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MICROSCOPIC STUDY OF TESTICULAR TISSUE STRUCTURE AND SPERMATOGENESIS FOLLOWING LONG TERM DOSE DEPENDENT ADMINISTRATION OF MONOSODIUM GLUTAMATE IN ADULT DIABETIC RATS

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Abstract

Background and aims: *Diabetic hyperglycemia leads to structural and functional alterations in body organs including testis. Monosodium glutamate (MSG) is a food additive which has toxic effects on human and animal's tissues. The aim of this study was to evaluate the effects of MSG on diabetic complications of testicular tissue.* ***Material and Methods:*** *48 adult rats were divided into six groups. Diabetes was induced by Streptozotocin (45 mg/kg i.p.). Monosodium glutamate was administrated in two doses (6 and 60 mg/kg) to control and diabetic groups. After eight weeks, the body weight was measured and blood samples were collected for analysis of the pituitary-testicular axis hormones. Formalin fixed tissue samples were prepared through routine histologic methods.* ***Results:*** *MSG led dose dependently to weight gain of control groups and prevented weight loss of diabetic rats. The blood levels of luteinizing hormone and testosterone were reduced in diabetic rats following administration of MSG. Reduction of cellular population of germinal epithelium, seminiferous tubules diameter decrement and decrease of indices of spermatogenesis were observed with more intensity in MSG treated diabetic rats.* ***Conclusions:*** *The results of this study suggest that the long term administration of MSG may induce in a dose dependent manner structural and functional alterations of testicular tissue in diabetic rats.*

key words: *Diabetic rats, Monosodium glutamate, Testicular tissue*

Background and Aims

The acute and chronic complications of diabetes are the major problems that occur in diabetic patients [1]. Increase of blood glucose levels leads to structural and functional changes in various target tissues and organs [2]. Induction of diabetes in male rats is associated with alteration in functions of reproductive system [3]. Diabetes is one of the most important risk factors for male infertility. In this regard, *in vivo* experiments on adult rats indicated that, induction of diabetes is associated with cellular alterations in testicular microenvironment [4].

Diabetes is a condition of increased oxidative stress and impaired energy metabolism. Recent experimental and clinical studies suggest that oxidative stress and

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production of reactive oxygen species (ROS) play a key role in the pathogenesis of both types of diabetes mellitus and subsequently development of diabetes complications [5,6].

The metabolism of glucose and production of lactate (the preferred energy source for germ cells) occur in the cytoplasm of Sertoli cells. In this regard, the microvascular damages of testicular tissue following diabetic hyperglycemia can alter the transport of glucose and subsequently lead to structural and functional changes of spermatogenesis due to alteration of cellular nutrition [7,8].

Monosodium glutamate (MSG) is the sodium salt of the L-form of glutamic acid. Glutamic acid is one of the most widespread amino acids found in natural products [9]. Some studies indicated that monosodium glutamate has toxic effects on human and animal's tissues [10]. Male infertility, testicular hemorrhage and alteration of sperm production and morphology, reduction of body growth, obesity and hypogonadism are the most often reported changes in cases of male infertility after administration of MSG [10-13]. Monosodium glutamate can induce oxidative stress through production of oxygen radicals and hydrogen peroxide which subsequently lead to oxidative DNA damage, cell membrane peroxidation and cellular death [14]. Oxidative stress is one of the most important factors involved in tissue damages following diabetic conditions and consumption of monosodium glutamate.

Taking into account the growing prevalence of diabetes, increased consumption of MSG containing foods and the importance of normal fertility for both physical and mental health, the aim of this study was to evaluate the long term and dose dependent effects of monosodium glutamate on microscopic structure of male gonads and spermatogenesis in diabetic conditions in a rat animal model.

Materials and Methods

Treatments and chemicals: Streptozotocin, STZ, (Sigma, ST. Louis, MO, USA) was used for induction of diabetes. The STZ was dissolved in 0.1 M citrate sodium buffer (*p*H 4.5) and was injected intraperitoneally (45 mg/kg body weight) in overnight fasting animals. Diabetes was confirmed 48 hours after injection of STZ with an 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 [15]. Monosodium glutamate (Sigma-Aldrich, St Louis, MO 63178, USA) was administrated intraperitoneally in two (low and high) dose levels (6 and 60 mg/kg body weight) [16].

Animals' procedures and experimental design: 48 adult male *Wistar* rats with mean body weight 193.83 g were divided into six experimental groups: 1) *Control group (Cont.):* normal and healthy rats that did not receive any type of treatment; *2) Low dose monosodium glutamate administrated control group (Cont+MSG6)* which received MSG 6 mg/kg body weight for eight weeks; *3) High dose monosodium glutamate administrated control group (Cont+MSG60.)* which received MSG 60 mg/kg body weight for eight weeks; 4) *Diabetic group (Diab.):* in this group, diabetes was induced and the animals were euthanized eight weeks after induction of diabetes; *5) Low dose monosodium glutamate administrated Diabetic group (Diab+MSG6):* in this group, two weeks after induction of diabetes, MSG was given in low dose (6 mg/kg) to diabetic animals for a period of eight weeks; *6) High dose monosodium glutamate administrated Diabetic group (Diab+MSG60):* this group consists of diabetic rats which received the high dose of MSG (60 mg/kg) for eight weeks after induction of

diabetes. The experimental period was performed two weeks after induction of diabetes.

All animals used for testing were housed under a 12 hour light-dark cycle with room temperature of 23-25°C and had access to food and water *ad libitum*. All animal procedures used in this study were approved by the University of Tabriz standards for human care and use of laboratory animals, in accordance with the Ethical Research Committee of the Ministry of Health and Medical Education of Iran (adopted in April 17, 2006) based on the Helsinki Protocol (Helsinki, Finland, 1975).

Measurement of Body Weight: Mean body weight of each group was measured twice a week from the beginning to the end of study.

Analytic procedures in plasma samples: the blood glucose levels were determined by spectrophotometery according to the glucose oxidase method (Unico 1200, Japan); plasma testosterone levels were measured by an enzyme-linked immunosorbent assay (ELISA) method using a commercial kit (Diaplus Inc. USA); plasma Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) levels were determined by ELISA assays using specific commercial kits (DRG Instruments GmbH, Germany).

Tissue preparation and histological techniques: The testicular tissues were immediately fixed in 10% formaldehyde in buffered solution containing 54 mM NaH2PO4 and 28 mM Na2HPO4 (pH 7.4) and kept at 4°C. After 48 hours, the transverse section was made on the middle part of each testis and kept immersed in the fixative solution for the completion of tissue fixation. Then, formaldehyde-fixed samples were embedded in paraffin and sliced with thickness of 6-7 micrometer and were mounted onto albumin-pre-coated glass slides. The mounted tissue samples were stained by the Hematoxylin and eosin method (H&E) for histological observations by light microscopy.

Morphometric analysis: For morphometric assessment of seminiferous tubules, the slides were studied at 200× magnification. To get extra precise results, only the seminiferous tubules (STs) that were sectioned transversely were studied and the shortest diameter of seminiferous tubules was considered for measurement.

Evaluation of spermatogenesis in testicular tissue: For estimation of spermatogenesis in testicular tissue, three different indices were used. Tubular differentiation index (TDI), repopulation index (RI) and spermiogenesis index (SPI). To determine the tubular differentiation index, the number of seminiferous tubules with more than three layers of germinal cells derived from type A of spermatogonia was calculated. To find out the repopulation and spermiogenesis indices, the ratio of active spermatogonia and inactive cells and respectively, the ratio of the number of seminiferous tubules with spermatozoids to the empty tubules, were calculated [17].

Statistical analyses: The obtained results were analyzed using the GraphPad PRISM® software version 5.04 (GraphPad Software, Inc. USA). All data were reported as mean ± Standard Error of the Mean (SEM). The comparison of means between experimental groups was evaluated by using the one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Differences were considered to be statistically significant if $p < 0.05$.

Results

Body weight changes: Table 1 shows the mean body weight at the end of study. Accordingly, the body weight of the three diabetic groups decreased significantly ($p<0.0001$) in comparison to *Cont+MSG6* and

Cont+MSG60 groups whereas, the body weight of control group was significantly different only in comparison to the diabetic group. In this regard, treatment of control rats with MSG led to more body weight gain in comparison to the control group but this increase was not significant (p>0.05).

Table 1. The mean of body weight in experimental groups.

	Mean	Std. Error
Control	194.3[£]	1.128
Cont+MSG6	203.3[*]	1.764
Cont+MSG60	206.1[*]	3.652
Diabetic	180.3	4.995
Diab+MSG6	181.6	3.412
Diab+MSG60	184.7	2.746

[*] Significant different in comparison to all diabetic groups;
[£] Significant different in comparison to diabetic group.

Figure 1 shows the changes of mean body weight in experimental groups during the period of study. According to this figure, the mean of body weight increased in all control groups and the administration of MSG was dose dependently led to a dose dependent increment of weight gain in the control groups. Furthermore, the body weight of diabetic groups decreased during the study. Moreover, treatment of diabetic rats with MSG led to an improvement of weight loss in comparison to the non-treated diabetic group.

Blood glucose and hormonal assays: Figure 2 shows the mean blood glucose in the experimental groups. In diabetic groups the blood glucose levels were significantly high in comparison to control groups (p<0.0001).

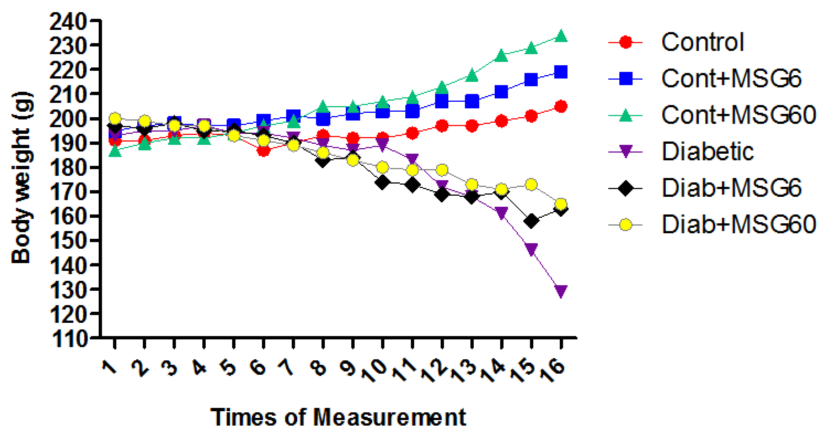

Figure 1. *Diagram of body weight during the period of study.*

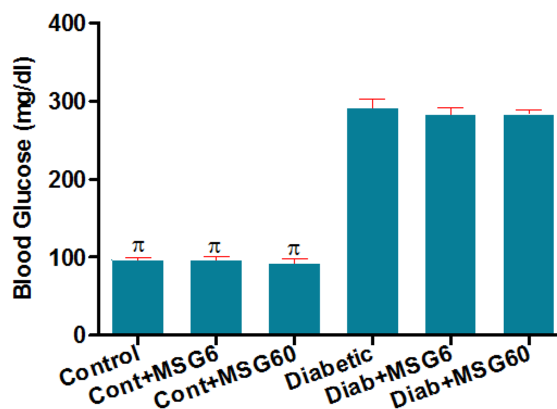

Figure 2. *The mean of blood glucose in experimental groups.*
Data are presented as mean ± SEM. [π] *Significantly different in comparison to diabetic groups.*

Figures 3, 4 and 5 show the blood concentration of pituitary gonadotropins and testosterone in the experimental groups. Administration of MSG led to insignificant decrease of FSH levels in control groups in comparison to non-treated control group (Figure 3). The levels of FSH were reduced significantly in diabetic groups in comparison to the control group ($p<0.0001$). The administration of MSG to diabetic groups led to a higher (but non-significant) decrease of FSH levels. The administration of MSG led to decrease of LH levels in both control and diabetic groups (Figure 4). In this regard, significant reduction of LH levels was seen in diabetic groups in comparison to the control group. MSG treated diabetic rats had significantly lower levels of LH in comparison to MSG treated control groups. Additionally, the administration of MSG dose dependently led to reduction of LH levels. However, this reduction was only significant between diabetic and diab+MSG60 groups. As Figure 5 shows, the changes of testosterone levels were similar with LH alterations. In this regard, testosterone levels were significantly lower in diabetic groups in comparison to control groups ($p<0.0001$). Likewise, the administration of MSG dose dependently led to reduction of testosterone levels in diabetic groups. This reduction of testosterone levels was seen between high dose MSG treated diabetic group and non-treated diabetic rats.

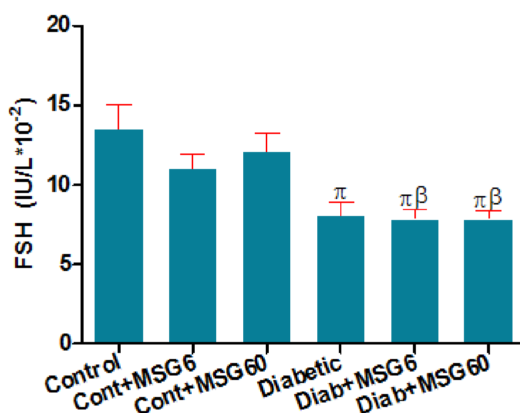

Figure 3. *Blood concentrations of FSH in experimental groups. $^{\pi}$ Significant different in comparison to control group; $^{\beta}$ Significant different in comparison to Cont+MSG60 group. Data are presented as mean ± SEM.*

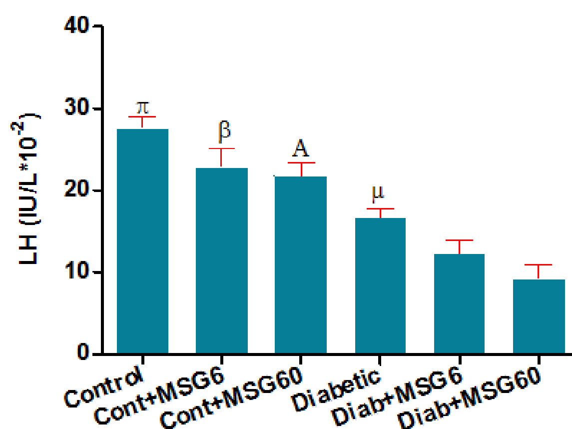

Figure 4. *Blood concentrations of LH in experimental groups. $^{\pi}$ Significant different in comparison to diabetic groups; $^{\beta}$ Significant different in comparison to MSG treated diabetic groups; ^{A} Significant different in comparison to MSG treated diabetic groups; $^{\mu}$ Significant different in comparison to Diab+MSG60 group. Data are presented as mean ± SEM.*

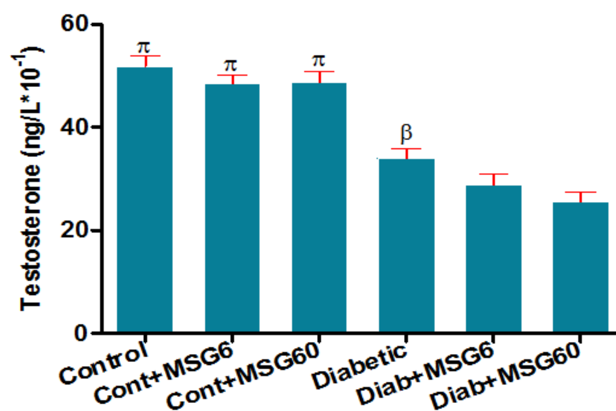

Figure 5. *Blood concentrations of testosterone in the experimental groups.* $^{\pi}$ *Significant different in comparison to diabetic groups;* $^{\beta}$ *Significant different in comparison to Diab+MSG60 group. Data are presented as mean ± SEM.*

Table 2. Cellular population of germinal epithelium of seminiferous tubules in experimental groups. Data are presented as mean±SEM

	Sertoli Cells	Spermatogonia	P.Spermatocyte	Spermatids
Control	$21±0.62^{*}$	56.67±0.89	$59.05±0.83^{*}$	$176±1.88^{*}$
Cont+MSG6	$19.57±0.43^{*}$	57.90±1.07	$58.14±0.81^{*}$	$172.9±3.37^{*}$
Cont+MSG60	$18.76±0.55^{\pi}$	55.95±1.24	$57.71±0.94^{*}$	$170±2.50^{*}$
Diabetic	16.29±0.51	57.90±1.38	$48.33±1.21^{\pi}$	$122.1±2.56^{\pi}$
Diab+MSG6	16.76±0.67	57.24±1.25	$51.57±1.04^{\pi}$	115.6±2.18
Diab+MSG60	16.24±0.46	58.95±0.93	42.95±1.16	105.3±2.90

Sertoli cells: * Significant difference in comparison to diabetic groups; $^{\pi}$ Significant difference in comparison to *Diabetic* and *Diab+MSG60* groups. Primary Spermatocyte: * Significant difference in comparison to diabetic groups; $^{\pi}$ Significant difference in comparison to *Diab+MSG60* group. Spermatids: * Significant difference in comparison to diabetic groups; $^{\pi}$ Significant difference in comparison to *Diab+MSG60* group.

Cellular and morphometric analysis of seminiferous tubules: Table 2 shows the number of various cell types of germinal epithelium in seminiferous tubules. The administration of MSG did not led to a significant change in the number of Sertoli cells in MSG treated control and diabetic groups. The number of Sertoli cells was reduced in diabetic groups. This reduction was significant between *Control* and *Cont+MSG6* groups and all diabetic groups (p<0.0001). There was no significant change in the number of spermatogonia cells in experimental groups. Induction of diabetes led to a significant reduction of primary spermatocytes in diabetic groups in comparison to control groups (p<0.0001). The administration of MSG did not led to any change in the population of primary spermatocytes in control groups. However, the high dose of MSG led to a significant decrease of primary spermatocytes in diabetic rats (p<0.0001). As Table 2 shows, the administration of MSG led to decrease of spermatids in MSG-treated control and diabetic groups. This reduction was not significant among the control groups but this reduction was significant between *Diabetic* and *Diab+MSG60* groups. In this regard, all diabetic groups significantly had a lower population of spermatids in comparison to control groups (p<0.0001).

Figure 6 shows the external diameter of seminiferous tubules in the experimental groups. The administration of MSG led to a decrease of ST diameter in both control and diabetic groups. This reduction was not significant between control groups. However, high dose of MSG led to significant reduction of ST diameter in the Diab+MSG60 group in comparison to other

diabetic groups (p<0.0001). In all diabetic groups the external diameter of ST was reduced significantly in comparison to *Control* and *Cont+MSG6* group while, this reduction was not significant between *Cont+MSG60* and *Diabetic* groups.

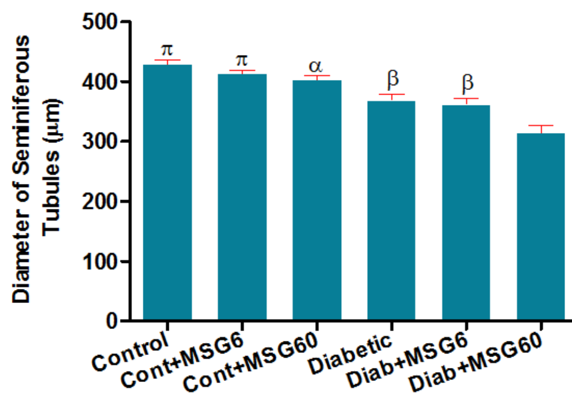

Figure 6. *Mean of seminiferous tubules external diameter in experimental groups. [π] Significant difference in comparison to diabetic groups; [α] Significant difference in comparison to Diabetic and Diab+MSG60 groups; [β] Significant difference in comparison to Diab+MSG60 group.*

Table 3. Indices of spermatogenesis in experimental groups. Data are presented as mean±SEM.

	TDI	SPI	RI
Control	0.84±0.030*	0.73±0.030*	0.79±0.023*
Cont+MSG6	0.77±0.036	0.72±0.032*	0.74±0.026*
Cont+MSG60	0.75±0.040	0.71±0.031π	0.71±0.031π
Diabetic	0.67±0.039	0.59±0.023	0.63±0.021
Diab+MSG6	0.62±0.029	0.57±0.030	0.61±0.027
Diab+MSG60	0.64±0.056	0.56±0.026	0.58±0.024

TDI: * Significant difference in comparison to diabetic groups. SPI: * Significant difference in comparison to diabetic groups; π Significant difference in comparison to *Diab+MSG6* and *Diab+MSG60* groups. RI: * Significant difference in comparison to diabetic groups; π Significant difference in comparison to *Diab+MSG60* group.

Indices of spermatogenesis: The administration of MSG led to reduction of tubular differentiation index in both control and diabetic groups (Table 3). This reduction was significant between control group and diabetic groups (p<0.0001). As Table 3 shows, index of spermiogenesis was reduced in MSG-treated control and diabetic groups in comparison to non-treated groups but this reduction was not significant. In diabetic groups, administration of MSG led to more reduction of spermiogenesis index. The alterations of repopulation index were similar to that of the index of spermiogenesis.

Histologic structure: The most obvious changes in testicular tissue of diabetic rats were tubular atrophy, disarrangement of spermatogenic cells and increase of intercellular spaces (Figure 7). The specified histologic changes in diabetic groups increased dose dependently following the administration of monosodium glutamate in MSG-treated diabetic groups.

Discussions

Monosodium glutamate can induce oxidative stress through production of oxygen radicals and hydrogen peroxide which subsequently lead to oxidative DNA damage and cell membrane peroxidation and cellular death [14]. Oxidative stress is one of the most important mechanisms involved in tissue damages in diabetic conditions and similarly following usage of monosodium glutamate. Induction of diabetes is associated

with cellular alterations in testicular microenvironment [4]. Moreover, MSG has toxic effects on human and animal's tissues and male infertility alterations were reported after administration of MSG [10-13]. According to expression of glutamate receptors in testicular tissue, this organ can be affected by monosodium glutamate [18,19].

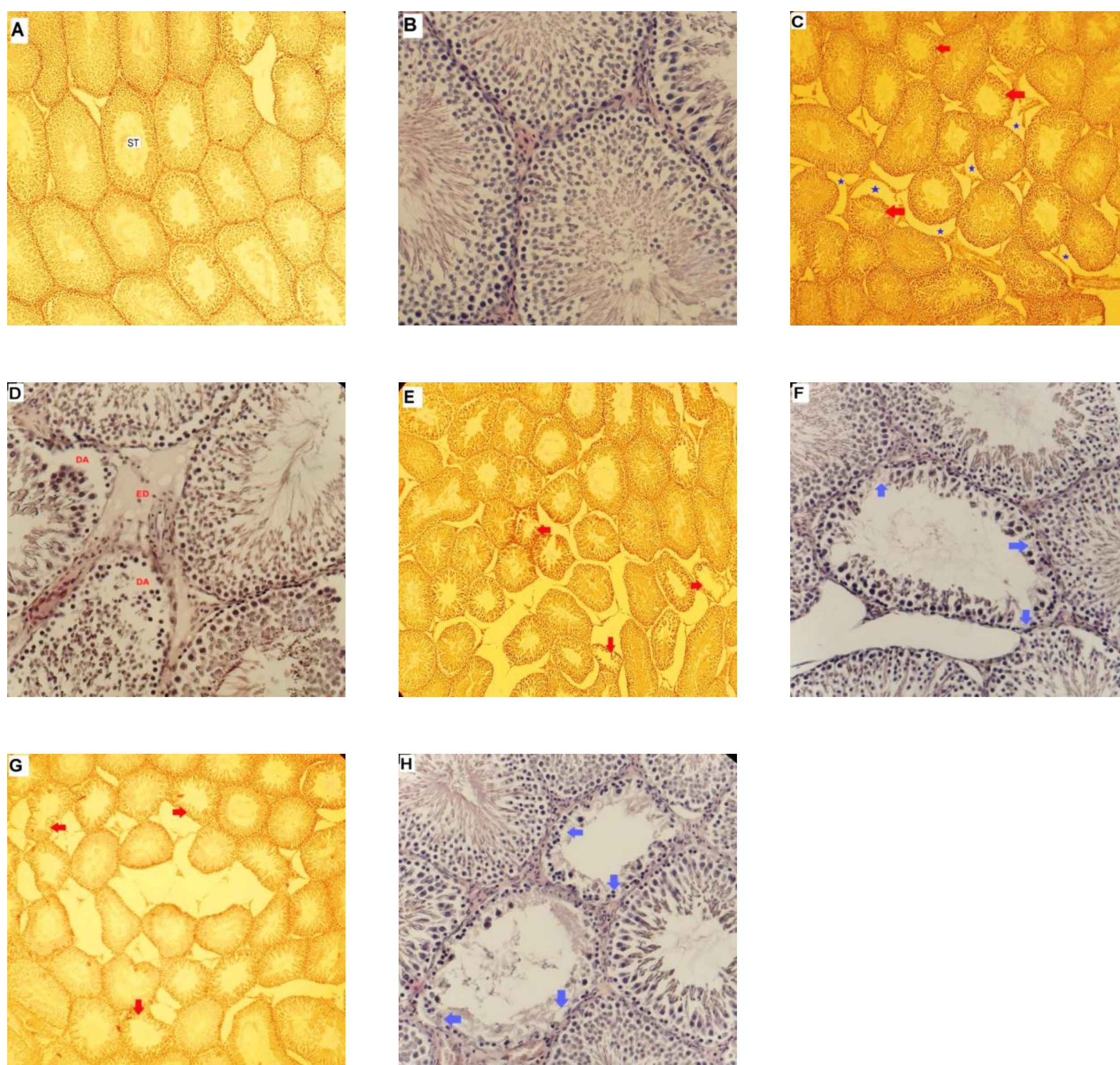

Figure 7. *Cross section of testicular tubules in various groups. A, B: Control; Seminiferous tubules (ST) in control groups have normal structure and germinal epithelium have normal cellular arrangement. C, D: Diabetic; Tubular atrophy (red arrows), increase of intercellular space (blue asterisks), connective tissue edema (ED) and disarrangement of spermatogenic cells (DA) was seen in sections from diabetic groups. E, F: Diab+MSG6; Tubular atrophy (red arrows) and reduction of spermatogenic cells population (blue arrows) was seen. G, H: Diab+MSG60. Higher dose of MSG led to more tubular atrophy (red arrows) and increase of the number of tubules with depopulated germ cells (blue arrows). Hematoxylin and Eosin staining. Magnification: A, C, E & G 100×; B, D, F & H 400×*

Glutamate has several roles in biological systems such as regulation of gene expression, of antioxidant reactions and immune responses [20]. The most discussed effects of MSG intake are neurotoxic effects in brain, increased risk for obesity and metabolic defects, Chinese restaurant syndrome and detrimental effects on sex organs [21]. Our knowledge about the effects

of MSG on the reproductive system is limited and usually based on the animal studies. Spermatogenesis is regulated by pituitary gonadotropins [22,23]. Imbalance of gonadotropins may lead to structural and functional alterations of testicular tissue [17]. Increased amounts of glutamate are associated with augmentation of intracellular calcium levels which may lead to neuronal cell death [24,25]. Hypothalamic neuronal cell death is specified following the administration of MSG to adult mice [26]. Hypothalamic-pituitary-gonadal axis dysfunction can lead to testicular functional alterations [27]. High doses of MSG cause neuronal necrosis in hypothalamic arcuate nuclei in neonatal rats [28].

Laboratory routes of MSG administration such as subcutaneous or intraperitoneal in rats may be hardly compared with the oral intake of MSG. However, it has been reported that low dose of MSG (3 g/kg/day) mixed with male rats foods for 14 days was led to degenerative changes of cerebellar cortex such as pyknotic Purkinje and granule cells with areas of degeneration surrounded by inflammatory cells in granular layer [29]. According to our results, MSG-treated control rats had more weight gain compared to the non-treated control group. In this regard, MSG-treated diabetic rats had a lower weight loss in comparison to non-treated diabetic animals. The reduction of body weight can be due to defect in glucose metabolism and excessive breakdown of tissue proteins following induction of diabetes [1,30,31]. The administration of MSG leads to increase of body weight in rats [32]. In this study, similar to our previous study [33], the analysis of body weight showed that higher dose of MSG leads to more body weight gain. One of the possible mechanisms of MSG-mediated body weight increment may be related to disrupting the hypothalamic signaling of leptin action [34]. Thus, obesity after MSG administration in rats may be the result of leptin resistance [35]. Increment of mRNA expression of interleukin-6, tumor necrosis factor-alpha, resistin and leptin in visceral adipose tissue, insulin, resistin and leptin levels in serum and impairment of glucose tolerance are reported related to the effects of MSG on increasing of body weight [36]. However, the mechanisms leading to obesity in animals and humans are different. In this way, it has been reported that, in humans, MSG-mediated obesity could be due to the increase in the intake of high caloric savory foods [37]. Streptozotocin specifically destroys the β-cells of pancreatic islets, leading to a decline in insulin levels and hyperglycemia [38]. Increment of insulin levels in serum and glucose tolerance impairment was reported following administration of MSG [36]. Accordingly, in our study the blood glucose levels were reduced following the administration of monosodium glutamate and this reduction was seen in more degrees for the higher dose of MSG, though this did not reach statistical significance.

Spermatogenesis is regulated by pituitary gonadotropins [22,23]. Imbalance of gonadotropins may lead to structural and functional alterations of testicular tissue [17]. In our study, slight decrement in FSH levels was seen after administration of MSG. Moreover, induction of diabetes and subsequently decline of insulin may lead to reduction of blood FSH levels. 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 [39].

In our study, MSG-treated groups had lower levels of LH compared to non-treated groups. It was shown that reduction of insulin secretion resulted to LH decrement and subsequently malfunctions of Leydig cells [40]. Insulin has a key role in the metabolism of Leydig cells (as

main target cells for LH) and maintenance of LH receptors on these cells [3]. The administration of MSG is associated with the reduction of LH and testosterone levels [41]. According to our results and previous studies it seems that use of MSG in diabetic conditions could lead to a further reduction in blood LH levels. MSG mediated reduction of testosterone levels that has been mentioned in previous studies, was also observed in our work. It is reported that, the number of LH binding sites in Leydig cells from diabetic rats is severely lowered after induction of diabetes [4]. These processes lead to depression of synthesis and secretion of testosterone by Leydig cells. Eventually, it seems that MSG and diabetes, by affecting the pituitary hormones, alter testosterone secretion.

The decrease in diameter of seminiferous tubules following induction of diabetes has been reported in previous studies [2,4,42]. Our results showed that treatment with MSG also led to reduction of STs diameter. This finding indicates that the seminiferous tubules became atrophied following administration of MSG. Moreover, administration of MSG in diabetic rats reduced further the diameter of seminiferous tubules. Depopulation of germ cells can lead to the atrophy of seminiferous tubules. In our study, a decrease of the number of primary spermatocytes and spermatids indicates the reduction of cell divisions. Accordingly, this reduction was seen in more degrees in MSG-treated diabetic animals. These findings indicate that MSG and diabetes can induce some alterations in spermatogonial cell division and production of primary spermatocytes and spermatids. The reduction of spermiation and repopulation indices in MSG-treated diabetic rats confirms these alterations. The histological observations in STs, illustrate depressed cellular activity of spermatogenic cells. Male infertility, testicular hemorrhage, alteration of sperm production and morphology and hypogonadism has been reported following the administration of MSG [10-13].

Conclusions

In conclusion, the results of this study suggest that the long term administration of monosodium glutamate may induce in a dose dependent manner structural and functional alterations of testicular tissue in diabetic rats. Finally, further studies are needed to better understand the mechanism of action of monosodium glutamate on the structure and the functions of reproductive system and despite the fact that these results are based on animal studies and cannot be extrapolated automatically to humans; they can be helpful in specifying therapeutic and nutritional strategies. However,

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