{7,8}. In therapeutic concentrations, GCs are strongly immunosuppressive and anti-inflammatory, which has made them one of the most frequently prescribed drugs worldwide⁹. Since GCs such as Dexamethasone (Dex) affect testis homeostasis by decreasing of testosterone level, and its high rate prescriptions, it seems essential to find a suitable medication for neutralizing or decreasing GCs negative side effects.

Minocycline is a semisynthetic second-generation tetracycline with proven safety, which is used in humans for the treatment of acne and urethritis. It may also be considered for the treatment of severe chronic inflammatory diseases, such as rheumatoid arthritis, as it exerts anti-inflammatory effects that are completely separate and distinct from its antimicrobial action^{10,11}. Minocycline crosses the blood-brain barrier and protects the neurons from a wide variety of neurodegenerative diseases¹². Minocycline delays the progression of amyotrophic lateral sclerosis (ALS) and Huntington's disease in humans and animal models¹³⁻¹⁵ and prevents the neuronal apoptosis induced by ionizing radiation, glutamate excitotoxicity and nitric oxide¹⁶.

Although there are the extensive reports of the neuroprotection of minocycline, less attention has paid on its protective effects on other tissues. In addition, since the testes also have a barrier (similar to brain) that prevents the perme-

ation of many medications, we examined minocycline as a possible agent for suppressing germ cell apoptosis.

Materials and Methods

This study was conducted according to the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Briefly, NMRI male mice weighing 25-30 g were kept in individual stainless steel cages under standardized conditions (constant temperature and humidity, 12-hour light-dark cycle).

Experimental Design

Thirty-two male mice were divided randomly into 4 groups of 8 mice each. Group M, received 100 mg/kg minocycline (Daru Pakhsh, Iran) daily by p.o. (gavage method) at 10.00 h for 7 days; group D, treated with 7 mg/kg Dex (Daru Pakhsh, Iran) by i.p. injection at 10.00 h for 7 days for 7 days; group D+M, treated with Dex (7 mg/kg) and Minocycline (100 mg/kg) at the same time; group C, treated with saline given daily by p.o. at 10.00 h for a week. One day after the final injection, the mice were sacrificed, and right testis from each animal was fixed in 10% formalin. The testis cut vertically into two pieces and embedded in paraffin and sectioned (5 μ m). In each of the two tissue blocks from each testis, five sections were used to histological assessment of spermatogenesis (hematoxylin and eosin [H&E] staining) and one section was used for deoxy-UTP-digoxigenin nick end labeling (TUNEL). The left testis was homogenized for assessment of testicular sperm head counts.

Histologic Examination of Spermatogenesis

Thirty round tubular cross-sections (with H&E staining) were studied per section with regard to the quality of the seminiferous epithelium. The Johnsen method^{17,18} was used to assess the quality of seminiferous epithelium. Johnsen's method applies a score of 1 to 10 for each tubule cross-section, according to the following criteria: 10, complete spermatogenesis and perfect tubules; 9, many spermatozoa present and disorganized spermatogenesis; 8, only a few spermatozoa present; 7, no spermatozoa but many spermatids present; 6, only a few spermatids present; 5, no spermatozoa or spermatids but many spermatocytes present; 4, only a few spermatocytes present; 3, only spermatogonia present; 2, no germ cells but only Sertoli cells present; and 1, no germ cells and no Sertoli cells present. The Johnsen score per tubule was expressed as Mean \pm SEM for each group.

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Testicular Sperm Counts

Testicular sperm head numbers were assessed by the procedure of Blazak¹⁹ to evaluate the numbers of mature elongate spermatids in the testis. Briefly, testes were homogenized in an 8-ml solution of 0.9% NaCl and 0.05% Triton X-100, and sperm heads were counted using a hemocytometer. Each sample was counted four times and averaged. To minimize error, the count was repeated at least five times for each mouse by 2 or 3 workers.

Measurement of Apoptosis

The deoxyuridine nick-end labeling (TUNEL) assay for apoptotic cell detection was performed with the In-Situ Cell Death Detection kit (POD kit, Roche, Germany). Briefly, dewaxed tissue sections were predigested with 20 μ g/ml proteinase K for 20 min and incubated in phosphate buffered saline solution (PBS) containing 3% H₂O₂ for 10 min to block the endogenous peroxidase activity. After incubating in 0.1% triton x-100 in 0.1% sodium citrate for two min on ice (4°C), the sections were incubated with the TUNEL reaction mixture, fluorescein-dUTP for 60 min at 37°C. The slides were then rinsed three times with PBS and incubated with secondary antiluorescine-POD-conjugate for 30 min. After washing three times in PBS, diaminobenzidine-H₂O₂ (DAB Santa Cruz, USA) chromogenic reaction was added to sections and counterstained with hematoxyline. As a control for method specificity, the step using the TUNEL reaction mixture omitted in negative control serial sections, and nucleotide mixture in reaction buffer was used instead. A cell was considered TUNEL-positive when the nuclear staining was brown and homogenous. Thirty seminiferous tubules on circular cross sections in each testis were evaluated. The number of TUNEL-positive nuclei per tubule was counted and expressed as Mean \pm SEM for each group.

Statistical Analysis

Statistical analysis was performed using the nonparametric Kruskal-Wallis analysis of variance test, with $P < 0.05$ considered statistically significant.

Results

Histological Assessments

The results of the Johnsen scoring are reported in Table I. In C group, normal spermatogenesis was observed and the mean Johnsen score was 9.1 ± 0.2 . M group demonstrated a normal architecture of the seminiferous tubules and intact germinal epithelium. There was no difference in the mean Johnsen score of C and M groups ($P > 0.01$). In D group, all sections of testes contain several tubules in which spermatogenesis were abnormal. The mean Johnsen score was significantly decreased ($P < 0.01$). In D+M group, most sections of testes contain a few tubules with poor spermatogenesis. The mean Johnsen score was slightly less than control group ($P > 0.01$).

Testicular Sperm Counts

The results of the number of testicular sperm heads per gram of testis are reported in Table I. The number of sperms head were significantly decreased in D group ($P < 0.01$). The sperm head numbers were significantly increased in D+M animals compared with the D group ($P < 0.01$). The difference between D+M and C groups was no significant ($P > 0.01$).

Assessment of Germ Cell Apoptosis

The results of the number of TUNEL-positive germ cells/tubule are reported in Figure 1. No detectable change was observed in the frequency of germ cell apoptosis between C and M group. Compared with C or M group, a marked increase in germ cell apoptosis was seen in D group ($P < 0.01$). The number of apoptotic germ cells in D+M group were significantly reduced in comparison to D group ($P < 0.05$). However, a signifi-

cant difference in the frequency of TUNEL-positive germ cells was found between D+M and C animals ($P < 0.05$). The decrease seen in the frequency of TUNEL-positive germ cells in D+M treated mice corroborates the results obtained from the histologic analysis. Figure 2 is the representative photomicrograph of testicular sections from mice processed for TUNEL staining. Spermatogonia and primary spermatocytes were rarely seen to undergo apoptosis in some seminiferous tubules of C group (Figure 2-A). In M group, a few numbers of seminiferous tubules were TUNEL-positive. In these tubules, TUNEL reactivity was observed only in a few numbers of spermatogonia (Figure 2-B). The majority of seminiferous tubules and all types of germ cells showed TUNEL-positive reaction in D group (Figure 2-C). In D+M group, TUNEL-reactivity was considerably lesser than D group. Primary spermatocytes showed the most reactivity. Staining pattern of spermatogonia was similar to C group (Figure 2-D).

Discussion

Glucocorticoids are extraordinary hormones that influence the activity of almost every cell in the body. They modulate the expression of approximately 10% of our genes, and are essential for life. They are also increasingly implicated in the pathogenesis of disease and produce many unwanted effects when given therapeutically²⁰.

This study demonstrated that Dex induces degeneration of germ cells and spermatogenesis defect. The Johnsen score and testicular sperm head numbers decreased significantly in the testis of Dex treated mice. The measurement of apoptosis demonstrated an increase in germ cell apoptosis after Dex treatment. The present data demonstrated that Dex induced testicular germ cell apoptosis in rats²¹. Histological assessments and the measurement of apoptosis in our study demonstrate beneficial effects of Minocycline on spermatogenesis through decreases in germ cell apoptosis.

Mark et al²² showed that Minocycline significantly prevented GnRH-A-induced germ cell apoptosis. They reported that Minocycline-mediated protection occurred at the mitochondria, involving the restoration of the BCL-2 levels and, in turn, suppression of cytochrome- *c* release from mitochondria.

Table I. Comparison of the number of testicular sperm head per gram of testis and Johnsen score between control and experimental groups.

Goups	Sperm head/testis ($\times 10^6$)	Johnsen score
C	22.71 ± 0.78	9.1 ± 0.2
M	23.32 ± 0.69	9.2 ± 0.3
D	9.43 ± 0.75	4.5 ± 0.3
D+M	20.98 ± 0.73	8.6 ± 0.1

Values are expressed as Mean \pm SEM for 8 mice, $P < 0.01$.

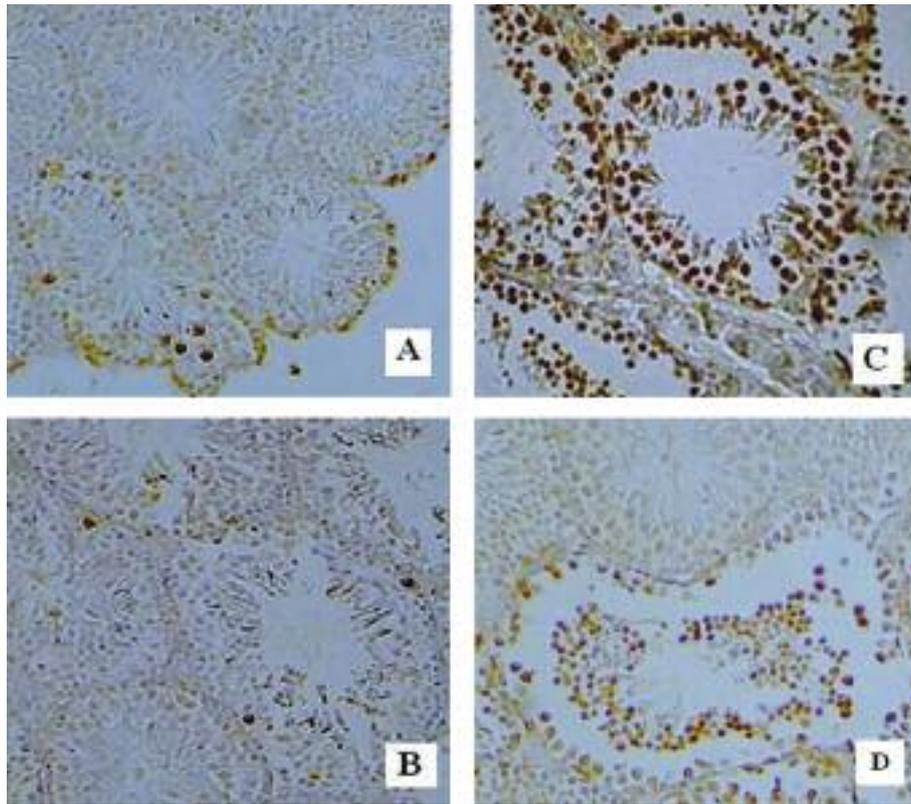


Figure 2. TUNEL stained testicular cross sections from control and experimental animals. **A**, C group (20 ×): only a few TUNEL-positive germ cells were observed. **B**, M (25 ×): TUNEL reactivity was rarely observed in spermatogonia. **D** group (25 ×): massive TUNEL-positive reaction in all types of germ cells was seen. **D**, D+M group (25 ×): TUNEL-positive germ cells were considerably decreased in comparison to D group.

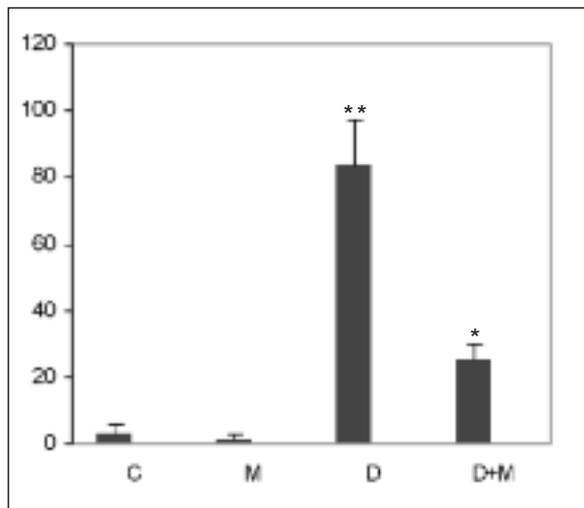


Figure 1. The number of TUNEL-positive germ cells/tubule in the testes cross sections of control and experimental groups. Values are expressed as Mean ± SEM for 8 mice, * $P < 0.05$; ** $P < 0.01$.

Minocycline protected kidney epithelial cells against apoptosis induced by hypoxia, azide, cisplatin, and staurosporine. The protection occurred at mitochondria, involving the suppression of Bax (an important proapoptotic protein) accumulation, outer membrane damage, and cytochrome-*c* release²³. The reason responsible for the protective effect of Minocycline on apoptosis induced by Dex in testicular germ cells, which revealed in this study, is not known. Possibility mechanisms are that Minocycline induces the expression of Bcl-2, an important antiapoptotic protein in testicular germ cells and suppresses of cytochrome-*c* release from mitochondria and Bax expression.

In conclusion, our findings demonstrated that Minocycline may have protective and modulating effect against Dex induced apoptosis and spermatogenic defects in mice. Further experiments are needed to clarify the mechanisms of the ef-

fect of Minocycline on different germ cell apoptosis signaling pathways. Extrapolation of these data to the human situation is not appropriate. However, this information does provide a stimulus for true clinical investigations.

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