Original Article

The role of androgen receptors in erectile dysfunction due to Diabetes Mellitus: An experimental study

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Abstract

Objective: To investigate the role of serum total testsoterone levels and androgen receptors in diabetic rats with erectile dysfunction and to contribute to the development of new treatment strategies for these diseases based on a common pathogenesis.

Material and Method: In the study, we used 8 rats in the control group, 8 rats in the diabetes mellitus (DM) group, and 8 rats in the castration group. In the control group (Group 1), rats were entirely healthy male rats. In the DM group (Group 2), DM was induced by streptozotocin injection. In the castration group (Group 3), rats underwent bilateral orchiectomy. In all the groups: weight, serum total testosterone level, intracavernosal pressure, and mean arterial pressure ratio (ICP / MAP) were measured. Rats were sacrificed at the 3rd week of the study in group 1, at the 3rd week of castration in group 3, and at the 6th week of the DM formation in group 2. Pancreas, prostate, corpus cavernosum, and testicular tissue were dissected and immuno histochemical staining for androgen receptor examination was performed.

Result: The median serum total testosterone levels for group 1, 2 and 3 were 2.5±0.92, 0.46±0.17, and 0.1 ng/ml, respectively. groups 2 and 3: serum total testosterone levels were significantly lower compared with group 1 ($p<0.01$). No statistically significant difference was seen between group 2 and 3 ($p=0.41$). AR expression was compared between groups 1 and 2; no changes in the corpus cavernosum and the prostate, increased expression in pancreas langerhans islets, and decreased expression in the testes were found.

Conclusion: According to our results, serum total testosterone level decreased significantly in diabetic rats with ED. AR expression did not change in the prostate and corpus cavernosum; it increased in the pancreatic langerhans islet cells and decreased in the testes.

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Introduction

Diabetes mellitus (DM) is a public health problem with a prevalence of 6.4% in the world[1]. Patient life expectancy can increase with further clarification of the pathophysiology of diabetes and new treatment options. However, the rate of late complications related with DM also increases. Erectile dysfunction (ED) is one of these late complications and its incidence has increased over the years[2].

In clinical practice, the measurement of serum total testosterone level has an important place in the evaluation of ED, except for history and physical examination[3]. Testosterone is the main steroidal androgen responsible for reproductive system maturation with important physiological functions in the formation of male characters. Testosterone is secreted by the Leydig cells in the testes and is transported by binding to the albumin and sex hormone binding globulin in the blood. Only 2% of testosterone is present in free form. It shows its effects by binding to the androgen receptor (AR)[4]. After the androgen / AR complex interaction, AR enters the nucleus and binds to the cell deoxyribonucleic acid (DNA) which results in androgenic effects after transcription of messenger ribonucleic acid (mRNA). The presence of a healthy AR is necessary as well as the level of testosterone in the emergence of these effects. In the structural and numerical disorders of AR, if the androgenic effects of testosterone change then various diseases occur[5].

In many studies in patients with erectile dysfunction, total testosterone level has been shown to decrease[6,7]. There are also studies showing that AR expression has changed[8]. In contast, it has been shown that hypogonadism symptoms occur as a result of mutations in the AR gene and an increased susceptibility to DM by causing insulin resistance[9]. In other words, there is a close and complex relationship between disease and pathogenesis.

The aim of our study was to determine how, and at what level, the serum total testosterone level and AR expression affected DM rats with ED. Our purpose was to shed light on the treatment of diabetic patients with ED, treatment of hormonal replacement strategies, and gene therapy, which is our future treatment horizon.

Material and Method

Preparation of Study Groups

In this study, we used a total of 24 Sprague-Dawley rats weighing about 350-400 g each. The rats in the control group (Group 1, n=8) were completely healthy male rats. This group was followed for weekly blood glucose monitoring and the risk of developing diabetes during the operation was excluded. In the diabetic group (Group 2), 8 rats were injected with 60 mg / kg Streptozotocin (STZ) through an intraperitoneal route, after 12 hours of fasting[10]. Blood glucose level (Accu-Check Advantage, Roche, Germany) was measured after three days and rats with a blood glucose level above 250 mg/dl were accepted as DM[11]. The blood sugar levels of the rats in this group were measured every 7 days in order to prevent the break down of the experimental group. Rats with DM were followed for 6 weeks. In the castration group (Group 3, n=8), the rats were first anesthetized by intramuscular injection of xylazine 7 mg/kg + ketamine at a dose of 100 mg/kg. The testes were found after a 3-cm incision made from the midline to the scrotum, following appropriate field cleaning and covering. The testes were cut from the spermatic cord. Following bleeding control, the operation area was primary sutured. After anesthesia, the rats in the operating room were put back into their cages after they had awakened from anesthesia. As in the control group, this group was also followed for weekly blood glucose monitoring and the risk of developing DM during the study was excluded. Rat wound sites were followed daily.

In addition to the first 3 groups, weight, serum total testosterone level, and intracavernosal pressure to mean arterial pressure ratio (ICP/MAP) were measured in Group 2 before the formation of DM (Group 4) and in Group 3 before castration (Group 5).

Study Plan

Rats in the castration group and control group (groups 1 and 3) were anesthetized using xylazine and ketamine based anesthesia in the third week of the study. The tail of the rat was cleaned and the veins were dilated with hot water. Since the rat did not feel pain, blood was obtained by dripping and entering the tail vein with a 30-Gauge needle. Serum total testosterone level was measured in blood samples. Rats under anesthesia were euthanized with cervical dislocation. Pancreas, prostate, corpus cavernosum, and testicular tissues were dissected in the control group. The pancreatic, prostate and corpus cavernosum tissues were dissected in the castration group. In the sixth week of the study, the diabetic group was anesthetized and euthanized using the protocol of the previous groups. Blood samples were taken from the rats in this group for hormonal and biochemical examination. The pancreas, prostate, corpus cavernosum and testicular tissues were dissected. All tissues were placed separately in tubes containing 10% formaldehyde. In this study, the groups were examined using coding to prevent possible bias.

Evaluation of Erectile Function in Rats

At the beginning of the study, all the rats in group 1, followed by the rats in groups 2 and 3, were anesthetized by intramuscular injection of xylazine 7 mg/kg + ketamine 100 mg/kg 1 day prior to the sacrification. The trachea was cannulated to provide air circulation. For the mean systemic arterial pressure measurement, the carotid artery was cannulated (PE-50, polyethylene tube). Systemic arterial pressure was measured. The left jugular vein was cannulated for fluid replacement. After the penis shaft was degloved from the skin and subcutaneous tissues, the ischiocavernous muscle was dissected and reached the corpus cavernosum. A 25G needle filled with 250 U/ml heparin was connected to the PE-50 tube and entered into the corpus cavernosum. Subsequently, the pressure transducer was ligated and intracavernosal pressure was measured. The bladder and prostate were described after an incision made from the midline of the abdomen. Major pelvic ganglion and cavernosal nerves were found at the posterolateral aspect of the prostate. Bipolar electrical stimulator was placed around the cavernosal nerve. Cavernosal nerve stimulation was performed at a frequency of 15Hz with a 30-second wave width and 5-Volt electrical stimulation. The stimulation period was set to be 1 minute following a rest period of 2-3 minutes. The ratio of the highest intracavernosal pressure detected during the measurement of the mean arterial pressure (ICP/MAP) was calculated[12].

Tissue Preperation

The preparations were washed for 30 minutes in Phosphate-buffered saline (PBS). Then dehydration was carried out for 80 minutes with 80% ethanol, 60 minutes 95% ethanol I, 60 minutes 95% ethanol II, 60 minutes 100% ethanol I, 60 minutes 100% ethanol III respectively. Apply 30 minutes with xylol I and 30 minutes with xylol II in the transperancy phase. After these phases, the components taken into the paraffin at 60°C and kept in paraffin I for 30 minutes and in paraffin II for 30 minutes. The next day the pieces were blocked (13,14). Preparations were made in 5 μm sections on microtome (Leica RM 2145) for light microscopic examination. Tissue sections were taken on the slides. All the sections were kept in the oven at 37°C for a night to adhere on the slides. The sections were kept in 62°C incubator for 120 minutes and then they were kept in xylol 2 for 60 minutes and removed from the paraffin for 60 minutes[13].

Immunohistochemistry

Sections were kept in the oven at 60°C overnight. After cooling, sections were kept in xylol 3 times for 30 minutes. Sections were kept in ethyl alcohol, 95%, 80%, 70% and 60%, respectively for 2 minutes. Sections were washed with distilled water

for 10 minutes and contours were plotted with a bounding pen [peroxidase anti peroxidase (PAP) pen]. The slides were washed in PBS 3 times for 5 minutes, transferred to 0.01 M citrate buffer (Sigma-aldrich, S1804) , and heated in a microwave oven (30 minutes) for antigen retrieval[15]. After cooling, the samples were incubated in 0.25% Triton X-100 (Sigma-aldrich, Triton™ X-100) PBS for 5 minutes. Endogenous peroxide blockage was performed and diluted with distilled water for 5 minutes. After washing 5 times with PBS solution for 5 minutes, block solution (or 1% BSA-PBS) (non-immune serum) was dropped on the sections for 60 minutes. The block solution was removed, then 50 μl primary antibody (Bioss Androgen Receptor antibody bs-12472R, 1/100 dilution), which had been properly diluted, was dropped and kept in a closed humid box at 4°C overnight. Washed 3 times with PBS solution for 5 minutes. The biotinylated secondary antibody (ScyTek, SPH125) was incubated at room temperature for 30 minutes in a closed humid box. Then washed 3 times with PBS solution for 5 minutes. The streptavidin-labeled secondary antibody was incubated at room temperature for 30 minutes in a closed humid box. Washed 3 times with PBS solution for 5 minutes. A solution of 3,3-diaminobenzidine (DAB) (ScyTek, ACK 500) was dropped, incubated for 8 minutes in a closed humid box and washed again with PBS solution 3 times for 5 minutes and with distilled water. Checked with Mayers' hematoxylen (Merck Millipore, 109249) for nucleus staining for 2 minutes. Washed with distilled water. Sections were kept in ethyl alcohol, 80%, 95%, 100%, respectively for 1 minute. The sections were cleared 2 times 5 minutes after drying. Sections were covered with medium[16]. The stained sections were examined with the Olympus BX-51 photomicroscope and photographs were taken with the Olympus C-5050 camera. The sections were examined under 40 and 100 magnification. The intensity of staining was based on the study of Mori et al[17].

Blood Samples

Serum total testosterone level was measured using the ELISA (enzyme-linked immunosorbent assay) method and the total testosterone kit (Beckman Coulter, DSL104000) with measuring instrument (Unicel, Dxl800). While the sensitivity of the kit is 0.1 ng/mL, the measurement range is 0.1-16 ng/mL.

Statistical Analysis

All data were analyzed using the Statistical Analysis for Social Sciences for Windows (SPSS, Chicago, USA) version 18.0. In our study, all values were calculated as median ± standard deviation and at least-maximum values. Data were calculated using one-way ANOVA, the Bonferroni post-hoc test. $p<0.05$ was considered statistically significant.

Result

The ages and weights of rats in groups 1, 2 and 3 are summarized in Table 1. The weight of the rats in group 2 was significantly lower compared to group 1 and 3 ($p<0.001$). There was no significant difference between the weight of the rats in groups 1 and 3 ($p=0.25$).

The median serum total testosterone level of all the groups are shown in Table 2. There was no statistically significant difference in serum total testosterone level between group 1 and 4 and group 1 and 5 ($p>0.05$). Serum total testosterone level was significantly lower in group 2 and 3 compared to the other groups ($p<0.01$). There was no statistically significant difference in serum total testosterone level between groups 2 and 3 ($p=0.41$).

When the ICP/MAP ratios of group 1, 2 and 3 were compared, the ICP/MAP ratio in group 1 was found to be significantly higher than in the other 2 groups ($p<0.001$). When Group 2 and 3 were compared, the ICP/MAP measurements of group 2 were significantly higher than group 3 ($p=0.003$). The ICP/MAP measurement results are shown in Figure 1.

Table 1. Median age and weight values of rats.

	Group 1 (N=8)	Group 2 (N=8)	Group 3 (N=8)
Age (day)	115.5±4.01 (110-121)	143±1.58 (140-145)	134±1.85 (132-138)
Weight (gr)	362.5±15.46 (342-385)	295±12.52 (275-310)	351±13.37 (326-371)

Table 2. Median serum total testosterone levels' of rats.

	Group 1 (N=8)	Group 2 (N=8)	Group 3 (N=8)	Group 4 (N=8)	Group 5 (N=8)
Median serum total Testosterone level (ng/mL)	2.50±0.92 (1.5-4.6)	0.46±0.17 (0.36-0.89)	0.1	2.60±0.37 (2.05-3.2)	2.55±0.34 (2.05-3.12)

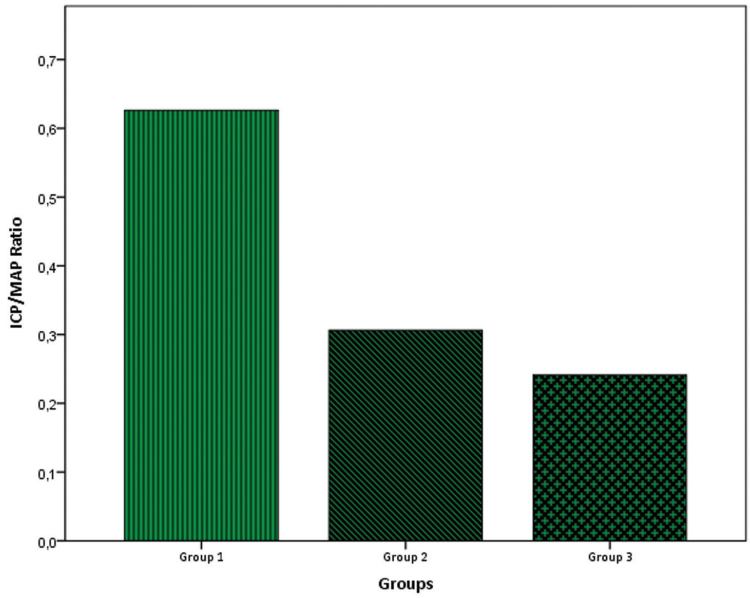

Figure 1. ICP/MAP ratios of study groups.

AR staining in testis, corpus cavernosum, prostate and pancreatic tissues are shown in Figure 2. AR expression was found to be very weak (+) in the corpus cavernosum in group 1. Moderate (++) staining was observed in the connective tissue and smooth muscle cells. In group 2, AR expression in the penis corpus cavernosum was found to be very weak (+). Moderate (++) staining was observed in the connective tissue and smooth muscle cells. In group 3 corpus cavernosum, AR expression was very weak (+). Connective tissue and smooth muscle cells showed moderate (++) staining. No significant difference was observed between AR staining in all 3 groups.

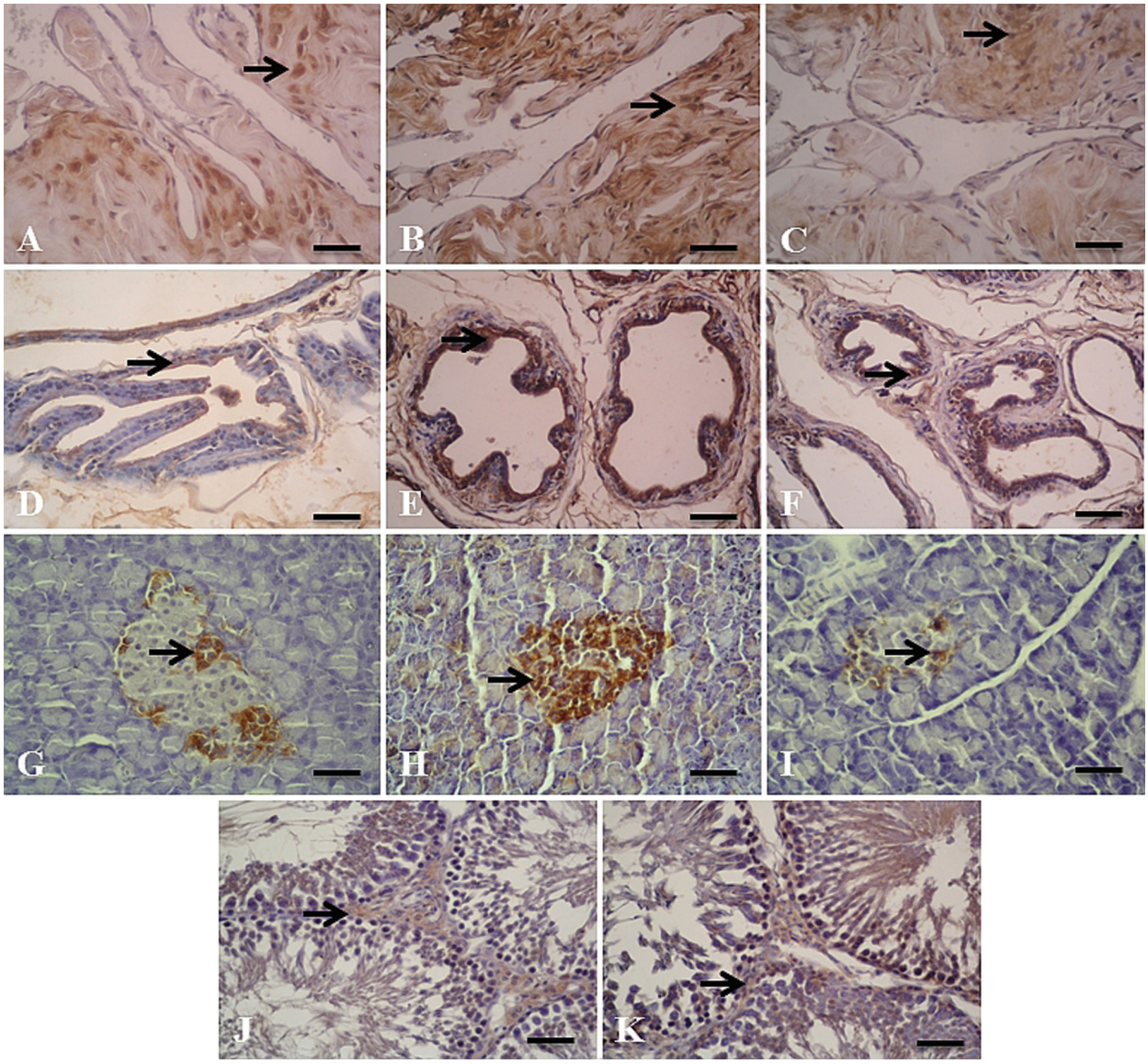

Figure 2. Images of the immunohistochemical staining of the androgen receptor at 40X magnification. Androgen receptor marked with arrow.
(A) Group 1; corpus cavernosum; (B) Group 2; corpus cavernosum; (C) Group 3; corpus cavernosum; (D) Group 1; prostate tissue, (E) Group 2; prostate tissue, (F) Group 3; prostate tissue, (G) Group 1; pancreatic tissue, (H) Group 2; pancreatic tissue, (I) Group 3; pancreatic tissue, (J) Group 1; testicular tissue, (K) Group 2; testicular tissue

AR expression was strong (+++) in Group 1 and 2 prostate gland pseudostratified prismatic epithelium and very weak (+) in group 3. No staining was seen in the connective tissue.

AR expression was detected in only the langerhans islet cells in the pancreas. AR staining was moderate (++) in group 1 pancreatic tissue at the margins of the langerhans islet cells. In group 2, AR staining was found to be strong (+++). In group 3 pancreatic tissue, a very weak (+) staining was detected on the margins of langerhans. In group 1 and 3, the sizes of the langerhans islet cells were normal, whereas in group 2, the size of the cellswas diminished.

Group 1 testis tissue, spermatogenic series cells also knownas spermatogonium show weak (+), spermatocytes and spermatitis show moderate (++) and Leydig cells show strong (+++) staining. In group 2 testis tissue, moderate (++) staining on the spermatogonyum cell line, weak (+) staining in spermatocytes and spermatitis and the Leydig cells, which are found in the intersititium, show extremely weak (+) staining. In general, AR expression in group 2 was found to be decreased compared to group 1.

Discussion

DM is an important health problem affecting approximately 6% of the world population[1]. It is estimated that it will affect about 10.1% of the world population by 2035[18]. Due to the prolonged survival of these patients, late complications are also increasing. According to the report of the Massachusetts Male Aging Study (MMAS), men with DM have a 75% risk of developing ED during their lifetime. In the same study, serious ED was found in approximately 28% of male patients treated with DM and this rate is approximately 3 times higher thanin healthy men[19]. In a study on rats by Vernet et al, erectile functions were shown to be lost in 95% of rats after DM. They also linked this loss to the decrease in penile nitric oxide synthetase (NOS) activity and level[20]. In our study, in accordance with the literature, erectile function was preserved completely in the control group rats, while lost in all rats in the DM group.

Another important cause of ED is hypogonadism. Numerous studies have shown the relationship between hypogonadism and DM. Androgens are critical for the development and survival of penile erectile tissue. In animal experiments, cavernosal parasympathetic fibers with endothelial NOS (eNOS) have been shown to depend on testosterone[21]. After androgen deprivation, apoptosis in penile erectile tissues increased, atrophy developed and ultrastructural form deteriorated[22,23]. Corona et al.[6] found low levels of testosterone in 24.5% of patients with ED and DM. Ghazi et al[24] in their study, 391 diabetic men with ED were examined and the total testosterone level was low in 33.2% of these patients. Many other studies have shown that low total testosterone level coexits with erectil dysfunction, ranging from 20-51%[25]. In a study by Chamness et al.[26], it was shown that NOS activity was reduced by about 45% in the penis of castrated rats, and this decrease was reversed by testosterone replacement therapy. In our study, serum total testosterone level was significantly lower in the DM group than the control group ($p<0.01$). The median serum total testosterone level was 2.5 ± 0.92 ng/mL in the control group, although with approximately 80% lower values in the diabetic group (0.46 ± 0.17 ng/mL). There was no significant difference in the serum total testosterone level between the castration group and the diabetic group ($p=0.41$). In the diabetic rats with ED, mean serum total testosterone level was almost reduced to the castration level. Therefore, even if all the other factors (such as endothelial dysfunction, peripheral neuropathy etc.) are preserved in diabetic rats, this decrease in the serum total testosterone level alone may cause ED. These results show that the serum total testosterone level is important inthe diagnosis, follow-up, and treatment of ED in patients with DM.

A healthy AR is required for testosterone to demonstrate its effects[27]. There is a CAG repeat region consisting of 10 to 35 repetitions in the AR gene exon 1 region. In studies, an inverse relationship was found between the number of repetitions in this region and the activity of AR[28]. In light of the literature, it has been shown that AR function and structure should be normal, as well as serum total testosterone level in order to maintain penile erectile function. Suzuki et al.[29] show the intensity of AR in the prostates of castrated rats. On the first day, AR expression decreased to 50% and was only 4% by the end of the 7th day. This study shows the importance of androgens in maintaining the presence of AR. In our study, AR expression was found to be strong in the pseudostratified prismatic epithelium of the prostate in the control and DM group. Although there was no decrease in AR staining against 6-week DM and low testosterone levels in the prostates of the rats in the DM group, AR expression was found to be very weak in the castration group pseudostratified prismatic epithelium of the prostate gland. One of the weaknesses of our study is its lack of an examination of AR functions. However, it can be predicted that AR functions will decrease due to low serum total testosterone levels in the diabetic group whenthe AR number is not decreased.

In a study by Yamanaka et al.[30], they examined the transcription of AR mRNA in a diabetic rat penis. No significant difference was found in AR mRNA transcription compared to the control group at the 4th and 8th week of diabetic rats with ED. However, AR mRNA transcription increased in rats with DM under insulin treatment. The expression of AR inan immunohistochemical study was similar in the group with DM and the control group. Takane et al.[31] studied AR in the rat's penis and showed that AR expression did not change in penile tissue after castration. In our study, AR expression in all 3 groups was very weak in the corpus cavernosum. Connective tissue and smooth muscle cells were found to be moderate. There was no significant difference between the 3 groups when compared to each other, which are similar and low. In light of our findings, it can be concluded that AR density in penile cavernosum, which is the primary responsible tissue, did not affect penile erectile functions.

As in many tissues, it is possible to find the androgen receptor in the pancreas. In a study by Li et al.[32], the degree of AR expression during DM was compared with the apoptosis in pancreatic beta cells in diabetic rats. The authors concluded that AR may be responsible for apoptosis of the pancreatic beta cells. Morimoto et al.[33] reported in their study that testosterone has a protective effect on pancreatic beta cells and that it is mediated through AR. In our study, AR expression in the pancreas was seen only in the langerhans iŞlet cells. AR expression was moderately observed in langerhans islet alpha cells in the control group. In the diabetic group, strong expression was observed in both alpha and beta cells. In the castration group, a very weak expression was observed in the alpha cells. Langerhans islet cells were normal in the control and castration groups, whereas in the diabetic group, cell size decreased. In light of these data, unlike previous studies, the expression of AR was strong inthe pancreatic langerhans islet cells in DM. In addition, as a result of the decrease in AR expression, we can say that the protective effect of AR disappeared while the risk of developing DM was increased in the castration group.

Conclusion

In the literature, the role of AR and testosterone level have been discussed in numerous studies in order to clear up the association between DM and ED. DM, MS, prostate cancer, ED, spinal bulbar atrophy, and breast cancer have a common pathogenesis as seen in the etiology of diseases, and these diseases can occur as a result of structural or functional changes in AR. Although serum total testosterone levelis normal in patients with DM, the occurence of ED shows the importance of AR function in the common

pathogenesis.

According to our study, which aimed to investigate the multiorgan effects of AR, it was found that serum total testosterone level decreased significantly in diabetic rats with ED. AR expression did not change in the prostate and corpus cavernosum; it increased in the pancreatic langerhans islet cells and decreased in the testes. Our study is an important study because it is based on the common pathogenesis of ED and DM at the hormone and receptor level. However, the lack of measurement of bioavailable testosterone level and AR function are the deficiencies of our study. Further studies and animal experiments are needed to elucidate the relationship between ED and DM.

Conflict of interest

The authors declare that they have no conflict of interest.

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