The Effects of Prednisolone on the Testes of Rabbits: Immunohistochemical and Ultrasonographic Observations

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Abstract: Objective: The aim of the study was to investigate the side effects of long-term high-dose administration of prednisolone on the testes of rabbits. Material and Methods: Twelve white male New-Zealand rabbits were included in our study. Prednisolone was administered intramuscularly to the first six rabbits at a dose of 20 mg/kg/week and Sodium Chloride solution (0.9%) to other six rabbits for 26 weeks. Rabbits were sacrificed under anesthesia. Testes were examined with ultrasonography and seminiferous tubules (ST) and capillary of testes were examined histopathologically. Results: Ultrasonographic examination of the testes revealed heterogeneous testis parenchyma invaded by numerous hyperechoic foci, representing testicular degeneration. Histological examination revealed thickening of the ST and basement membrane (BM) of capillary in the testes of rabbits and immunohistochemical examination revealed an increase of collagen IV fibers in the prednisolone-treated groups. The average thickness of the STBM of prednisolone-treated group and control group was 225.68 and 80.96 microns, respectively. Conclusion: In our study, a significant thickening of the BM and an increase in collagen IV fibers were noticed in rabbits administered with prednisolone. These features demonstrate that prolonged administration of prednisolone may affect BM in tissues. These results indicate that ultrasonography of the testis may be useful in the differential diagnosis of testicular degeneration.

Key words: Testes, seminiferous tubule basement membrane, high-dose prednisolone, ultrasonography

INTRODUCTION

Glucocorticoids are hormones that have many effects on the human body. They bind to specific intracellular receptors in the target tissue. The glucocorticoid receptors are widely distributed through the body. Glucocorticoids generally promote intermediate metabolism, and have also anti-inflammatory action. The antiinflammatory effects on basement membrane (BM) are especially apparent. It is suggested that glucocorticoids decrease the collagen storage and immunocomplexes level by crossing over the BM. The BM contains several proteins including laminin, type IV collagen, heparin sulphate, proteoglycans, and eactin/nidogen [2].

BMs are thin sheet-like extracellular structures that compartmentalize tissues. They are substrata for cells of various organs and provide important signals for differentiation, maintenance, and remodeling of tissues. The BM functions may alter in many acquired and genetic diseases, that includes Goodpasture syndrome, an autoimmune disorder, Alport syndrome which is a form of hereditary disease that characterized by benign proliferation of smooth muscle. The major component of the BMs is type IV collagen, it is important in the pathogenesis of the Goodpasture and Alport syndrome.
Seminiferous tubule basement membrane (STBM) has a special role in spermatogenesis. Ultrastructural abnormalities of the STBM may cause infertility. Type IV collagen is a family of complex polypeptides and it is a major component of mammalian BM that has been localized to both the inner and the outer extracellular matrix (ECM) layers of the STBMs. This collagen is secreted by myofibroblasts and Sertoli cells. Collagen IV is essential for the formation of testicular cords.

The increased thickness of BM is associated with an increase in the ECM components and the irregular configuration of myofibroblasts, facing the germinal epithelium. Glucocorticoids have been used for many autoimmune or other conditions. But, there are a few studies about the effects of these drugs on the testes.

It may also be possible to use the ultrasound technique in the veterinary andrology. Important results of the ultrasonic methods may increase the diagnosis ratio of male genital organ problems. Diagnostic ultrasound is the most common imaging technique used to supplement the physical examination of the scrotum and an accurate mean of evaluating many scrotal disease. We aimed to explore the effects of the glucocorticoids on the testes of rabbits and we used ultrasonography and immunohistochemical methods at the same time for this purpose.

**MATERIALS AND METHOD**

**Animal Preparations:** In our study, New Zealand White male rabbits, (6 for study and 6 for control) age of 18 months, and weighed 2000 ±100 gr, were used and were bred on a 12 h light-12 h dark schedule with diet and water available ad libitum. Prednisolone, 20 mg/kg per week was injected to subject rabbits (Group 1) intramuscularly, every morning a.m. 08.00 o’clock. During the 26 weeks period, only normal saline was given to control group at the same time with prednisolone.

**Ultrasonography:** The rabbit is examined in the supine position. The scrotal sac is supported by the examiner’s hand. Images of both testes are obtained in transverse and sagittal planes. We used Toshiba Echocce ultrasonography (Tokyo, Japan) with 7.5 MHz linear probe. Rabbits were examined in room (soundless, quiet and lightlessness) at p. m. 7 o’clock. US light were used during the examination. Both groups were examined by per week every evening at the same time. High-frequency transducers (linear-array and convex) are commonly used because it provides, increased resolution of the scrotal contents. A direct-contact scan is performed using acoustic coupling gel. The rabbit is examined in the supine position.

**Immunohistochemical Staining:** For immunohistochemical analysis, animals were perfused through the ascending aorta with immunofix [4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and 0.01 % Triton X 100] under anesthesia (sodium pentobarbital, 100 mg/kg, i.p.) at the end of the 26 week period. At the end of the perfusion, animals’ testes were removed and post fixed in the same solution. Tissues was fixed overnight at 4°C. Testes were dissected and cut in 2 mm slices. Then, tissues were embedded in paraffin. Tissues were sectioned at 5 mm and collected on to Poly-L-lysine-coated slides. After deparaffinization, the sections were treated with 0.3 % H2O2 in methanol for 30 min for inactivation of endogenous peroxidase, then incubated in blocking solution for 10 min. Section were incubated with collagen IV (ready use, monoclonal mouse anti-human collagen IV). The immunostaining was carried out using Zymed following the manufacturer’s instructions (incubating with a mixture of biotinylated goat anti-mouse IgG for 10 min, followed by incubation with horseradish peroxidase-labeled streptavidin for 5 min.) The immunostaining was visualized by incubating with AEC (3-amino-9-ethylcarbazole). During the nucleus stain, opposite Mayer’s Hematoxylin stain was applied. Controls for the specificity of the immunohistochemistry involved omission of the primary antibody.

**Image Analysis:** Staining samples was estimated with light microscopy (Carl Zeiss Axioplan II). Images were transferred with camera (Carl Zeiss Axio MRC5) to computer. At the end of the method, each group’s STBMs diameter was measured by Zeiss Carl AxioVision and KS 300 analysis system. Basal membrane and tubule thickness was measured by microscopy (Carl Zeiss Axioplan II)!!! Staining samples was occurred macrophage, Leyding cells, fibroblast, capillary vessels!!!!.

**Anlamadım:** Statistiki analiz: Her durumun ortalama ± standart sapma değeri olarak ifade edilmiştir. Mann-Whitney U testi kullanılmış ve farklılıkların istatistiksel olarak anlamlı olduğu belirlenmiştir. P değeri (<0.05) kabul edilmiştir.

**RESULTS AND DISCUSSION**

**Ultrasonographical Findings:** Ultrasonographic observations of group 1 (Figure 1) and group 2 (Figure 2) were explained. Ultrasonographically, the testicular parenchyma was heterogeneous and mineralization foci were represented by hyperechoic areas in the Group 1 rabbits. The testicular parenchymas were ultrasonographically normal, in Group 2 rabbits.
Histological Findings: Maturation arrest, lumen loss and degeneration at the germinative epithelial cells were observed in the tissues of the group 1 rabbits. Interstitial tissue cell increase was also found. No histopathological change was observed in the group 2 with the H.E. staining.

Immunohistochemical Findings: Collagen IV level increased in the BM of ST and capillary in group 1 (Figure 4) after the immunohistochemically staining.

Fig. 1: Control group; ultrasonographical observation of rabbit testis

Fig. 2: Experimental group; ultrasonographical observation of rabbit testis. Uncharacteristically heterogeneous areas(0.50 cm²)

Fig. 3: Histopathologic features of Group2; monoclonal mouse anti-human collagen IV immunohistochemical stain. 40 x (Original magnification) st : Seminiferous tubule, gec : Germinative epithelial cell, c : Capillary l : Seminiferous tubule lumen, arrow-head: Capillary basement membrane, arrow : Seminiferous tubule basement membrane seminiferous tubules.

Fig. 4: Histopathologic features of group1; monoclonal mouse anti-human collagen IV immunohistochemical stain. 100 x (Original magnification) bl : Basement membrane, st : Seminiferous tubule, ge : Germinative epithelium.

Image Analysis: STBM thickness was measured as 5 mm in group 1 while it was 2.5 mm in group 2. By the way seminiferous tubules diameters (STDs) of group 1 were 225.68 ± 6.79 mm, and in the group 2 were 80.96 ± 7.32 mm.

Statistical Analysis: In the H.E. staining of rabbit testes, results showed that STDs were significantly decreased in group 2 (mean ± standard deviation: 80.96 ± 7.32), (t= 12.164, P= 0.0001) when compared with group 1 (mean ± standard deviation: 225.68 ± 6.79).

Discussion: In the present study, we aimed to determine effects of long-time high-dose prednisolone on the rabbit testes by immunohistochemical and ultrasonografic analysis.

Concerning spermatogenesis, Contreras et al. (16) showed that chronic supraphysiologic glucocorticoid dose administration effects gonadial function, reduces sexual glucocorticoids without changes in baseline and stimulates LH secretion. Wels et al. (17) concluded that glucocorticoids directly suppress Leydig cell glucocorticoidogenesis by decreasing gonadotropin stimulation of cAMP production and the activity of 17 alpha-hydroxilase. Glucocorticoids are inhibitors of protein synthesis. Glucocorticoids increase endurance of lysosome membranes. Hence, they decrease release of collagenase. They inhibit capillary growth, fibroblast proliferation and activity, and collagen deposition. They increase vascular permeability and fibrin deposition.
Five decades of experimental and clinical experience have changed corticoid therapy thoroughly. Glucocorticoids have two modes of action. First one is a genomic effect; it forms anti-inflammatory proteins which inhibit pro-inflammatory cytokines. This effect is initiated even by small doses, but late of onset is. The use of high doses initiates non-genomic effects through alterations of the cell membrane; these effects are found early after initiation of treatment. The adverse effects of corticoids are extremely rare, if modern application forms and therapy regimens are used. Very high doses for a short time in case of acute illness, very low doses in long-term therapy of chronic illnesses, and the use of topical substances wherever this is possible.

Interactions between Sertoli cells, peritubular myoid cells, Leydig cells, and germ cells are thought to be essential for spermatogenesis. Each of these interactions must be communicated through the ECM of the BM. In a study of adolescents with varicocele, type IV collagen displayed peritubular areas of annular thickening of immunostaining alternating with areas of interrupted or reduced immunoreactivity. Distribution of immunoreactivity appeared as irregular, wavy lines, following the contour of the peritubular basal lamina as observed with a transmission electron microscope, bordering irregular arrays of myoid cells that invaginated into the BM. These deep invaginations may be a consequence of increasing deposition of ECM components leading to thickening of the BM and decreased spermatogenesis. Some reports have demonstrated that as sclerosis of seminiferous tubules progresses, tubule diameter decreases and BM thickness increases.

A thickened BM is observed in atrophied testes. Over expression of the α1 (IV collagen) chain was correlated with an abnormally thickened BM and is related to spermatogenic dysfunction. Among the more sensitive criteria of testicular function is the minor diameter of essentially round ST. In the rabbit, diameter of the tubular wall increases considerably. The ECM of testes might play an important role in the process of spermatogenesis. Talu et al. reported that instillation of local anaesthetic and corticosteroid combination into the tunica vaginalis significantly decreased the postoperative scrotal pain, scrotal swelling, peritesticular fibrosis and prolonged the postoperative pain-free period with no apparent adverse effects upon wound healing and infection.

Currently, scrotal sonography is used for evaluation of the location, characteristics of scrotal masses, extratesticular pathologic lesions, scrotal trauma; for detection of an occult primary in patients with known metastatic disease, varicoceles and follow-up patients with previous testicular neoplasm, leukemia, lymphoma, testicular microlithiasis.

In our study, STDs significantly increased in group 1 and meantime ultrasonographically, the testicular parenchyma was observed as heterogeneous hyperechoic area. In our rabbits treated with long-time high-dose prednisolone, IV collagen was expressed very more intensely than in normal testes. The STBM in rabbits treated (group 1) with long-time high-dose prednisolone was thicker than control rabbits. In all rabbits treated with long-time high-dose prednisolone, as BM thickness increases, the germ cell numbers decreased significantly. Our findings suggest that the increase in collagen IV level at the BM of ST and capillary was due to glucocorticoids. Our study show that prednisolone may cause dysfunction in tissues with thick collagen IV level. We observed this affect in the rabbit testes. Further studies should be conducted on the affects of prednisolone on other tissues. During the study, subject male rabbits were copulated female rabbits. Consequent was copulated, leveret was died. Besides, studies should be advised fertilization and living chance of leveret.

We think that the excellent association between ultrasonographic findings and gross and histological changes suggested that testicular ultrasonography might be a valuable diagnostic tool for assessing testicular degeneration in domestic animals. Ultrasonographic examination of the testes should be considered part of the routine investigation of the male reproductive tract.

REFERENCES


