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INVESTIGATION OF OPIOID GROWTH FACTOR PATHWAY INHIBITION ON THE HISTOLOGIC STRUCTURE OF TESTICULAR TISSUE AND MICROSCOPIC INDICES OF SPERMATOGENESIS IN ADULT DIABETIC MICE

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Abstract

Background and aims: *Endogenous opioids function as negative factors affecting the growth has been established. The most influential factor in the growth and differentiation of the proliferating cells is the opioid growth factor (OGF). Recently, some studies have been completed about the effects of opioid growth factor in the pathogenesis of diabetes and the beneficial effects of inhibition of this growth pathway have been demonstrated. The aim of this study was to investigate the effect of inhibition of opioids growth pathway, in proliferation and growth of testicular germ cells and spermatogenesis following experimental diabetes in adult mice.* ***Material and methods:*** *Diabetes was induced in adult mice by Streptozotocin. Diabetic animals were treated with Naltrexone 15, 30 and 60 mg/kg for 60 days. At the end of the study, testicular and body weight was recorded, tissue samples were collected and histomorphometrical studies were performed under light microscope.* ***Results:*** *The results showed that the use of naltrexone has a little effect on preventing diabetic weight loss. Histomorphometric indices such as tubular diameter and germinal epithelium height were improved dose dependently in naltrexone treated diabetic mice. The number of tubular germ cells was increased non-significantly in diabetic animals following administration of naltrexone. Improvement of microscopic indices of spermatogenesis was observed in naltrexone treated diabetic mice.* ***Conclusions:*** *According to the results of this study and the role of naltrexone as OGF-OGF receptor inhibitor and up-regulating activity of naltrexone which leads to increased DNA synthesis and cell division process, the administration of naltrexone could be effective in reduction of diabetic induced alterations of spermatogenesis.*

key words: *Endogenous opioids, Diabetic mice, Spermatogenesis, Testicular tissue*

Background and aims

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]. Previous studies reported that diabetes is one of the most

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important risk factors for male infertility problems. In this regard, cellular alterations in testicular microenvironment are associated with diabetes mellitus [3,4]. Diabetes is a condition of increased oxidative stress and impaired energy metabolism. Recent experimental and clinical studies suggest that oxidative stress and 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 by affecting of various cellular activities [5,6].

Endogenous opioid peptides are known to be potent regulators of growth as well as neuromodulators/ neurotransmitters [7]. One native opioid peptide, [Met^5]-enkephalin, has been identified as a negative growth regulator [8-10]. This peptide was termed opioid growth factor (OGF). OGF has various actions and functions in development, cellular renewal, cancer, wound healing, and angiogenesis [11]. This peptide can influence the reduction of cell proliferation through delaying of G_0/G_1 phase of the cell cycle [11]. In this regard, the application of OGF antagonists such as naltrexone (NTX) can interfere with OGF activity and subsequently can accelerate growth by removal of inhibitory signals [12]. According to the reduction of germinal cell populations associated with diabetes and the inhibitory role of OGF on the growth and proliferation of cells in most tissues, the aim of this study was to investigate the effects of the administration of OGF antagonists on the reduction of tissue alterations and improving of cell population in testicular tissue following induction of experimental diabetes.

Material and methods

Chemicals and treatments: Streptozotocin, STZ, (*Sigma, ST. Louis, MO*) 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 (160 mg/kg body weight) in overnight fasting animals. Diabetes was confirmed 48 hours after injection of STZ with an automated glucose analyzer device (EASY GLUCO, Infopia Co., Ltd., Korea). The animals with blood glucose levels above 400 mg/dl were considered diabetic and were used in this study [13]. Naltrexone hydrochloride (Sigma-Aldrich Chemie GmbH, Germany) was dissolved in distilled water and injected intraperitoneally in three doses of 15, 30 and 60 mg/kg once a day for six weeks after induction of diabetes. Metformin hydrochloride (GLUCOPHAGE, Merck Sante s.a.s., LYON - FRANCE) dissolved in distilled water and administrated with a dose of 150 mg/kg by oral gavage method for six weeks was used as active control [14].

Animal procedures: 60 adult male mice with mean body weight 28.50 g were divided into six experimental groups: 1) *Control group:* normal and healthy mice that did not receive any type of treatment; 2) *Diabetic group:* in this group, two weeks after confirmation of diabetic state, the normal saline was injected intraperitoneal for six weeks and the animals were euthanized at the end of eight weeks of study; 3) *15 mg/kg NTX administrated diabetic group (Diab+Nalt15)* diabetic mice which received 15 mg/kg of NTX for six weeks; 4) *30 mg/kg NTX administrated diabetic group (Diab+Nalt30)* diabetic mice which received 30 mg/kg of NTX for six weeks; 5) *60 mg/kg NTX administrated diabetic group (Diab+Nalt60):* in this group, naltrexone was administrated with dose of 60 mg/kg daily for six weeks and the animals were euthanized eight weeks after induction of diabetes; *6) Metformin administrated diabetic group (Diab+Met):* in this group, two weeks after induction of diabetes, metformin was given orally to diabetic animals for a period of six weeks.

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 and testicular weight: At the end of study, the weight of each animal was recorded. Afterward, the right and left gonads of each mouse were separated from the body and their weights were recorded with digital scale.

Tissue preparation and morphometric analysis: Testicular tissues were immediately fixed in 10% buffered formaldehyde solution. Paraffin embedded samples were cut with thickness of six micrometers and were stained with hematoxylin and eosin (H&E) method. For morphometric assessment of seminiferous tubules, the slides were studied at 400× magnification. The analyses were performed from images obtained and digitalized using Dino-Eye Eyepiece Camera AM7023B (Dino-Lite Digital Microscope). The images were processed by Dino-Lite image analysis system software. 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 [15].

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 deviation (SD). 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

Blood glucose: following the administration of STZ, the mean of blood glucose levels was significantly increased in diabetic groups (Table 1). Accordingly, the mean blood glucose decreased following the administration of metformin, but did not reduce to the levels of the control group. In all diabetic groups treated with NTX, the mean blood glucose was significantly higher in comparison to control and Diab+Met groups.

Body weight and testicular weight: there was no significant difference for initial body weight between groups (Table 1). The comparison of final body weight showed that, in NTX and metformin treated diabetic mice, the mean of body weight was reduced. This reduction was significant in the Diab+Nalt30 and Diab+Met groups in comparison to control mice. According to the results, the administration of NTX dose dependently leads to body weight decrement. The mean testicular weight increased in the

diabetic group in comparison to other groups. Moreover, the administration of NTX in diabetic mice, dose dependently induced the increase of testicular weight, but this increase was not statistically significant.

Table 1. Mean blood glucose, body weight and testicular weight in the experimental groups.

	Blood glucose (mg/dl)	Initial body weight (g)	Final body weight (g)	Testicular weight ($\times 10^{-3}$ g)
Control	93.80±16.42*	29.50±3.00	34.40±3.71	180.0±15.23
Diabetic	468±74.22	26.00±5.83	28.50±5.42	202.3±23.84
Diab+Nalt15	427.80±132.70	27.00±4.24	27.00±7.07	164.0±1.414
Diab+Nalt30	491.70±30.88	27.33±2.42	25.33±3.72	181.0±47.35
Diab+Nalt60	477.80±152.50	33.50±3.41	30.00±1.63	189.8±41.82
Diab+Met	199±110.00*	28.00±5.29	24.00±2.00	192.0±17.09
P value	<0.0001	-	<0.019	-
F ratio	18.77	-	3.423	-

Blood glucose: * significant different in comparison to all diabetic groups; Final body weight: * significant difference in comparison to control group. Data are presented as mean±SD

Table 2. Testicular morphometry and microscopic indices of spermatogenesis in the experimental groups.

	Tubular diameter (mμ)	Germinal epithelium height (μm)	TDI (%)	SPI (%)	RI (%)
Control	202.0±20.76	71.56±11.60	93.33±10.33	83.33±15.06	79.10±8.439
Diabetic	160.9±7.025*	37.16±6.06*	50.00±20.98*	30.00±30.33*	56.70±13.52*
Diab+Nalt15	140.0±29.31*β	40.15±13.11*	53.33±16.33*	36.67±23.38	59.90±9.183*
Diab+Nalt30	151.1±23.75*	52.64±5.83*β	63.33±15.06	50.00±32.86	62.00±10.66*
Diab+Nalt60	177.2±18.88	60.24±5.86 βα	66.67±16.33	50.00±35.21	65.70±12.98
Diab+Met	158.4±13.65*	51.92±6.68*	66.67±27.33	76.67±23.38	64.70±12.18
P value	<0.0002	<0.0001	<0.0059	<0.0114	<0.0012
F ratio	7.052	12.79	4.104	3.602	4.724

Tubular diameter: * significant difference in comparison to control group; β significant difference in comparison to Diab+Nalt60 group. Germinal epithelium height: * significant difference in comparison to control group; β significant difference in comparison to diabetic group; α significant difference in comparison to Diab+Nalt15 group. TDI, SPI and RI: * significant difference in comparison to control group. Data are presented as mean±SD

Table 3. Cellular population of seminiferous tubules in experimental groups.

	Sertoli Cells (#/20 tubules)	Spermatogonia (#/20 tubules)	Spermatocytes (#/20 tubules)	Spermatids (#/20 tubules)
Control	12.20±2.09	31.40±11.25	34.60±8.208	127.7±25.71
Diabetic	9.80±2.25	31.70±6.00	29.00±5.774	103.5±19.11
Diab+Nalt15	10.90±2.58	33.80±4.26	31.10±5.065	99.80±15.53
Diab+Nalt30	10.70±2.62	32.10±6.91	32.20±5.245	108.5±23.72
Diab+Nalt60	11.50±2.06	32.70±4.59	33.30±5.539	117.8±30.55
Diab+Met	11.80±1.93	30.20±5.77	32.60±6.293	118.1±23.43

Data are presented as mean±SD

Testicular morphometry: according to Table 2, the diameter of STs was reduced significantly in all diabetic groups (except Diab+Nalt60) in comparison to control mice. In this regard, the administration of NTX in diabetic mice, dose dependently lead to an increase of the diameter of STs. Changes observed in the germinal epithelium height in experimental groups were similar to the results of tubular diameter.

Microscopic indices of spermatogenesis: all indices of spermatogenesis were increased after the administration of NTX in diabetic groups (Table 2).

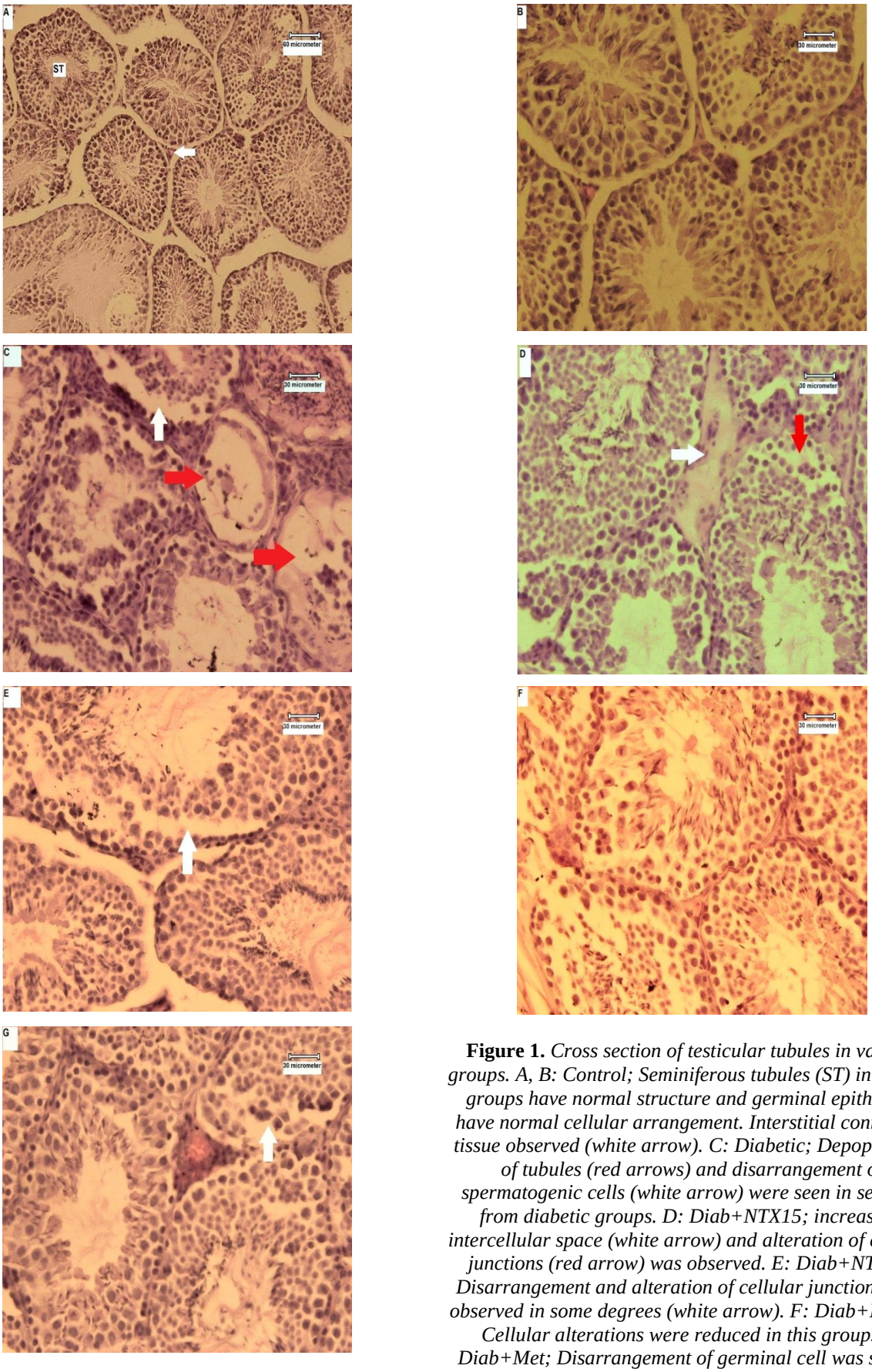

Figure 1. *Cross section of testicular tubules in various groups. A, B: Control; Seminiferous tubules (ST) in control groups have normal structure and germinal epithelium have normal cellular arrangement. Interstitial connective tissue observed (white arrow). C: Diabetic; Depopulation of tubules (red arrows) and disarrangement of spermatogenic cells (white arrow) were seen in sections from diabetic groups. D: Diab+NTX15; increase of intercellular space (white arrow) and alteration of cell-cell junctions (red arrow) was observed. E: Diab+NTX30; Disarrangement and alteration of cellular junctions were observed in some degrees (white arrow). F: Diab+NTX60; Cellular alterations were reduced in this group. G: Diab+Met; Disarrangement of germinal cell was seen in some degrees (white arrow). Hematoxylin and Eosin staining. Magnification: A 100×; B, C, D, E, F & G 400×*

Cellular population of STs: as Table 3 shows, with the exception of spermatogonia, the number of various cell types in testicular STs was reduced following induction of diabetes. A non-significant increment was observed in cell populations of STs following the administration of NTX in diabetic groups.

Histologic findings: the most noticeable changes observed in the histologic evaluation of testicular seminiferous tubules in diabetic mice were tubular atrophy; disarrangement and depopulation of spermatogenic cells, alteration of cell-cell junctions and increase of intercellular space (Figure 1). In NTX treated diabetic groups, the above mentioned microscopic changes were observed with various intensities. Moreover, cellular and histologic changes were reduced following dose dependent administration of naltrexone in diabetic animals. In metformin treated diabetic mice, the histologic alterations of testicular tissue were improved in comparison to diabetic animals.

Discussions

Increase of blood glucose levels leads to structural and functional changes in various target tissues and organs [2]. Diabetes is one of the most important risk factors for male infertility. In this regard, cellular alterations in testicular microenvironment are associated with diabetes mellitus [16]. Endogenous opioid peptides (OGF; [Met^5]-enkephalin) act as growth factors in neural cells and other cells and tissues [11]. They are potent regulators of growth and known as opioid growth factors [8-10]. The relationship between opioid peptides and diabetes has been considered in different studies [17-26]. OGF activity was targeted to cells that were developing, renewing, or repairing. Testicular tissue possesses potent spermatogenic cells which mitotic and meiotic cell divisions occur permanently in this microenvironment. Our study is the first to report about the role of OGF and the effect of its inhibition on the cellular and tissue alteration of the male gonads in hyperglycemic condition in laboratory animal model.

In this study, decrease of body weight was observed in NTX and metformin treated diabetic mice in comparison to control and untreated diabetic groups. The reduction of body weight could be due to the breakdown of tissue proteins in diabetic conditions [1,27]. It can be deduced that, NTX had no effect in reduction of blood glucose levels and could not prevent diabetic weight loss. Consequently, severely increased blood glucose should be associated with weight loss while preserved blood glucose with weight maintenance. The administration of metformin to diabetic animals led to decline of blood glucose levels however, metformin treated mice showed weight loss similar to other diabetic groups. Metformin can reduce the adipose tissue mass, therefore, does not cause weight gain in diabetic mice [28,29]. However, our result showed that, mean testicular weight was increased in all experimental groups in comparison to control mice. In this regard, the ratio of testicular weight to body weight was not significant between groups (data not shown).

OGF has been identified as a negative growth regulator [11]. The biological effects of OGF can be blocked by naltrexone [30]. An increase of OGF levels leads to the reduction of repopulation and wound healing, and reduces the DNA synthesis, cellular migration and tissue organization [11]. Accordingly, blockade of interaction between OGF and its receptor (OGFr) through opioid antagonists could lead to improvement of cell growth and tissue organization. It has been reported that, consumption of NTX in diabetic mice leads to reduction of wound size from 13 to 30 percent in comparison to diabetic mice treated with normal

saline [31]. The presence of opioid receptors (mu, kappa and delta) in Sertoli and Leydig cells and the existence of opioid peptides synthesis pathways in Leydig cells have been proven so, it has been realized that these compounds have an important regulatory effects on cellular and hormonal activity of testicular tissue [32]. It has been shown that, the alteration of testicular functions related to the administration of opioid receptors agonists (morphine) has been reduced and inhibited after using of NTX [33].

In this study, all histomorphometric indices (STs diameter, germinal epithelium height and atrophy of STs) were reduced in the diabetic group. Treatment of diabetic mice with NTX, dose dependently leads to an improvement of these indices. Increase in population of germinal epithelium was observed following the administration of NTX in diabetic mice. Also, we observed an improvement of microscopic indices of spermatogenesis and histologic modifications.

Conclusions

In adult diabetic mice, the induction of diabetes is associated with structural and functional alteration of testicular tissue through the reduction of spermatogenic cells population and alteration of cell proliferation. Naltrexone, an OGF antagonist, could be effective in reducing diabetic alterations of spermatogenesis.

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Conflicts of interest: The authors declare that they have no conflict of interest.

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