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Protective Effect of Plants Extracts Mixture on Sperm Abnormalities, Testicular and Epididymal Tissues in Diabetic Male Rats

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Abstract
Background: Diabetes has been associated with reproductive impairment in both men and women. About 90% of diabetic patients have disturbances in sexual function, including a decrease in libido, impotence and infertility. Attention has been paid to the search of effective drugs in the field of traditional medicine.
Objective: The aim of the present study was to investigate the effect of diabetes mellitus type 2(T2DM) on the sperm abnormalities, testicular and epididymal tissues of male rats. Also to evaluate the efficiency of the mixture of methanol-watery extracts composed of five medicinal plants (*Trigonella faenum-graecum* seeds, *Nigella sativa* seeds, *Zingiber officinale* rhizomes, *Olea europeae* leaves and *Fraxinus ssp*.seeds) in reducing harmful effect of DM on the percentage of sperm abnormalities, testicular and epididymal tissues.
Methods: Twenty-four mature male rats were used in six groups (n=4), the first group kept as a control. The second group received alloxan (120 mg/ kg body weight i.p.) for 3 days to induction of T2DM while the third, fourth, and fifth groups were diabetic rats received the plants extracts mixture orally for 45, 60, 75 days, respectively after induction of T2DM. The sixth group received the plants extracts mixture for 60 days. By the end of the experiment, animals were sacrificed and the testis and epididymis were excised and sperm was collected for morphological abnormalities of the sperm shape. Also histopathological examination of the testes and epididymis were examined.
Results: The sperm shape abnormalities significantly increased ($p<0.05$) in alloxan – induced diabetic rats compared with normal control group. Histopathological examination showed degenerative changes of both testes and epididymis in diabetic rats which include significant decrease ($p<0.05$) in both diameter of seminiferous tubules and their germinal epithelium heights and non-significant decrease ($p>0.05$) in both diameters of cauda epididymal tubules and height of epithelium of cauda epididymal tubules. On the other hand, atrophied seminiferous tubules, atrophied spermatogenic cells and the lumen of semineferous tubules free from sperm was detected. Furthermore, expansion of interstitial space between seminiferous tubules were seen . In contrast , oral administration of the plants extracts mixture to the diabetic rats at three period of study 45, 60, 75 days, respectively statistically decreased ($p<0.05$) most sperms abnormalities compared with diabetic group and caused non-significant differences compared with control group besides enhancing the histological changes of both testes and epididymis.
Conclusion: DM has negative effect in male testis and epidydimis of alloxan-induced rats and may contribute in reduction of fertility while the plants extracts mixture used in this study can reduce most degerenative changes occurred in testes and epidydimis in diabetic groups treated with the mixture of plants extracts. Therefore, plants extracts mixture can be effectively used by DM patients for therapeutic purpose.
Key words: Alloxan- induced diabetes, Sperm Abnormalities, Histopathological Changes of testis and epididymis, rats.

1. Introduction

Diabetes mellitus (DM) is a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins and an increased risk of complications from vascular diseases (Davis, 2006) which are attributed to an insufficient supply of insulin (Khalil, 2004). Diabetes has been associated with reproductive impairment in both men and women. About 90% of diabetic patients have disturbances in sexual function, including a decrease in libido, impotence and infertility (Feng *et al* .,2001). DM is an epidemic metabolic disease concurrent with falling fertility rates, which provokes severe detrimental blood testis barrier (BTB) alterations. It induces testicular alterations, disrupting the metabolic cooperation between the cellular constituents of BTB, with dramatic consequences on sperm quality and fertility (Alves *et al*.,2013). The toxic side effects, contraindication and sometimes diminutions in response after prolonged use of antidiabetic drugs encouraged to search for therapeutic herbal remedies for safety, efficacy and economy. Many plants were known for their activity as antidiabetic agents (Khalil, 2004). Many of the currently available drugs have been directly or indirectly derived from them and they are useful in the correction of metabolic disorders such as DM (Rathnakar *et al*.,2011).

Several studies showed that hyperglycemia and oxidative stress play a central role in diabetic tissue damage (Kuhad *et al*., 2008; Rabbani *et al*., 2009). High level of blood sugar determines overproduction of reactive oxygen species (ROS) by the mitochondria electron transport chain (Mastrocola *et al*., 2005; Rabbani *et al*., 2009). ROS virtually damages all cellular components, leading to DNA and protein modification (Rabbani *et al*., 2009). Hyperglycemia lead patients to increased oxidative stress because the production of several reducing sugars (through glycolysis and the polyol pathway) is enhanced. These reducing sugars can easily react with lipids and proteins (nonenzymatic glycation reaction), increasing the production of ROS (Palmeira *et al*., 2001). In consequence, the diabetic patients suffer from an increased risk of oxidative stress-related diseases not only in the present generation but can also transmit the nuclear defects to their progeny (Blasiak *et al*., 2004). Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic DM (Palmeira *et al*., 2001).

Moreover, Sandra *et al*. (2008) concluded that the neuropathy and vascular insufficiency which caused by diabetes may be related to sexual dysfunction. Hyperglyceamia-induced oxidative stress has been reported to be associated with testicular failure leading to sexual dysfunction, impotence and infertility. Increasing evidences indicate that the oxidative damage to the sperm nucleus results in mutation and often these changes in the DNA sequences are transmitted to the offspring, contributing in the heritable disorders in newborns. As reported, identifying the changes in the sperm shape and sperm count could be a useful method to study the influence of a disease/drug on the male germinal cells (Taylor, 2001; Aitken and Baker, 2006).

Testicular and sperm cells have increased susceptibility to free radical damage due to higher level of polyunsaturated fatty acid (PUFA), low oxygen tension and lack of antioxidant defence mechanism (Aitken and Roman, 2009). This may explain the possible aetiologies for increasing cases of infertility among males (Saalu, 2010). Infertile male diabetics tend to suffer from decreased sex libido, diminishing sperm count, endocrine disturbance, impaired penile erection and ejaculation (Mallick *et al*., 2007; Zhao *et al*., 2010).

2 Materials and methods:

2.1 Animals:

Males of adult albino rats weighing 200-300 g were used, bred in the Animal House Lab, Collage of Science, University of Babylon, Iraq. These animals were maintained under standard laboratory conditions and provided a standard diet and water *ad libitum*.

2.2 Induction and assessment of diabetes:

A single dose of alloxan monohydrate (120 mg/kg b.wt.) daily for 3 days was prepared in normal saline solution and injected intraperitoneally to induce T2DM (Al-Joubori,2012). Diabetes was confirmed after 48 h of alloxan injection, Blood samples were collected from the tail vein and fasting glucose levels were estimated by using an automated blood glucose analyzer (Rossmax). The rats were fasted for (12-18) hours and the rats had fasting glucose level above 200 mg / d1 were selected and used for the present study (Kuhad *et al*., 2008). Also, fasting blood glucose values were determined just prior to killing the animals at the end of experiment.

2.3 Plants extracts preparation

The plant powder for each plant was extracted with mixture of methanol and distilled water in a ratio of 20 % methanol: 80% distilled water (V/V) in average of 1 gm of plant powder :3 gm of mixture using blender for 30 min at room temperature. The suspension were filtered by guase and the filtrate concentrated in oven at 45 ^{o}C. The crude extracts were stored at 4 ^{o}C until use (Sato *et al*.,1990).

2.4 Experimental design:

Male rats were randomly selected and divided in six groups (n=4). First group consisted of normal control animals. Also, diabetic male rats were randomly selected and divided in four groups : diabetic group, diabetic groups treated with the plants extracts mixture for 45, 60, 75 days, respectively. While the sixth group were normal rats received the plants extracts mixture for 60 days.

2.5 Evaluation of sperm abnormalities

For sperm-shape analysis, the epididimus excised and minced in physiological saline. The tail of the epididymis was cut into small pieces in normal saline. Briefly the smears of sperm suspension were made on clean glass slides and stained with methylene blue according to the (Shalaby and Mouneir, 2010). The stained smears were observed under a light microscopic with 40X objective. Sperms were classified into normal and abnormal. At least 1000 sperms per animal were assessed for morphological abnormalities. The total sperm abnormality was expressed as percentage incidence. The sperm abnormalities were evaluated according to standard method of Narayana (2008).

2.6 Histopathological Examination:

Testes and epididymis of the normal and treated rats were taken and fixed in 10% formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. These specimens were

cleared in xylene, embedded in paraffin, sectioned at 5 microns thickness and stained with Hematoxylen and Eosin (H&E) then examined microscopically (Presnell and Sctlreibman, 1997).

2.7 Statistical analysis:

Statistical analysis was performed with SPSS software (version 17). Data were analyzed using one-way analysis of variance (ANOVA) for sperm abnormalities while t-test was used for comparison between diameters of tubules and epithelium for different groups. Results were reported as mean ± S.E. and differences were considered as significant when $P<0.05$.

3 Results

3.1 Sperm-shape abnormalities:

Sperm examination (Table-1) and (figure-1) showed that the sperm abnormalities (head and tail) were more frequent in diabetic male rats than those of the normal control. Statistical analysis showed significant increase ($P<0.05$) between diabetic and normal rats. On the other hand, the sperm abnormalities significantly decreased ($P < 0.05$) in normal group treated with the plants extracts mixture for 60 days and diabetic groups received the plants extracts mixture for 45, 60, 75 days, respectively compared to those of the diabetic animals. These abnormalities were non-significant when compared with control group.

3.2 Histopathological study

Histopathological examination of the testes of normal rats showed normal histological structure of active mature functioning seminiferous tubules associated with complete spermatogenic series as demonstrated in fig.2A. The testes of alloxan –induced diabetic rats revealed marked degeneration of most seminiferous tubules include atrophied seminiferous tubules with absence of spermatogenic series and sperms in tubular lumen (fig.2B) and expansion of interstitial spaces between seminiferous tubules. Also, significant decrease ($p<0.05$) in both diameter of seminiferous tubules (296.7±17.30) and their germinal epithelium heights (41±1.66) compared with negative control (507.4±23.96) and (81.3±4.14) respectively as shown in fig.3. The testes of diabetic rats given the mixture for 45 days orally showed mild degeneration of some seminiferous tubules associated with partially recovery and normal spermatogenic series of another seminiferous tubules as demonstrated in fig 2C. Microscopic examination of the testes of diabetic rats given the plants extracts mixture for (60,70 days) revealed normal histological structure of most seminiferous tubules with normal spermatogenic series as illustrated in fig. 2D and E. Examination of the testes of normal rats given the mixture for 60 days revealed normal histological structure of most seminiferous tubules with normal spermatogenic series as demonstrated in fig.2 F.

. Moreover, histopathological study revealed degenerative changes in epididymal tissues in alloxan-induced diabetic rats include damage of epididymal walls (fig 4B) and presence of non-significant decrease ($p>0.05$) in both diameters of cauda epididymal tubules and their epithelium heights as seen in fig .5. The oral administration of the plants extracts mixture caused enhancing the degenerative changes in epididymal tissues in diabetic rats (fig.4).

4 Discussion

In the present study, the diabetic condition of male rats significantly increased the sperm shape abnormalities compared to those in the normal negative controls. These effects appeared to be mediated through the oxidative stress and inducing of ROS generated due to hyperglycemia (Vikram *et al*., 2007; Shrilatha and Muralidhara, 2007). The generation of ROS in diabetes was considered to be the major cause for mutagenecity including chromosome aberrations, DNA fragmentation, micronuclei and sperm abnormalities (Otton *et al*., 2004; Rabbani *et al*., 2009). Several studies reported that suggested mechanism for inducing damage of nuclear component and sperm abnormalities in diabetic condition include the activation of several damaging pathways by the ROS such as accelerated formation of advanced glycation end production (AGE), polyol pathway, hexosamine pathway, protein kinase (PKC) or increase of lipid peroxidation (LPO) (Piconi *et al*., 2003; Valko *et al*., 2007; Rabbani *et al*., 2009). LPO occurs when ROS attacked the polyunsaturated fatty acid residues of phospholipids of cell membrane which is extremely sensitive to the oxidation. The spermatozoa are highly susceptible to the damage by excess concentrations of ROS due to high content of polyunsaturated fatty acid within their plasma membrane. Increased LPO and altered membrane can affect the sperm function through impaired metabolism, motility, acrosome reaction as well as oxidative damage to sperm DNA leading to increase of morphological changes in sperm and decrease of caudal sperm count (Sanchez *et al*., 2006; Rabbani *et al*., 2009).

Mammalian sperm cells present a specific lipidic composition, with a high content of polyunsaturated fatty acids, plasmalogens and sphingomyelins. The lipids in the spermatozoa are the main substrates for the ROS mediated peroxidation and damage to it is reported to affect the motility and fertilization capacity of sperms (Doreswamy *et al*., 2004). The oxidative damage initiates sperm plasma membrane destruction, apoptosis and germ cell death (Prasad *et al*., 2012). Furthermore, free radicals induce genotoxicity by initiating sperm DNA denaturation and fragmentation with limited DNA repair. Damage of genetic material in spermatogonia and

spermatocytes has been related to the increased sperm abnormalities. These alterations bring about possibilities of genetic disorders if passed down to offspring (Silva and Gadella, 2006).

Our results are in agreement with (Abd El-Rahim *et al*., 2010) who reported that the diabetic condition of male rats significantly increased the sperm shape abnormalities besides significant reducing of caudal sperm count. Also, the experimental T2DM after the administration of streptozotocin (STZ) had significantly (P<0.001) increased the sperm shape abnormality and decreased the sperm count compared to the control animals (Rabbani *et al*., 2010). Induction of diabetes led to significant decrease in the studied sperm quality parameters (Erukainure *et al*., 2012).

In contrast, the oral administration of the plants extracts mixture to the alloxan diabetic rats in the present study had reduced alloxan mediated changes in the sperm shape abnormalies and improved testicular antioxidant activities suggesting its protective potential against spermatotoxic and testicular toxicity in diabetic. This effect of the plants extracts mixture may due to the antioxidant property and ability to decrease the alloxan mediated defects in germinal cells . Al-Joubori (2012) reported that this plants extracts mixture had many antioxidant bands which were 3,1,2,2 and 3 in methanol–watery extract of fenugreek seeds, black cumin seeds, olea leafs, ginger rhizome, fraxinus seeds respectively. Also, this mixture had reduced the mitotic index and chromosomal abnormalities besides enhancing the serum levels of glucose, insulin and urea compared to diabetes control. So they suggested that the plants extracts mixture possessing glucose lowering property along with an antioxidant effect play a beneficial role in preventing the ROS mediated DNA damages. This results are consistent with Krishnamoorthy *et al*. (2007) who reported that antioxidants limit the nuclear damage by preventing the free radical action.

Histopathological changes revealed marked degeneration of most seminiferous tubules include atrophied seminiferous tubules with absence of spermatogenic series and sperms in tubular lumen (fig.2B) and significant decrease ($p<0.05$) in both diameter of seminiferous tubules and height of germinal epithelium of testes and epididymis compared to those in the normal controls. These changes may due to DM which induces subtle molecular changes that are important for sperm quality and function and alters conventional sperm parameters. In addition, DM causes histologic damage of the epididymis, with a negative impact on sperm transit. Various mechanisms may explain the sperm damage observed in patients with DM. These include endocrine disorders, neuropathy, and increased oxidative stress (La Vignera *et al*., 2012). These effects may due to DM decreases serum testosterone levels (Shalaby and Mouneir, 2010; Shahreari *et al*.,2010) which associated with a steroidogenetic defect in Leydig cells. Furthermore, DM is associated with an increased oxidative stress, which damages sperm nuclear and mitochondrial DNA. Finally, spermatogenesis derangement and germ cell apoptosis in type 1 DM may relate to a local autoimmune damage, whereas insulin resistance, obesity, and other related comorbidities may impair sperm parameters and decrease testosterone serum levels in patients with T2 DM (La Vignera *et al*., 2012).

Moreover, intraperitoneal administration of alloxan (120 mg.kg b.wt./day) to normal male rats for 3 days decreased the weight of testes, seminal vesicle and prostate glands, induced marked testicular degeneration, lowered semen quality and quantity, increased blood glucose and decreased serum insulin and testosterone levels. It is well known that diabetes is positively associated with lowered male fertility and sexual dysfunction (Shalaby and Mouneir, 2010). The cytoplasmic carbohydrates (mainly glucose) are the preliminary origin to supply required energy to the most of biochemical activities such as mitosis and miosis. Any disruption in carbohydrates metabolism and/or transport through germinal epithelium is able to influence their mitotic and biological activities, which in turn can lead to spermatogenesis arrest in seminiferous tubules (Bustos-Obregón and González-Hormazabal,2003; Malekinegad *et al*.,2010).

Oral administration of the plants extracts mixture to the diabetic rats at three period of study 45, 60, 75 days, respectively caused enhancing the histological changes of both testes and epididymis besides enhancing the diameter of seminiferous tubules , diameter of epididymal tubules and height of epithelium of testes and epididymus. These results indicate that methanol-watery extracts of five plants used in this study act to attenuate the degenerative changes in testes and epididymIs because it containing many compounds act separately or synergistically to enhance testes function and retards to normal value. This may due to presence of some phytochemical compounds such as saponin, flavones, tannins and terpenes and its action can be related to possessing insulin-like action and had ability to induce DNA repair systems due to antioxidant activities which reduce or prevent generation of free radicals. Also, the longer period of treatment (60 and 75) days is more effective in reducing degenerative changes of T2DM due to enhancing insulin level (Al-joubori, 2012) which plays a role in regulating pulsatile GnRH secretion (Tanaka *et al*.,2000).

Sertoli cells dysfunction in turn could be able to result in germinal cells degeneration and dissociation (Dutta and Meijer, 2003). As Sertoli cells are involved in the regulation of spermatogenesis, providing nutritional support for germ cells, any metabolic alteration in these cells derived from DM may be responsible for spermatogenesis disruption, playing a crucial role in fertility/subfertility associated with this pathology. These

cells have a glucose sensing machinery that reacts to hormonal fluctuations and several mechanisms to counteract hyper/hypoglycemic events. (Alves *et al*., 2013). The ultrastructural changes of Sertoli and Leydig cells, brought about by streptozotocin **i**nduced diabetes, because of the alterations in pituitary gonadotropins, and these changes influence the normal spermatogenesis in rats (Kianifard *et al*.,2010).

These result in agreement with previous studies which showed that oral administration of *Zingiber officinale* roots extracts to diabetic rats for 65 days increased the weight of testes and seminal vesicles; improved semen quantity and quality; decreased blood glucose level, but increased serum insulin and testosterone levels and ameliorated the degenerative lesions which seen in the testes of diabetic rats (Shalaby and Mouneir,2010). Also, Fenugreek steroids potentially unregulated the key steroidogenesis enzymes such as 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), malic enzyme, 3beta-hydroxysteroid dehydrogenase (3beta-HSD) and glucose-6-phosphate dehydrogenase (G6P-DH) activities as cholesterol rate in testis, which considerably enhanced testosterone and estradiol levels in the plasma of surviving diabetic rats and prevented the alteration of the key carbohydrate enzymes such as hexokinase and pyruvate kinase activities as well as testicular glycogen and seminal fructose contents in surviving diabetic rats. Furthermore, fenugreek steroids administration to surviving diabetic rats significantly decreased the sperm shape abnormality and improved the sperm count. Above all, the potential protective action of reproductive systems was approved by the histological study of testis and epididymis (Hamden *et al*., 2010).

5. Conclusions:

This paper indicates improved testicular and epidydimal and reduced sperm abnormalities by oral administration of the plants extracts mixture used in this study suggesting its protective potential against spermatotoxic and testicular toxicity in diabetic male rats. Based on the antidiabetic, antioxidant and reduced sperm abnormalities and histological changes exhibited by the mixture used in this study, it can be suggested that this mixture could be useful in reducing the male reproductive defects associated with DM. Therefore, plants extracts mixture can be effectively used by DM patients for therapeutic purpose.

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Table (1): Sperm-shape abnormalities in diabetic rats and diabetic-treated groups.

Treatment	Sperm Shape abnormalities (M±SE)					Sum of abnormalities	Total abnormalities %
	without tail	without head	aberrant mid piece	aberrant tail	without hook		
Negative control	2±0.40	0.67± 0.23	0.67± 0.21	0.00± 0.00	2.67± 0.47	6	0.6
Diabetic control	2.33± 0.23	13.67± 4.11 *	3.33± 0.56 *	1.67± 0.76 *	7.67± 2.25 *	28.6 *	2.86
Plant extracts mixture (60 days)	1.50± 0.64	1.33± 1.47 **	2.00± 0.73 * **	0.83± 0.31	3.25± 1.03 **	8.9 **	0.89
DM + Mixture of plants extracts (45days)	1.00± 0.37	2.33± 0.56 **	0.33± 0.21 **	1.00± 0.00	3.67± 0.94 **	8 **	0.8
DM + Mixture of plants extracts (60 days)	1.33± 0.21	0.33± 0.21 **	2.33± 0.42 *	0.67± 0.42	3.00± 1.08 **	7.66 **	0.76
DM + Mixture of plants extracts (75 days)	1.00± 0.21	1.33± 0.21 **	2.67± 0.21 *	1.00± 0.00 *	2.66± 0.47	8.66 **	0.86

Data were expressed as mean ± S.E.

* Significantly different (P<0.05) compared with negative control

** Significantly different (P<0.05) compared with diabetic control

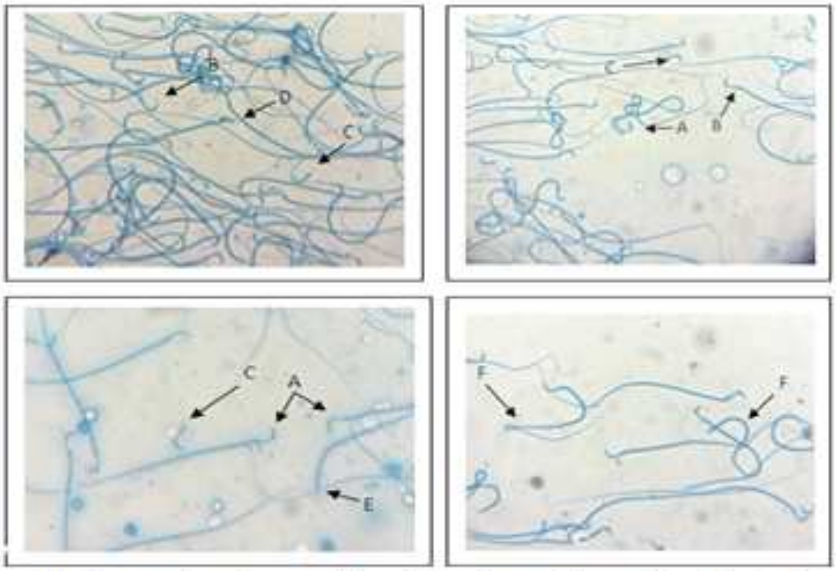


Figure (1): Sperm-shape abnormalities of diabetic control and treated rats showing: (A): aberrant midpiece, (B): normal sperm, (C): without tail, (D): hookless, (E): without head, (F): aberrant tail. (methylene blue - 400X).

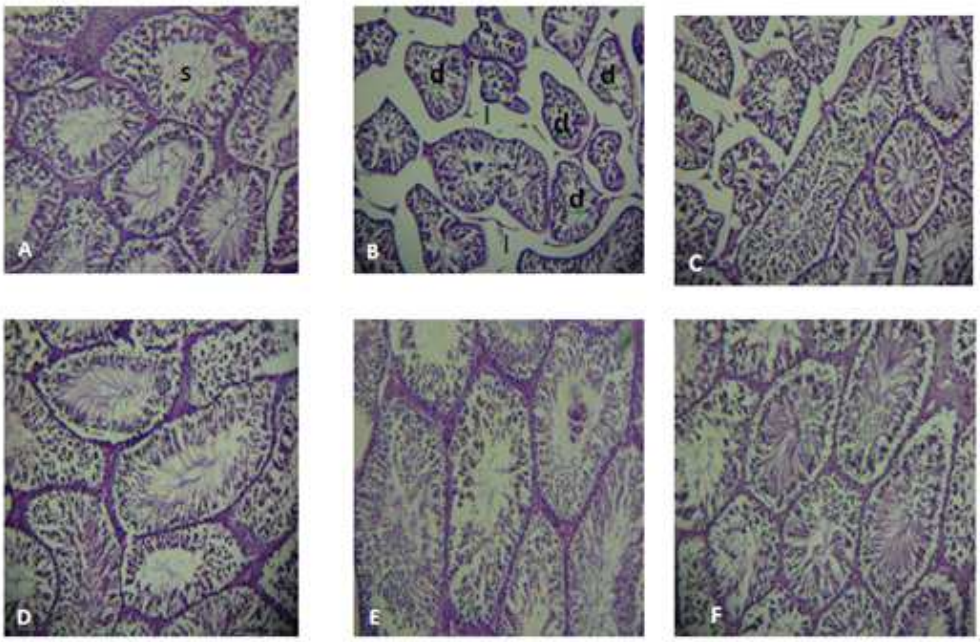


Fig. 2: Cross section from testis of :

(A): Negative control group showing normal histological structure of active mature functioning seminiferous tubules (S) associated with normal spermatogenic series and sperms.

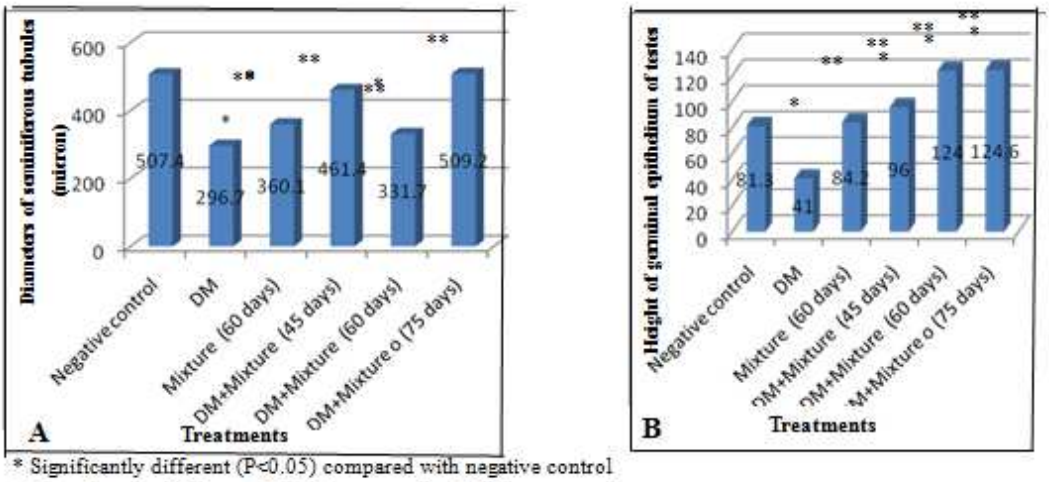
(B) : Diabetic control group showing marked degeneration (d) of most seminiferous tubules with absence of spermatogenic series and sperms in tubular lumen, atrophied seminiferous tubules and expansion of interstitial spaces (I) between seminiferous tubules.

(C): Diabetic group given the mixture for 45 days showing mild degeneration of some seminiferous tubules with partially recovery and normal spermatogenic series in another seminiferous tubules.

(D): Diabetic group given the mixture for 60 days showing normal histological structure of most seminiferous tubules and presence of sperms in the lumen of most seminiferous tubules.

(E): Diabetic group given the mixture for 75 days showing normal histological structure of most seminiferous tubules and presence of sperms in the lumen of most seminiferous tubules.

(F): Normal group given the mixture for 60 days showing normal histological structure of most seminiferous tubules and presence of sperms in the lumen of most seminiferous tubules (H&E, 400X).



* Significantly different (P<0.05) compared with negative control

** Significantly different (P<0.05) compared with diabetic control

Figure 3: Effect of different treatments on diameters of seminiferous tubules (A) and height of germinal epithelium of testes (B).

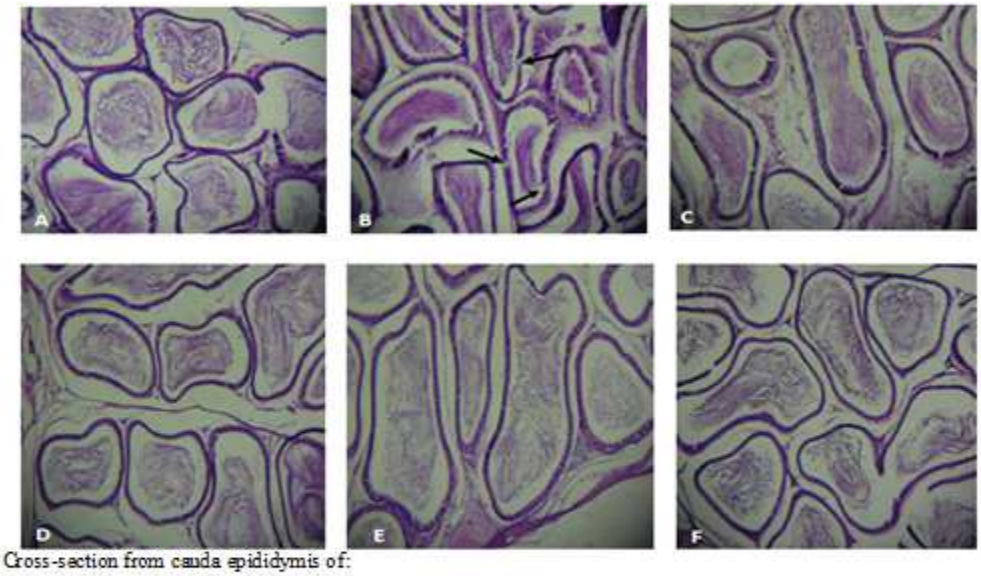


Figure 4: Cross-section from cauda epididymis of:
(A): Negative control group showing normal view for epididymal tubules.
(B): Diabetic control group showing damage in epididymal tubules walls (↗).
(C): Diabetic group given the mixture for 45 days showing mild degeneration of some epididymal tubules walls.
(D): Diabetic group given the mixture for 60 days showing normal histological structure of most epididymal tubules.
(E): Diabetic group given the mixture for 75 days showing normal histological structure of most epididymal tubules.
(F): Normal group given the mixture for 60 days showing normal histological structure of most epididymal tubules. (H&E, 100 X).

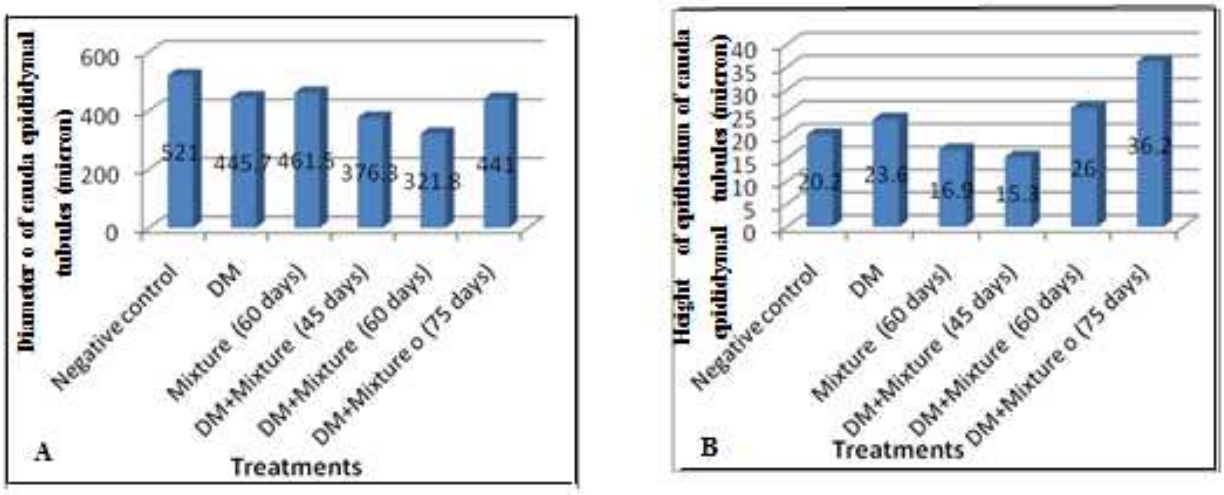


Figure 5: Effect of different treatment on diameters of cauda epididymal tubules (A) and height of epithelial cell of cauda epididymal tubules (B).