



Effects of Morus Alba Extract on the Microscopic Changes of Spermatogenesis in Experimentally Induced Diabetes Mellitus in Adult Rats

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Abstract

Diabetic hyperglycemia has a direct association with microscopic changes of testicular tissue and spermatogenic alterations. Most of these changes are mediated with oxidative stress. Use of herbal and chemical glucose lowering drugs is an important way for reduction of diabetes related structural changes of target tissues. This study was designed to investigate the protective effects of Morus alba extract on the microscopic structure of testicular tissue following induction of diabetes. Diabetes was induced by streptozotocin. Morus alba extract and metformin were administrated to various groups for period of 12 weeks. The blood glucose levels, pituitary gonadotropins and testosterone were measured. Histomorphometrical study and sperm analysis were performed for evaluation of the function of reproductive system. Reduction of body weight, pituitary gonadotropins and testosterone levels in diabetic groups, were improved after treatment. Decrease of spermatogenic indices was accompanied with the changes in histomorphology of seminiferous tubules. Moreover, the sperm analysis results were decreased in diabetic rats. These microscopic changes were enhanced after treatment in diabetic groups. The results of this study indicate that the administration of glucose lowering medical herbs only or in combination with chemical drugs, can preserve the positive effects in improvement of spermatogenic alterations related to diabetes.

Keywords: Diabetes, Morus alba, rat, spermatogenesis

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Introduction

Diabetes mellitus (DM) is a serious metabolic disorder with numerous complications [1]. It is well known that, increase of blood glucose level leads to structural and functional changes in various target tissues and organs [2]. The change of microscopic structure of male reproductive system in diabetic conditions has been reported in several studies [2-4]. In this regard, it has been reported, the mean testicular weight is decreased around 20 percent in comparison to healthy and/or streptozotocin (STZ) resistant rats [5]. Recent experimental and clinical studies suggest an oxidative stress and production of reactive oxygen species (ROS) play a key role in pathogenesis of both types of diabetes mellitus and subsequently development of diabetes complications [6, 7]. In addition to the role of oxidative stress, the occurrence of some alterations in production, secretion and/or function of certain hormones such as insulin and pituitary gonadotropins may influence some changes in microscopic structure and function of spermatogenic cells. Insulin insufficiency and impairment of regulatory action of this hormone on Leydig and Sertoli cells have an important role in testicular dysfunction in diabetic patients [8]. Likewise, the endocrine functions of testes are controlled by pituitary gonadotropins [8]. Follicle-stimulating Hormone (FSH) regulates the functions of Sertoli cells and luteinizing hormone (LH) controls the Leydig cells functions [9]. Insulin has an imperative role on the control of cell proliferation and metabolism of Leydig cells. The regulation of testicular function is the result of multiple mechanisms that include the combined effects of insulin/glucose, FSH, and LH. Testicular dysfunction and diminished testosterone production are related to diabetes and can influence the production of spermatozoid [10-12]. Diabetes associated microvascular disorders have a relationship with tissue damages and hyperglycemia plays a key role in diabetic vascular complications through many metabolic and structural derangements [13]. The spermatogenic activities of testicular seminiferous tubules, carried out by Sertoli cell mediated glucose uptake. The metabolism of glucose and production of lactate (the preferred energy source of germ cells) occur in cytoplasm of Sertoli cells. In this regard, the microvascular damages of testicular tissue following diabetic hyperglycemia can alter the transportation of glucose and subsequently lead to structural and functional changes of spermatogenesis due to derangement of cellular nutrition [14, 15]. The herbal drugs have gained importance because of their efficacy and cost effectiveness in treatment of several diseases. Mulberries (*Morus alba* L.) are reported to possess antidiabetic activity [16]. *Morus Alba* (*M. alba*) has long been used in treatment and

prevention of diabetes and as a general tonic to enhance health in traditional oriental medicine. The mulberry extract have potent antioxidant activity [17-19]. Moreover, the mulberry fruit contains high amounts of flavonoids and alkaloids and many of its bioactivities specifications were linked to presence of these compounds [19-21]. In diabetes, many of structural and functional alterations in spermatogenesis are related to hyperglycemia and oxidative damages [6, 7]. On the basis of the antioxidant and antidiabetic activities of *Morus alba*, in the present research we examine the protective effects of *Morus alba* on the structural and functional alterations of spermatogenesis after induction of diabetes.

Materials and Methods

Animal Procedure

The 56 adult male Wistar rats with mean body weight 196 ± 11.28 g were used in this study. The animals were procured by the Center of Animal Housing and Breeding of the Faculty of Veterinary Medicine, Urmia University. The animals were placed in standard cages under 12:12 h light: dark cycle with 23-25°C room temperatures. Animals were fed on standard laboratory animal's chow and water ad libitum during the whole period of experiment. All animal procedures were approved by the standards for humane care and use of laboratory animals, University of Tabriz in accordance with Research Ethical Committee of the Ministry of Health and Medical Education of Iran (adopted in April 17, 2006) based on Helsinki Protocol (Helsinki, Finland, 1975).

Experimental Design

The animals were divided into seven groups each holding eight rats as described following. A) Control (Con.): normal and apparently healthy rats that did not receive any type of treatment during the 12 weeks of study; B) Control + *Morus alba* extract (Con.MA): the animals of this group were treated with *Morus alba* extract with dose of 100 mg kg⁻¹ per day for a period of 12 weeks and then euthanized at the end of study; C) Control + Metformin + *Morus alba* extract (Con.Met.MA): in this group, the extract of *Morus alba* (100 mg kg⁻¹) and Metformin (100 mg kg⁻¹) were administered together to animals for 12 weeks; D) Diabetic (Dia.): in this group, experimental diabetes was induced by single intraperitoneal injection of streptozotocin with dose of 45 mg/kg of body weight. The animals of this group were euthanized after 12 weeks ; E) Diabetic + *Morus alba* extract (Dia.MA): this group

consisted of STZ-induced diabetic rats treated with Morus alba extract with dose of 100 mg kg⁻¹ of body weight per day by oral gavages method from two weeks after induction of diabetes till end of study; F) Diabetic + Metformin (Dia.Met.): the animals of this group were treated with metformin with dose of 100 mg kg⁻¹ of body weight per day by oral gavages two weeks after induction of diabetes till end of study; G) Diabetic + Metformin + Morus alba extract (Dia.Met.MA): in this group, the extract of Morus alba and Metformin were administered to animals with the same dose and treatment period as group C.

Treatments and Chemicals

Experimental diabetes was induced by Streptozotocin (Sigma, ST. Louis, MO, USA) with dose of 45mg/kg bodyweight. Streptozotocin dissolved in 0.1 M citrate sodium buffer (pH = 4.5) and injected intraperitoneally to overnight fasting animals. Induction of diabetes was confirmed 48 hours after injection of STZ by measurement of fasting blood glucose levels with automated glucose analyzer device (Glucometer, On Call EZ, SD, USA). The animals with blood glucose levels above 200 mg/dl were considered diabetic and were used in this study [22]. For metformin treatment, metformin hydrochloride (GLUCOPHAGE, Merck Sante s.a.s., LYON - FRANCE) tablets were dissolved in distilled water and gavaged with above mentioned dose.

Morus Alba Extract Preparation

Fresh Mulberry fruit (*Morus alba*) was collected from the trees. The collected samples selected to uniformity in shape and color. The fruits were dried at 70 °C in duration of four days and then ground to powder. For water extraction, each 50 gram of powder was mixed with 100 milliliters of boiling distilled water by magnetic stirrer for 15 minutes. The water extract was freeze dried and kept in 4°C.

Body Weight and Blood Collection

At the end of study, the weight of each animal was recorded. The overnight fasting rats were euthanized with CO₂ inhalation. The blood was immediately collected by cardiac puncture. All blood plasma samples stored at -20°C until further analyses. Blood glucose levels were measured by spectrophotometry using glucose-oxidase method (Unico 1200, Japan).

Analytic procedure in plasma samples

The serum concentration of FSH, LH and testosterone was assayed by enzyme-linked immunosorbent assay (ELISA) method with commercial diagnostic ELISA kits (DRG Instruments GmbH, Germany) for FSH, LH and (Diaplus Inc. USA) for testosterone.

Morphometric Analyses

Morphometrical studies of seminiferous tubules diameter (STD) and germinal epithelium height (GEH) were performed on formaldehyde-fixed testicular samples with thickness of 6-7 micrometer stained with Hematoxylin and Eosin method. The slides were studied at 200× magnification. To get approved results, the seminiferous tubules that sectioned transversely were studied and the shortest diameter of them was considered for measurement. The analyses were performed on the images obtained and digitalized with digital camera (Olympus DP70, Olympus Europe, Hamburg, Germany). The images were processed by computerized image analysis system (Olympus Soft Imaging Solutions GmbH, Munster, Germany). For each tissue sample, twenty tubules were measured in different microscopic fields.

Evaluation of spermatogenesis in testicular tissue

For evaluation of spermatogenesis, three different indices were used. Tubular differentiation index (TDI): the number of seminiferous tubules that have more than three layers of germinal cells derived from type A of spermatogonia; repopulation index (RI): the ratio of active spermatogonia to inactive spermatogonia and spermiogenesis index (SPI): the ratio of the number of seminiferous tubules with spermatozooids to the empty tubules [23].

Sperm Analyses

For analyses of sperm, the cauda epididymis was separated from testis and cut into small pieces in one milliliter of Ham's F10 culture medium. The epididymal sperm count was evaluated by hemocytometer with light microscope at 400× magnification. Sperm motility was assessed with phase contrast microscope at 400× magnification. Ten microscopic fields were observed and the mean of counted sperms was considered as sperm motility for each sample. To estimate the percentage of viability, twenty microliter of sperm suspension was mixed with equal volume of 0.05 percent eosin-Y. The prepared slides were viewed by bright-field microscope at 400× magnification. Two hundred sperms were considered for calculating of indices in experimental groups [24].

Statistical Analyses

The obtained results were analyzed with the SPSS Statistics software version 19. All data were reported as mean $\pm$ SEM. The comparison of means between experimental groups was evaluated with one way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Differences were considered to be statistically significant if $p < 0.05$.

Results

As figure 1 shows, administration of M.alba led to a little and nonsignificant increment in mean body weight of group B, whereas loss of body weight was seen in all diabetic groups ($p < 0.05$). In this regard, M.alba treated diabetic group had significant body weight increment in comparison to other diabetic groups.

Induction of diabetes led to significant increment ($p < 0.05$) of blood glucose levels in untreated diabetic group in comparison to other groups (Figure 2). Diabetic animals that were treated with M.alba extract, had lower blood glucose levels in comparison to metformin treated diabetic rats ($p > 0.05$). As figure 2 shows, following administration of both M.alba extract and metformin to diabetic rats, there was greater reduction in the mean blood glucose levels, compared to other treated diabetic groups. However, this reduction did not attain normal levels ($p < 0.05$). In all diabetic groups (except group G), mean blood level of FSH was significantly lower than control groups ($p < 0.05$) (Table 1). This significant difference in FSH levels was also seen between groups D and G. As table 1 shows, administration of metformin and/or M.alba extract, had no effect on the alteration of FSH levels in control groups whereas, use of metformin and/or M.alba extract, led to elevation of FSH levels in diabetic rats ($p > 0.05$). The blood level of LH was significantly low in all diabetic groups (except group G) in comparison to control groups. The mean blood level of LH was increased significantly in group G in comparison to groups D and F ($p < 0.05$). Blood testosterone level was not changed like other hormones. Treatment of diabetic rats, led to elevation of blood testosterone level in comparison to untreated diabetic group ($p > 0.05$). There was no significant difference in blood level of testosterone between treated diabetic groups and control groups. The blood level of testosterone was reduced in untreated diabetic group in comparison to groups A and C ($p < 0.05$).

The diameter of seminiferous tubules was reduced after induction of diabetes (Figure 4). This reduction was only significant between control groups and groups D and E (Figure 3). In

diabetic groups, the comparison of results showed a moderate increment in STs Diameter of treated diabetic groups in comparison to untreated diabetic rats (Figure 4). This increase in diameter was seen significantly in groups F and G. The height of germinal epithelium showed similar changes. In all diabetic groups (except group G), the height of germinal epithelium was reduced significantly in comparison to control group. Moreover, the height of germinal epithelium was increased significantly in group G in comparison to group D ($p < 0.05$).

All indices of spermatogenesis were reduced significantly in diabetic groups in comparison to control groups (Table 2). These indices were increased in all treated diabetic groups. This improvement was seen significantly between group D and group G for TDI between group D and groups F and G for SPI and between group D and groups E and G for RI index ($p < 0.05$).

As table 3 shows, the epididymal sperm count was reduced significantly in all diabetic groups in comparison to control groups ($p < 0.05$). Epididymal sperm count was increased significantly in all treated diabetic groups compared to untreated diabetic rats. The percentage of sperm motility and viability was reduced significantly in all diabetic groups in comparison to groups A and B (for sperm motility) and to control group (for sperm viability). There was no significant difference in sperm motility and viability between group G and group C (for sperm motility) and between group G and groups B and C (for sperm viability). In groups E and G, the percentage of sperm motility and viability was increased significantly in comparison to group D ($p < 0.05$).

Table 1. Blood concentration of gonadotropins and testosterone in experimental groups

	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/ml)
A	0.27±0.07 ^{d,e,f}	0.24±0.08 ^{d,e,f}	5.58±0.63 ^d
B	0.27±0.04 ^{d,e,f}	0.23±0.06 ^{d,e,f}	5.31±0.27
C	0.28±0.09 ^{d,e,f}	0.25±0.02 ^{d,e,f,g}	5.56±0.71 ^d
D	0.19±0.09	0.18±0.06	4.91±0.21
E	0.21±0.06	0.19±0.07	5.01±0.37
F	0.21±0.03	0.18±0.09	5.11±0.29
G	0.24±0.05 ^d	0.21±0.07 ^{d,f}	5.19±0.56

Values shown are mean ± SEM. Significantly different compared to ^aA, ^bB, ^cC, ^dD, ^eE, ^fF, ^gG groups (P < 0.05 for all). A: control; B: Control+M.alba; C: control+metformin+M.alba; D: diabetic; E: diabetic+M.alba; F: diabetic+metformin; G: diabetic+metformin+M.alba. FSH: follicle-stimulating hormone; LH: luteinizing hormone.

Table 2. Microscopic indices of spermatogenesis in testicular tissue of experimental groups

	TDI (%)	SPI (%)	RI (%)
A	90.75±2.35 ^{d,e,f,g}	89.71±4.11 ^{d,e,f,g}	83.49±1.87 ^{d,e,f,g}
B	89.12±4.17 ^{d,e,f,g}	90.23±5.25 ^{d,e,f,g}	82.97±6.93 ^{d,e,f,g}
C	90.27±7.26 ^{d,e,f,g}	89.12±6.16 ^{d,e,f,g}	84.29±4.14 ^{d,e,f,g}
D	81.66±4.11	74.91±5.40	67.50±3.81
E	83.57±6.27	76.51±4.99	72.19±5.56 ^d
F	83.66±5.14	78.07±7.40 ^d	71.44±4.17
G	85.22±5.61 ^d	79.46±6.56 ^d	74.20±6.06 ^d

Values shown are mean ± SEM. Significantly different compared to ^aA, ^bB, ^cC, ^dD, ^eE, ^fF, ^gG groups (P < 0.05 for all). A: control; B: Control+M.alba; C: control+metformin+M.alba; D: diabetic; E: diabetic+M.alba; F: diabetic+metformin; G: diabetic+metformin+M.alba. TDI: tubular differentiation index; SPI: spermiogenesis index; RI: repopulation index.

Table 3. Epididymal sperm analysis in experimental groups

	Sperm Count ($10^6/\text{ml}$)	Sperm Motility (%)	Sperm Viability (%)
A	83.51 $\pm$ 6.33 ^{d,e,f,g}	85.43 $\pm$ 8.19 ^{d,e,f,g}	88.45 $\pm$ 6.13 ^{d,e,f,g}
B	84.17 $\pm$ 5.94 ^{d,e,f,g}	85.17 $\pm$ 7.43 ^{d,e,f,g}	87.39 $\pm$ 7.41 ^{d,e,f}
C	83.97 $\pm$ 6.17 ^{d,e,f,g}	84.73 $\pm$ 7.21 ^{d,e,f}	87.71 $\pm$ 7.66 ^{d,e,f}
D	67.44 $\pm$ 5.91	74.49 $\pm$ 6.59	77.45 $\pm$ 6.99
E	73.21 $\pm$ 6.17 ^d	79.26 $\pm$ 7.17 ^d	81.65 $\pm$ 7.54 ^d
F	72.69 $\pm$ 7.28 ^d	77.89 $\pm$ 7.26	79.86 $\pm$ 8.11
G	74.29 $\pm$ 6.51 ^d	81.67 $\pm$ 8.01 ^d	82.27 $\pm$ 7.76 ^d

Values shown are mean $\pm$ SEM. Significantly different compared to ^aA, ^bB, ^cC, ^dD, ^eE, ^fF., ^gG groups ($P < 0.05$ for all). A: control; B: Control+M.alba; C: control+metformin+M.alba; D: diabetic; E: diabetic+M.alba; F: diabetic+metformin; G: diabetic+metformin+M.alba.

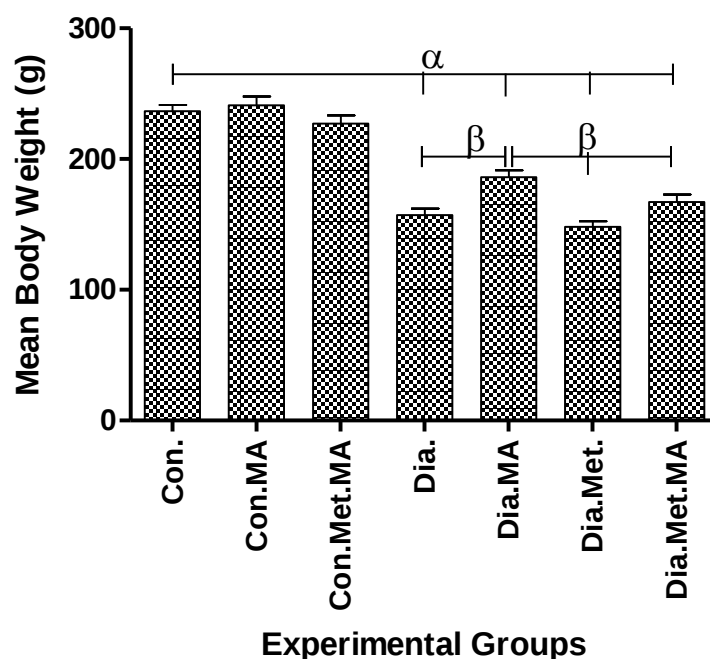


Figure 1. Mean body weight of experimental groups at the end of study. (α) Significant different in comparison to Control group. (β) Significant different in comparison to diabetic+M.alba group ($p < 0.05$).

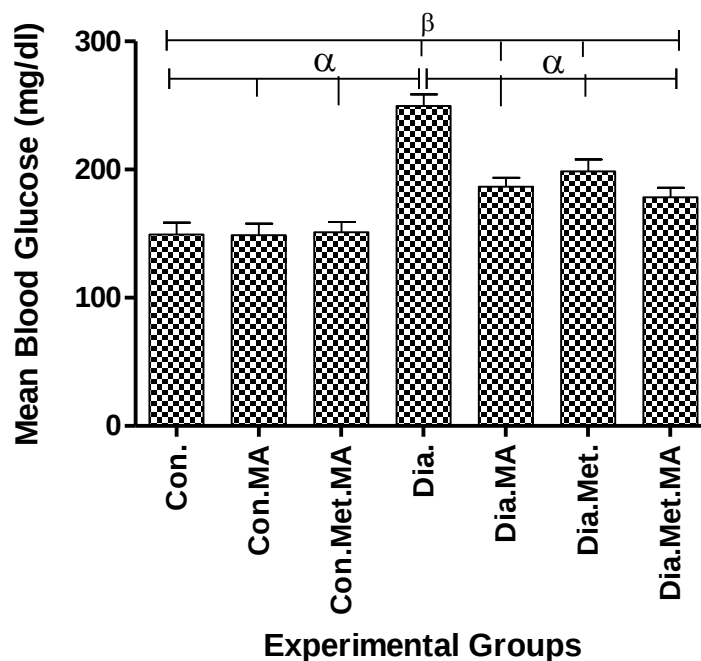


Figure 2. Mean blood glucose levels in experimental groups at the end of study. (α) Significantly different in comparison to diabetic group. (β) Significantly different in comparison to control group ($p < 0.05$).

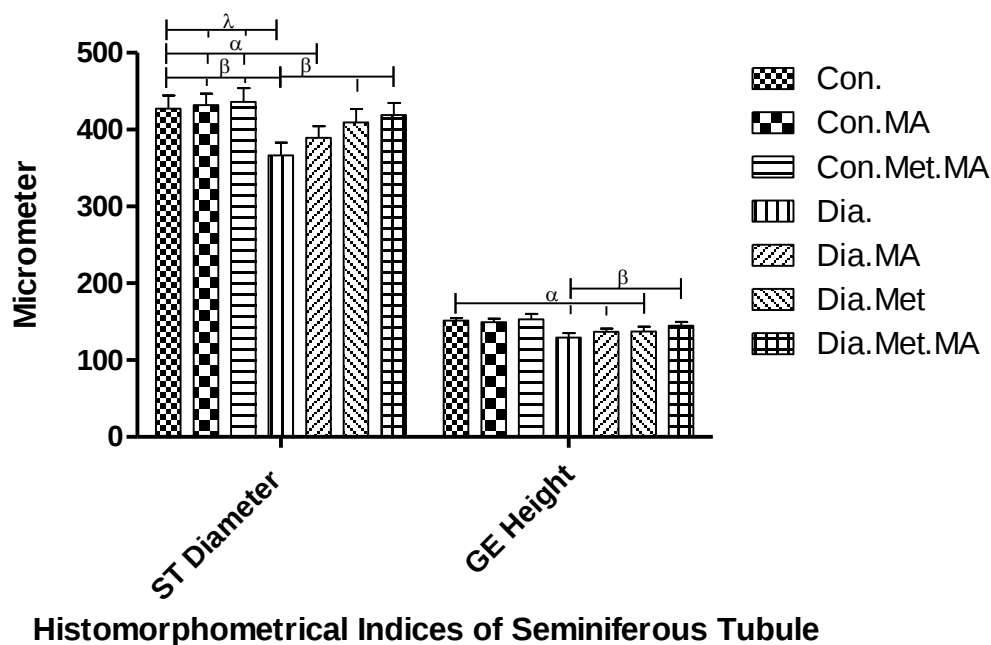


Figure 3. Histomorphometrical indices of testicular seminiferous tubules in experimental groups. ST diameter: (α) Significant different in comparison to diabetic+M.alba group. (β) Significant different in comparison to diabetic Group. (λ) Significant different in comparison to diabetic group. GE height: (α) Significant different in comparison to control Group. (β) Significant different between diabetic and diabetic+metformin+M.alba groups ($p < 0.05$).

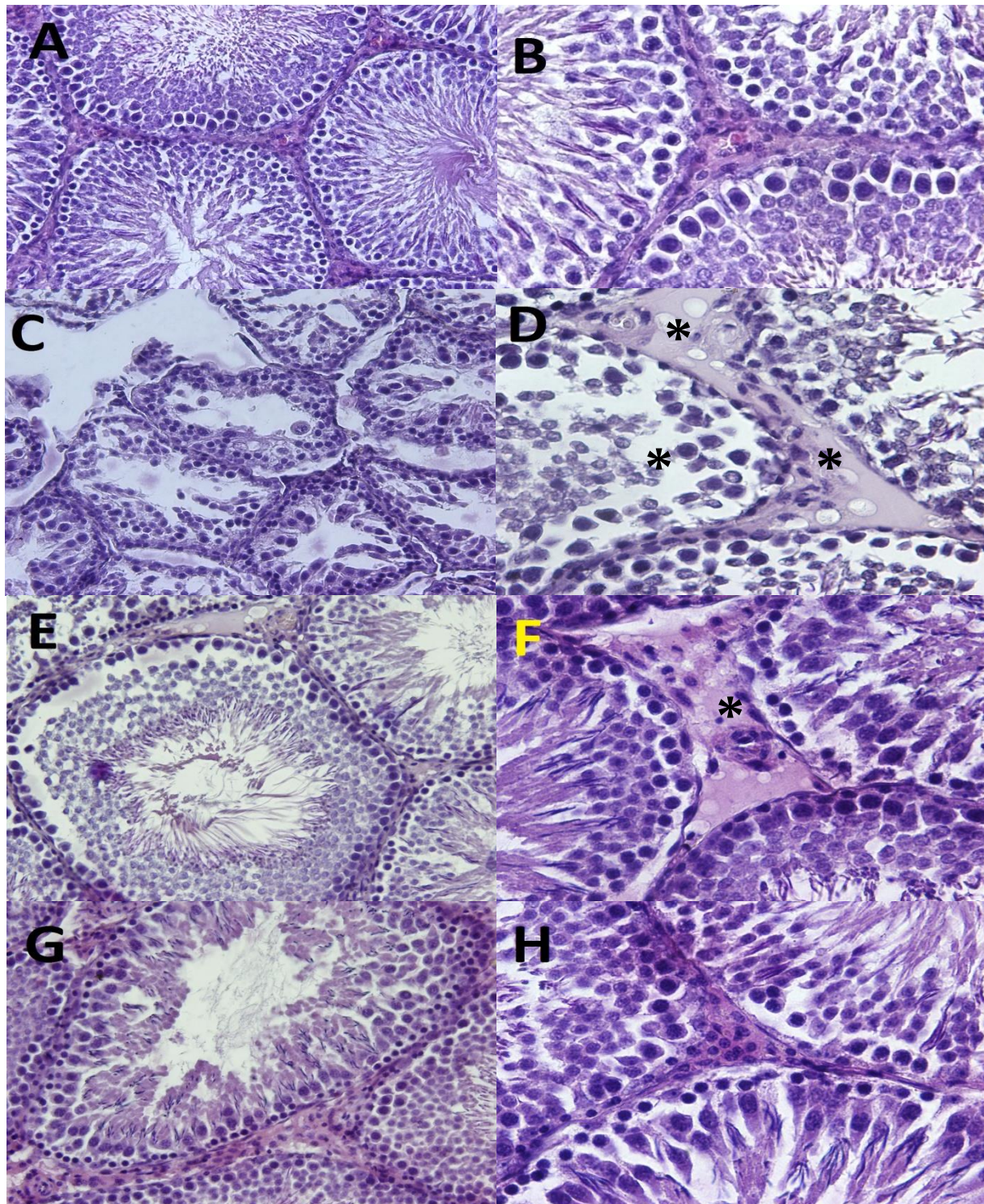


Figure 4. Cross section of testicular tubules in various groups. A,B: control; C,D: dibetic; E,F: diabetic+metformin; G,H: diabetic+metformin+M.alba. Malformation and atrophy of tubules with disarrangement of spermatogenic cells (black asterisk) and edema of interstitial connective tissue (red asterisks) was seen in sections from diabetic group. Disarrangement of cells was reduced in diabetic+metformin group but edema was seen. Spermatogenic cells population and arrangement was similar to control sections and edema was reduced in diabetic+metformin+M.alba group. Hematoxylin and Eosin staining. Magnification: Left column 200 $\times$, right column 400 $\times$.

Discussion

Following the induction of diabetes, the microscopic changes of testicular tissue and the alteration in normal spermatogenesis have been observed to increase [4, 5]. In this regard, the excessive production of reactive oxygen species and microvascular damages are reported to substantially contribute to the occurrence of structural and functional changes [25-27]. Our previous studies showed that induction of diabetes leads to both steroidogenic and spermatogenic dysfunctions and impairment of testicular function [28, 29]. Prevention of oxidative damage with natural antioxidants is considered as one of the most effective strategies in control and reduction of diabetic complications. In this way, the use of phenolic rich fruits and medical herbs can reduce the risk of developing chronic diseases by reduction of oxidative stress and inhibition of macromolecular oxidation [30]. In this study, the hypoglycemic effects of *Morus alba*, as one natural antidiabetic herbal drug, was evaluated. There is a paucity of data on the effects of combined forms of natural and chemical antidiabetic on reduction and prevention of diabetes associated tissue damages. In our study, the mean body weight in groups that were treated with *M.alba* increased whereas, groups that received both metformin and *M.alba* had a lower body weight gain. These results indicate that, use of metformin cause a greater body weight loss due to excessive reduction in adipose tissue. These results are in accordance with our previous report and other studies [1, 29]. Results of our study showed, the blood glucose lowering effect and capability of *M.alba* is equal to metformin. This ability of *M.alba* in reduction of glucose levels, improved when it was administered with metformin. Metformin decreases blood glucose, but does not reduce glucose levels below normal [31]. Insulin resistance is the most important factor in development of diabetes mellitus, and metformin can reduce the resistance of target cells to insulin [32]. This function of metformin explains the effectiveness and the ability of blood glucose lowering effect of combined form of Metformin/*M.alba*. Lack of insulin in diabetic rats can affect the serum FSH levels [8]. One of the most important functions of insulin is the modulation of blood FSH levels and strong correlation have been found between FSH and insulin levels in blood plasma [33]. The comparison of FSH levels between different groups in our study revealed that, treatment of diabetes with metformin and/or *M.alba* leads to increase of blood FSH levels to some extent. This increment was higher in group G. As regards to the relationship between changes of insulin and FSH levels, the results of this study showed that the effect of metformin and *M.alba* in improvement of insulin secretion is

comparable to each other. Like FSH, the alteration of blood LH levels was greater more in diabetic groups. Insulin has a key role in metabolism of Leydig cells (as main target cells of LH) and maintenance of LH receptors on these cells [34]. Reduction of insulin secretion resulted to LH decrement and subsequently malfunctions of Leydig cells. Our results showed that use of metformin and M.alba in combination has greater effect in elevation of blood LH levels. This effect is achieved through the improvement of insulin function in diabetic rats. The results of blood testosterone measurement in our study showed that, the ability of metformin and M.alba in elevation of blood testosterone levels is approximately equal to each other. Increase of blood testosterone levels in treated diabetic rats showed that the function of Leydig cells may have a direct relationship with blood glucose levels.

In this study, and in our earlier reports [28, 29], the reduction in diameter of seminiferous tubules and depletion in germinal cell population was seen following induction of diabetes (Figure 4). Decrement in diameter of STs was accompanied with depletion in the height of germinal epithelium which causes the atrophy of seminiferous tubules. These histological observations in STs, illustrate the depressed cellular activity of spermatogenic cells in diabetic conditions. Oxidative stress in testicular tissue has a direct relationship with abnormal spermatogenesis due to decrement of glutathione in male germ cells which lead to incomplete functional maturation and capacitation of spermatozoa [35, 36]. Diabetic hyperglycemia is one of the causes of increased oxidative stress in different tissues [37]. Accordingly, Use of blood glucose lowering herbs and/or chemicals can be helpful in reduction of oxidative stress and subsequently tissue damages. Our study showed that the administration of metformin and/or M.alba alone or in combination, leads to improvement of histomorphometry of testicular tissue. The evaluation of microscopic indices of spermatogenesis confirms these histologic results. Diminished tubular differentiation (TDI) and spermiation (SPI) indices in diabetic rats indicates that, conversion of spermatogonia to primary spermatocytes is reduced. Reduction of repopulation index in diabetic rats demonstrates the number of inactive spermatogonia increased after induction of diabetes. This process cause a decline of the number of primary spermatocytes derived from spermatogonia cells. These alterations in cellular conversion and/or activity lead to reduction in production of spermatozooids. In this regard, reduction of blood glucose levels following treatment with herbal and/or chemical drugs is related to improvement of cell differentiation in epithelium of seminiferous tubules. The results from different indices of sperm analysis in this study indicate that the number,

ability of movement and the mortality rate of spermatozoids have a relationship with cellular activity of testicular germinal epithelium. The structure of spermatozoids has large quantities of lipids. Hyperglycemic oxidative stress can influence the normal structure of developing spermatozoids due to induction of excessive lipid phosphorylation [36]. As the results of this study indicate, decrease of blood glucose levels following treatment of diabetes with M.alba and metformin leads to improvement of cellular activity indices of testicular tissue and sperm analysis. In conclusion, the results of this study revealed that, use of herbal hypoglycemic drugs single or with chemical drugs, can be used as one important strategy for control and reduction of diabetic complications in reproductive system.

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