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The Effects of Sildenafil Citrate and Vitamins A, C and E on Testicular Damage in Alloxan-Diabetic Rats

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Abstract: Sildenafil citrate is an active ingredient used successfully in the treatment of erection disorders caused by a variety of factors. The purpose of this study is to research the effects of Sildenafil citrate and vitamins A, C and E on testicular degeneration in rats with alloxan diabetes. The rats were divided into II groups; healthy control and diabetic groups. The diabetic rats were divided into X groups with ten animals in each group. Group I consisted of control diabetic rats that were given only distilled water, Group II was the diabetic group treated with glibenclamide; Group III was the diabetic group treated with insulin, Group IV was the diabetic group treated with Sildenafil citrate, Group V was the diabetic group treated with Vitamin A, Group VI was the diabetic group treated with vitamin C, Group VII was the diabetic group treated with vitamin E, Group VIII was the diabetic group treated with Sildenafil citrate and vitamin A, Group IX was the diabetic group treated with Sildenafil citrate and vitamin C and Group X was the diabetic group treated with Sildenafil citrate and vitamin E. Testis tissue samples were collected on the 3, 7 and 15th days of treatment in control and diabetic groups. In the histopathological examination of the testicles of diabetic rats, the seminiferous tubules and interstitium of testes in the control groups were normal and the complete spermatogenic cells in the seminiferous tubules were healthy and uniformly arranged. Severe degeneration and tubular atrophy was observed in the seminiferous tubules on the 3, 7 and 15th days in the group with induced alloxan diabetes given only distilled water (Group I). The lesions on day 3rd in the diabetic group given glibenclamide (Group II) were similar to those in the diabetic control group given only distilled water. All of the tubules had been affected by the 3rd day in group three which was given insulin. A small number of spermatogenic cells and giant cells were observed in some tubules. However, almost al of the degenerated tubules had healed on the 7 and 15th days. Even though, recovery on day 15th was very similar in Groups IV-X recovery was much more pronounced in Groups VIII-X beginning from day 3rd.

Key words: Alloxan, diabetes, infertility, sildenafil citrate, testes, rat, histopathology, TUNEL

INTRODUCTION

Diabetes Mellitus (DM), a major endocrine disorder and growing health problem in most countries is now emerging as a deadly disease (Hamden *et al*., 2009). There is increasing evidence that diabetes is closely associated with male reproductive dysfunction. Pathological changes related to diabetes have been observed in Leydig cells, interstitial connective tissue, seminiferous tubules, tunica albuginea and testes.

Compared with non-diabetic people, male diabetic patients show an increasing incidence of Erectile Dysfunction (ED), hypogonadism and infertility. Experimental diabetic animals tend to suffer from testicular dysfunction such as reduced sperm count, low serum testosterone levels and decreased fertility (Zhao *et al*., 2010; Mallick *et al*., 2007; Cai *et al*., 2000; Steger and Rabe, 1997; De Tejada *et al*., 1989). ED is the inability to achieve the erection required for sexual activity (NIH Consensus development panel on impotence). ED frequency in patients with DM is reported to vary between 35 and 75% (McCulloch *et al*., 1980). A number of mechanisms are affected in men with DM, contributing to the development of ED. The fundamental reasons for

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this occurrence are reported to be caused by vascular and nerve-related disorders (Murray *et al*., 1987; Utkan *et al*., 2001). Sildenafil citrate has bee n shown to be highly effective in the treatment of erectile dysfunction and it is used to treat impotence of various etiologies with good tolerance (Morales *et al*., 1998). Nitric oxide synthase (Lewis *et al*., 1996) and two distinct PDE (Phosphodiesterase) isoforms (PDE_1 and PDE_4) are present in human sperm cells (Fisch *et al*., 1998). Sildenafil is a specific and potent inhibitor of cGMP-specific PDE type-5 which is the predominant PDE isoenzyme responsible for the degradation of cGMP in the corpus cavernosum and has also minor inhibitory effects on PDE_6 and PDE_1 activities (Morales *et al*., 1998). Sexual stimulation is mandatory for sildenafil to increase nitric oxide production and stimulate cGMP production which in turn causes trabecular smooth muscle relaxation, dilatation of the cavernosal arteries, increased intracavernosal pressure and penile erection (Fabbri *et al*., 1999; Goldstein *et al*., 1998). ED can be successfully treated with sildenafil (Aversa *et al*., 2000).

Vitamin-A (Retinol) is required for male and female reproduction as well as to support many developmental processes. In the male, meiotic entry of germ cells occurs after birth and throughout adulthood. The vitamin A metabolite, all-trans Retinoic Acid (atRA), supports many functions of the vitamin including cellular growth and differentiation (Clagett-Dame and DeLuca, 2002). Severe vitamin A deficiency leads to reproductive failure in males. In male rats, spermatogenesis is blocked and most germ cells degenerate (Thompson *et al*., 1963).

Vitamin C (Ascorbic acid): The role of ascorbic acid in the regulation of sexual activity in females is significant as it has been observed that the inhibition of the estrus cycle in alloxan diabetes is corrected with Vitamin C (Deb and Chatterjee, 1963).

Vitamin E (Tocopherol): In recent years it has been shown that the most important factor increasing the production of free radicals in diabetes is the hyperglycemic state which can induce such damage as lipoperoxidation (Hamden *et al*., 2009). Vitamin E provides significant protection in the testes of rats against oxidative damage caused by diabetes (Naziroglu and Simsek, 2009).

Programmed cell death which is known as apoptosis, plays a critical role in normal spermatogenesis. Germ cell apoptosis occurs in normal physiologic spermatogenesis. However, the incidence of germ cell apoptosis increases when accompanied by non-physiological stresses such as exposure to various toxins and diabetes (Cai *et al*., 2000; Sinha-Hikim and Swerdloff, 1999). TdT-mediated dUTP Nick-End Labeling (TUNEL) is being used routinely to detect apoptotic cells in various tissues. The technique uses TdT to catalyze template-independent addition of digoxygenin-dUTP and dATP to 3'-OH ends of fragmented DNA generated by internucleosomal cleavage. The incorporated nucleotides form a random heteropolymer of digoxigenin dUTP and dATP. An anti-digoxigenin antibody conjugated to peroxidase is then added which generates an intense signal for chromogenic substrates. In this study, researchers compare the histopathological and apoptotic effects of Sildenafil citrate, Vitamin A, C and E on the testes of diabetic and non-diabetic male rats. On the 3, 7 and 15th days after administering alloxan and sildenafil, Vitamin A, E and C treatment, the histopathology of the testes was closely scrutinized and the levels of plasma testosterone were evaluated.

MATERIALS AND METHODS

Animals and groups: A total of 100 Swiss albino rats 250-300 g obtained from the central animal clearinghouse at Ataturk Univesity were used for the study in 2009. Rats were kept in individual cages maintained under standard conditions (temperature: 22±2°C; humidity: 60±5%; 12 h dark/light cycle) and fed with standard pellets and water *ad libidum*. The handling of the animals was approved by the local Ethics Committee for the care and use of laboratory animals. Prior to each procedure in the study, the animals were not fed for 18 h as required by the study procedure.

The rats were divided into X groups with ten rats in each group. The groups were arranged as follows; Group I: diabetic+distilled water (control); Group II: Glibenclamide reference; Group III: insulin reference; Group IV: Sildenafil citrate; Group V: Vitamin A; Group VI: Vitamin C; Group VII: Vitamin E; Group VIII: Vitamin A+Sildenafil citrate; Group IX: Vitamin C+Sildenafil citrate; Group X: Vitamin E+Sildenafil citrate.

Alloxan-induced diabetes: Diabetes was induced by the intraperitoneal injection of alloxan monohydrate (120 mg kg^{-1}) dissolved in distilled water (5%) for 3 consecutive days. Diabetes was confirmed 3 days after the administration of the last alloxan dose by determining the blood glucose concentration (day 6th). Only animals with blood glucose levels >250 mg dL^{-1} were used (Jaouhari *et al*., 2000).

Chemicals: Glibenclamide (Gliben tbl. Nobel) and Alloxan (Alloxan (5.6-dioxyuracil) monohydrate, Sigma (Steinheim, Germany) were used. Sildenafil citrate-Viagra™ was purchased from Pfizer (Pfizer Inc., New York). Glibenclamide and Alloxan were dissolved in serum physiologic.

Histopathological examination: Testicular sections from the rats were fixed in Bouin's solution for 24 h and were embedded in paraffin wax. About 4 μm thick sections were stained with Hematoxylin-Eosin (HE) for histopathological examination.

Apoptotic cells in the testis tissue were detected by terminal deoxynucleotidyl Transferase-mediated dUTP Nick End-Labeling (TUNEL) staining using a commercial ready-to-use kit (*In situ* cell death detection kit, AP, Roche Diagnostics, Germany).

Tissues from each rat were deparaffinized and dehydrated. Afterwards, the sections were incubated sequentially with cytonin (Trevigen) at 37°C for 30 min, hydrogen peroxide 3% for 5 min at room temperature, 200 mL of TUNEL mixture (TdT and label solution) at 37°C for 60 min and with POD converter at 37°C for 30 min. The sections were then treated with 3-Amino-9-Ethylcarbosol (AEC) for 5 min washed with phosphate buffer (pH 7.4) and counterstained with Mayer's hematoxylin. Control sections were stained using the same procedure but DNAse was used instead of the TUNEL mixture.

RESULTS

In the present study, the seminiferous tubules and interstitium of testes in control groups were normal and the complete spermatogenic cells in the seminiferous tubules were healthy and uniformly arranged (Fig. 1a). Severe degeneration and tubular atrophy was observed in the seminiferous tubules on the 3, 7 and 15th days in the group with induced alloxan diabetes given only distilled water (Group I).

In this group with severe destruction of the seminiferous tubules, there were multinucleated giant cells in tubules, vacuolization in cytoplasms of sertoli cells, degeneration, sloughing and depletion of the germ cell (Fig. 1b, c and Table 1).

A reduction in the diameter of the tubules was observed in relation to the degeneration of the seminiferous tubules. Most of the seminiferous tubules were either partially or totally atrophied. Many of the undamaged seminiferous tubules contained degenerated spermatids. In the diabetic group to which glibenclamide was administered (Group II), the lesions on day 3rd were similar to those in the diabetic control group given only distilled water. On the 7th day, however degenerated and partially healed tubules were found together (Fig. 2a). The few cells remaining in the degenerated tubules were TUNEL-positive apoptotic cells. The lumen of some of these cells contained degenerated spermatid and giant cells while some of them had no sperm cell at all. Very few tubules with severe lesions remained on day 7 and 15th as recovery was more noticeable (Fig. 2c).

On day 3rd almost all of the tubules had been affected in Group III which was given insulin. While some tubules had a limited number of spermatogenic cells, in most of them no spermatogenic cells remained and giant cells had formed (Fig. 3a, b). However, almost all of the tubules had healed on the 7 and 15th day. The diabetic rats in Group IV which were given sildenafil citrate had

Table 1: Severity of lesions in the study groups by day

		Days		
Groups	Compounds	3rd	7th	15th
I	Distilled water	+++++	+++++	+++++
II	Glibenclamide	++++	+++	++
III	Insulin	++++	+++	++
IV	Sildenafil citrate	++	+	-
V	Vitamin A	++	+	+
VI	Vitamin C	++	+	+
VII	Vitamin E	++	+	+
VIII	Vitamin A+ Sildenafil citrate	+	+	-
IX	Vitamin C+ Sildenafil citrate	+	+	-
X	Vitamin E+ Sildenafil citrate	+	+	-

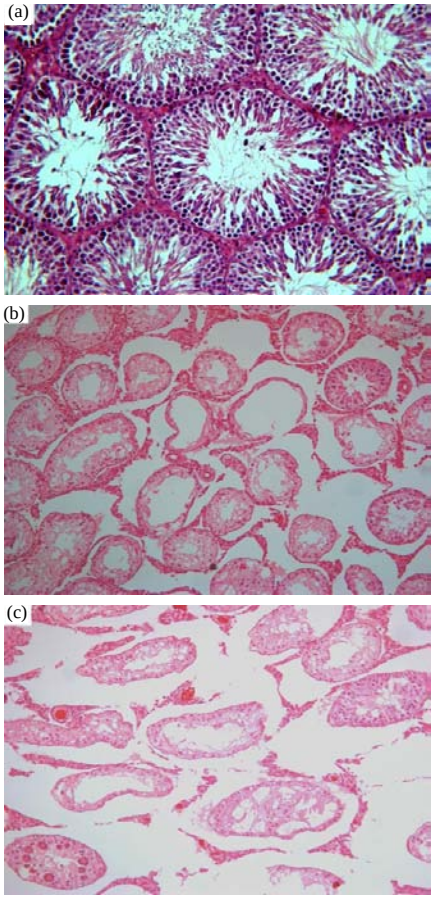

Fig. 1: a) Normal, x20; b): I group 7th day, atrophy and degeneration of tubules, x10 and c): I group 7th day, degeneration and giant cell, x10

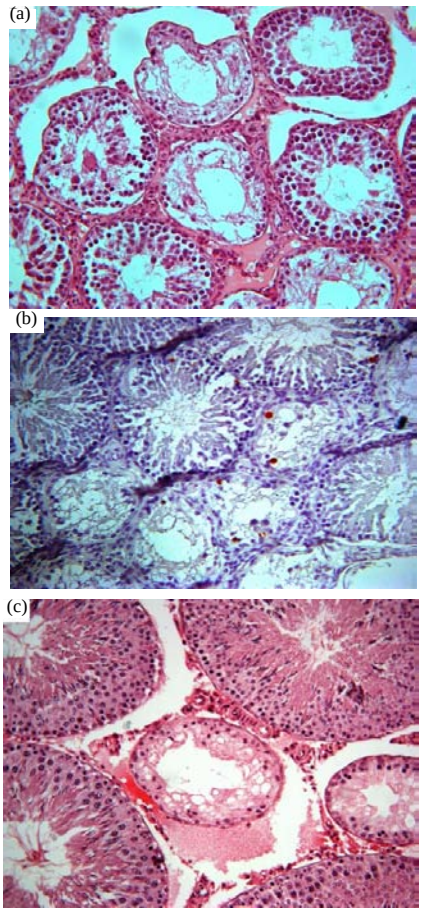

Fig. 2: a) II group, 3rd day, x20 ;b): II group, 3rd day, x20 TUNEL and c): II group, 15th day, x20

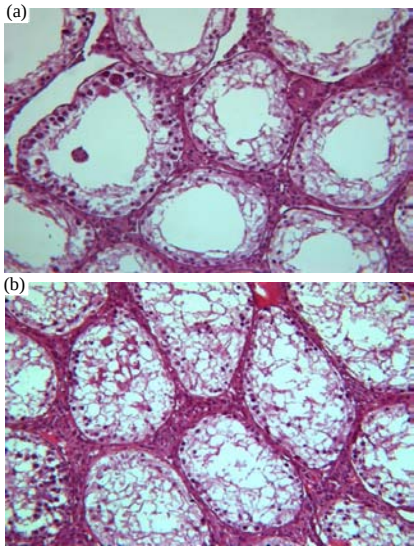

Fig. 3: a) 3rd day giant cell, x20 and b): III group, 3rd day, x20

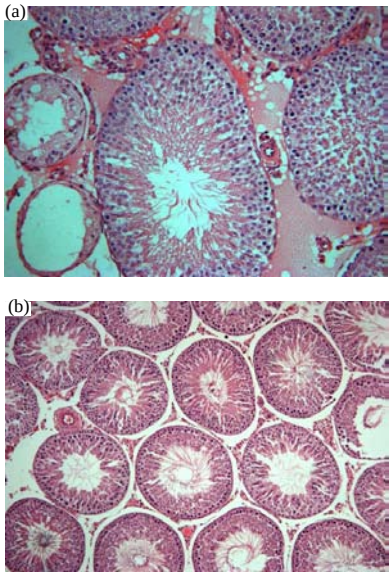

Fig. 4: a) IV group, 3rd day, x20 and b): IV group, 15th day, x10

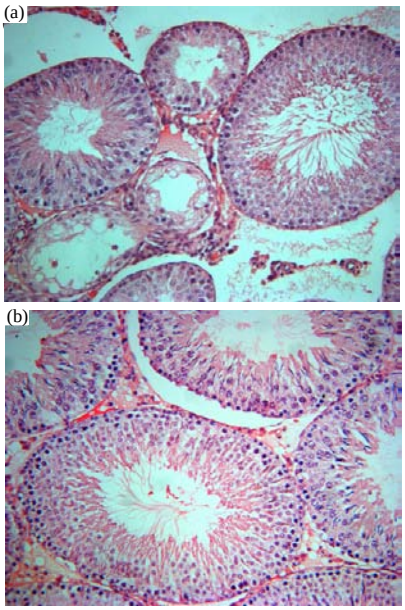

Fig. 5: a) 3rd day, x20 and b): 15th day, x20

both degenerated and sound tubules together on day 3rd (Fig. 4a). The recovery on day 15th was remarkable. There were very few degenerated tubules (Fig. 4b). In Groups V-VII which were given only Vitamins A, C and E, degeneration in the tubules on day 3rd was much less pronounced than the insulin and glibenclamide group (Fig. 5a). The recovery was as pronounced as that on day 15th with Sildenafil citrate (Fig. 5b). The recovery on

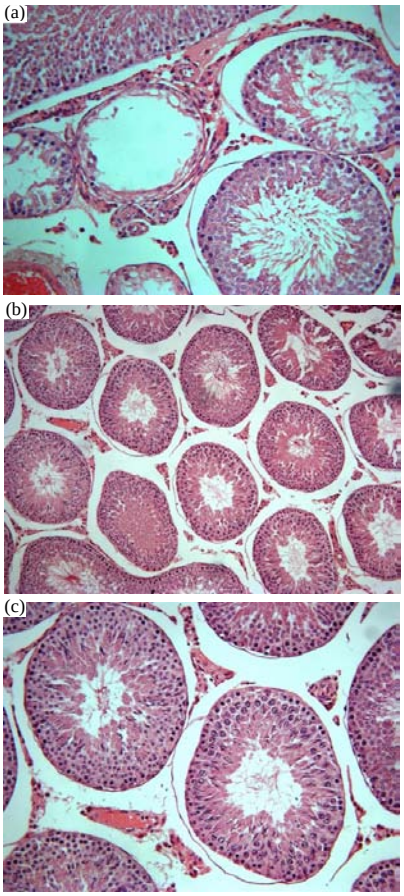

Fig. 6: a) VIII group, 3rd day, x20; b): VIII group, 15th day, x10 and c): VIII group, 15th day, x20

day 3rd in groups to which both Sildenafil citrate and vitamins were given was much better than that of the groups given only sildenafil citrate or only vitamins. On day 15th, tubules and spermatogenis were almost completely sound (Fig. 6a-c and 7a, b).

Very severe degradation was observed in the diabetic control group on the 3, 7 and 15th day and there was slight and delayed recovery in the glibenclamide and insulin groups but recovery began much earlier and degenerations was less pronounced in the groups given only Sildenafil citrate or vitamins A, C and E. However in the groups that were given sildenafil and vitamins together, the recovery on day 3rd developed much quicker than that of the groups which were given only sildenafil or vitamins. Even though, recovery on day 15th was very similar in Groups IV-X, recovery was much more pronounced in Groups VIII-X even on day 3rd.

TUNEL-positive cells were significantly more numerous in diabetic rats treated with distilled water than in the other groups. TUNEL-positive cells were similar in all of the treated diabetic groups. Insulin-treated diabetic

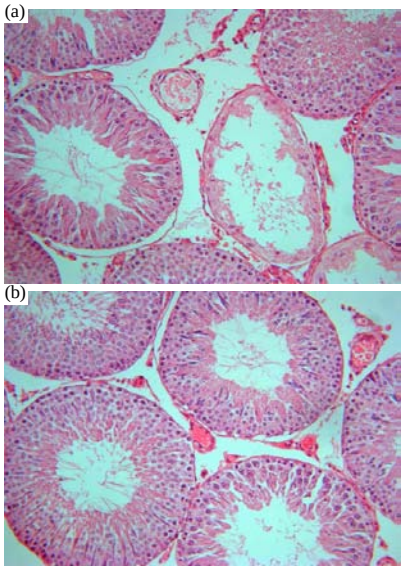

Fig. 7: a) X group, 3rd day, x20 and b): X group, 15th day, x20

group. The number of TUNEL-labeled cells was reduced in the insulin-treated diabetic group compared with the diabetic group.

DISCUSSION

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia due to defects in insulin production and function. The present study revealed that diabetes mellitus in male rats caused testicular dysfunctions and that treatment with sildenafil citrate and vitamins improved these functional deficits by providing protection against the impairment of seminiferous tubules and the loss of spermatogenic cell series. In addition, the number of TUNEL-positive cells in the germinal epithelium was significantly increased in diabetic rats, an indication of apoptosis induced by diabetes mellitus in the adult male rats. However, the number of TUNEL-positive cells was reduced after Sildenafil citrate and Sildenafil citrate+Vitamin A, C and E treatment in diabetic rats. The dysfunctional metabolism of glucose in diabetes is mainly attributed to diabetic oxidative stress caused by a variety of factors. Hyperglycemia leads to the overproduction of free radicals and the non-enzymatic glycation of proteins, which has a deleterious effect on the testes.

Researchers such as Uslu *et al.* (2009), Guneli *et al.* (2008), Cai *et al.* (2000), Murray *et al.* (1983) and Wright *et al.* (1982) have reported tubular atrophy in the testes with experimental diabetes they induced in rats and mice. The tubular atrophy observed in this study was more severe in diabetic control Group I and II but was less severe in all of the other groups due to the effects of

Sildenafil and the vitamins. In a study by Cai *et al.* (2000), they determined that the diameter of the seminiferous tubules was reduced and there was thickening in the testicular arteries in the rats with STZ-induced diabetes and that there were either no germ cells or only a few germs cells remaining.

In a similar study by Zhao *et al.* (2010) reported that apoptotic cell deaths in cells in the spermatogenic series (TUNEL-positive cells) were significantly ($p<0.05$) greater than the control group in a study where they examined testis tissue in rats exposed to low doses of radiation at the end of a 12 weeks trial period compared to the group not exposed to radiation. In this study, on the other hand, significantly more TUNEL-positive cells were found in diabetic control Group I when compared with the other groups.

Ricci *et al.* (2009) conducted a histopathological examination of the testes of both adult (SAI) and young (SPI) rats 50 days after diabetes was induced with STZ and they reported that the young rats were affected more that there was a significant reduction in the diameter of the seminiferous tubules and that there were far more abnormal tubules. In a similar study by Aybek *et al.* (2008) researched the impact of Vitamin E on oxidative stress in young and old rats that had diabetes induced with STZ but reported that no histopathological difference was found in any group. Guneli *et al.* (2008) studied how administering melatonin affected testes in rats that had diabetes induced with STZ and in the end they determined that the seminiferous tubule diameters and germ cells decreased and there was a thickening in the basal membrane in the diabetic rats. Furthermore, there were significantly more TUNEL-positive cells in the diabetic rats when compared with the control group. Melatonin significantly attenuated the diabetes-induced morphological changes and germ cell apoptosis in the diabetic rat testes. These results suggest that intraperitoneal administration of melatonin for 5 days is a potentially beneficial agent for reducing testicular damage in adult diabetic rats, probably by decreasing oxidative stress. When Naziroglu (2003) administered Vitamin C, Vitamin E, selenium and the three together to rats with STZ-induced diabetes and compared these with the control group, they reported that these vitamins and selenium provided significant protection against oxidative stress in the testes of diabetic rats.

The results of the study are parallel to the results of the study by Naziroglu (2003) and different from the results obtained by Aybek *et al.* (2008). In groups given Sildenafil citrate and Sildenafil citrate+Vitamins A, C and E, it was clearly demonstrated that the lesions became less severe starting on the 3rd day and recovery was accelerated. The decreased severity of the lesions in the groups given Sildenafil citrate and vitamins was remarkable. The reason for this could be viewed as Sildenafil enhancing NOS synthesis and accelerating regeneration with the vitamins.

The sertoli cells and spermatogenic series cells are nourished via diffusion from the blood vessels in the interstitial connective tissue. These blood vessels are important not just in terms of nourishing the Leydig cells but also for nourishing the tubules. It has been reported that thickening in the interstitial blood vessels could cause tubular atrophy by reducing nutrition to the tubules (Cameron *et al.*, 1985). In experimental diabetic studies, thickening of the interstitial blood vessel walls resulted in testicular degeneration and the onset of ischemia by causing the blood vessel walls in seminiferous tubules to become thicker (Kaya, 1986).

Uslu *et al.* (2009) demonstrated that the testes of rats that had alloxan-induced diabetes had both normal seminiferous tubules and degenerated tubules most of the seminiferous tubules had separated from each other and that edematous gaps had formed between them that there was pyknosis and marginal hyperchromasia in the nucleus of some tubule cells that there were vacuoles of varying severity in the cytoplasma that some of the spermatogenic cells in some of the more severely affected tubule lumens were sloughing off as necrotic tissue and that ones that remained intact had formed giant cells.

They reported that in some tubules there were giant cells in the tubule lumens even though the spermatogenic cells did not evidence severe degeneration and that in a small number of tubules the cells had disappeared entirely or consisted of a few sertoli cells and sometimes spermatogonium. They observed that there were no sperm cells in the lumens of tubules that had suffered complete or partial atrophy that the basal membranes of these tubules maintained their integrity and that there was sometimes hyaline-like thickening and that the many of the blood vessel walls were also thicker (Uslu *et al.*, 2009). In their study, Ozturk reported that spermatogenic cells were sloughed completely in some tubules while in some of them only the sertoli cells remained intact and that the sertoli cells were more resilient than the spermatogenic cells.

An examination of the cross-sections obtained in this study demonstrated that in the diabetic control group (Group I) and the glibenclamide reference group (Group II) where degeneration was more severe, most of the tubules in the testes had suffered degeneration. The seminiferous tubules had separated from each other. There was thickening in the tubule basal membrane, edematous gaps

had formed between the seminiferous tubules. There was pyknosis and marginal hyperchromasia in the nucleus of some tubule cells.

There were vacuoles of varying severity in the cytoplasma, some of the spermatogenic cells in some of the more severely affected tubule lumens were sloughed off as necrotic tissue and that the ones that remained intact had formed giant cells. There were more giant cells in Group I and fewer in Group II but none were observed in the other groups.

Even though it is not known exactly how multinucleated giant cells are formed, they are thought to form as a result of the fusion of spermatids (Kaya, 1986).

Multinucleated giant cells have been reported to form in some seminiferous tubules in cases of ischemia and cryptorchidisim and systemic, toxic and infectious agents that result in tubular atrophy (Kaya, 1986; Leon *et al*., 1987; Cernochova and Kamarad, 1992; Torgersen *et al*., 1982; Sasagawa *et al*., 1995).

The degenerations mentioned above were found to be very severe in Groups I and II. However in Groups III-X which were given Sildenafil citrate and Vitamins A, C and E degeneration was much less severe and giant cells were completely absent. It is thought that Sildenafil and the vitamins prevented severe degeneration by keeping free radicals out of the cell.

Furthermore, the groups that were given only vitamins A, C and E (V-VII) and those given Sildenafil citrate with vitamins A, C and E recovered much more quickly from the effects of diabetes. The reason for this could be viewed as vitamin C and E reducing the impact of diabetes in the cells as reported by Hamden *et al*. (2009). The fact that they bind free radicals and that they accelerate cell recovery by reactivating the antioxidant enzymes suppressed by administering alloxan.

CONCLUSION

The present study indicated that alloxan-induced diabetes in the testicular tissues of rats prevents spermatogenesis and leads to infertility by causing the thickening of blood vessel walls and the basal membranes of seminiferous tubules, the formation of multinucleated giant cells and severe degenerative damage.

The healing effect of Sildenafil citrate and vitamins A, C and E in the testes was studied in Groups VIII-X which were given a combination of the drug and vitamins and Groups V-VII were given only vitamins and this led to a better recovery from degeneration caused by diabetes, clearly demonstrating that the severity of the lesions decreased starting on day 3rd. In this study, it was concluded that for the purpose of reducing the effects of cell degeneration which is one of the causes of infertility in humans and animals with DM and as an adjunct treatment, a combination of Sildenafil citrate with vitamins A, C and E could be beneficial.

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