

## Morphological Evaluation of Testis Tissue of Rats in Various Time Points after Diabetes Type 1 Induction

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### Abstract

**Objective:** Previous studies have indicated the hyperglycemia-induced cell death in various tissues such as brain, kidneys, liver and especially in the testis. Recent studies have reported that diabetes can trigger male infertility. In this study we report the histological analysis of the testis tissue after diabetes type 1 induction in wistar rats.

**Materials and Methods:** At various time intervals after diabetes type 1 induction, testicular cell was assessed in the controls and in the diabetic rats. Hyperglycemia was induced in male wistar rats by intraperitoneal injection of drug streptozotocin (STZ). At different time points (4, 6, 8 and 20 weeks) post diabetes type 1 induction, rats were euthanized and testicular tissues were removed for histological analysis. Their testes were fixed in formaldehyde (37%), embedded in paraffin and then sectioned (4µm thick). They were further deparaffinized, stained with Hematoxylin-Eosin (H&E) and DAPI, and observed under light and fluorescence microscope, respectively.

**Results:** Histological results showed reduced cell density in testis, which indicates that diabetes type 1 and hyperglycemia conditions impair normal cell density in testis tissue. The changes in seminiferous tubules from 4 weeks to 20 weeks were also observed. The testicular histology of diabetic animals shows that the maximum reduction in cell density occurred after 20 weeks.

**Conclusion:** The induced diabetic condition provides evidence that hyperglycemia plays an important role in pathogenesis of diabetes, and also indicated that chronic hyperglycemia eventually leads to cell death and male infertility. Probably, the consequent of inflammatory condition of hyperglycemia resulted in apoptotic-related gene products and testicular dysfunction which has an important implication for infertility, and offer new chances for therapeutic interventions.

**Keywords:** Diabetes, Infertility, Streptozotocin, Testis

### Introduction

Infertility affects 13-18% of couples, and growing evidence from clinical and epidemiological studies suggests an increasing incidence of male reproductive problems (1). Diabetes has been associated

with reproductive impairment in both men and women (2-4). Although the pathophysiology of reproductive derangements in young diabetic women has been largely investigated (5,6), few studies have been

conducted in men. The defective spermatogenesis may be the consequence of a direct testicular effect from the diabetes (7,8). Testicular cell apoptosis is an important event of diabetes and hyperglycemia conditions (9,10). However, the mechanisms that cause inflammation and apoptosis are beginning to be emerged. Although several studies have been reporting the role of diabetes in the infertility (11,12) but the morphological changes and time course of cell death after diabetes type 1 induction have not been reported so far. The reduction of tissue cell density after diabetes induction is a major factor in infertility (13,14). Several mechanisms are proposed for testicular apoptosis and cell death in diabetes. For example, hyperglycemia can induce ROS production that leads to cell apoptosis in various tissues such as testis (2,15,16). These defects are related to early apoptosis in diabetic patient (17). In this study, we evaluated the effect of chronic hyperglycemia on morphological cell density of rat testes tissue in various time intervals after diabetes induction.

## Materials and Methods

### *Animals*

In this research, adult Wistar rats (6-8 weeks old and 200–250 g weight) were mated and the pups were grown to 6-8 weeks old. They were housed and maintained at a constant temperature of 20-22° C with a relative humidity of 55% and standard 12:12 h light-darkness cycles, and had free access to standard rat chow ad libitum and tap water, and allowed 1 wk acclimatizing to the laboratory conditions before experiments. All the procedures including diabetes induction and sacrifice operation were in strict accordance with Iranian legislation on use and care of laboratory animals.

### *Hyperglycemic rat model:*

Adult male rats (6-8 weeks old, weighing 200–250 g) were intra-peritoneally injected with a single dose of streptozotocin (STZ; Sigma-

Aldrich, Germany) at 55 mg/kg body weight, dissolved in 10 mM sodium citrate buffer (pH 4.5) after 12 h of food deprivation. Rats injected with citrate alone without STZ served as the normal control. On day 2 after STZ induction, a blood sample was obtained from the rat tail vein, and random glucose levels were measured using the One-Touch Ultra 2 blood glucose monitoring system (LifeScan, Mountainview, CA). For the present study, hyperglycemia was defined as a blood glucose level of 20 mM or higher. Citrate buffer-treated rats were used as a normoglycemic control (blood glucose <12 mM). Groups of 5 diabetes rats were sacrificed after CO<sub>2</sub> anesthesia at weeks 4, 6, 8 (early phase of diabetes progression), 20 (late phase of diabetes progression). Blood was collected by cardiac puncture for biochemical analysis, and testis tissues were removed for further analysis. Five control rats in each above-mentioned time point were also sacrificed and studied.

### *Tissue preparation and testicular histology analysis:*

In specified time points (4, 6, 8 and 20 weeks) post diabetes type 1 induction and also in control groups, rats were euthanized and after laparotomy, testis tissues were dissected. One of testicular samples was immediately fixed for 1 day at room temperature in formaldehyde (10%) for histological examinations. The tissues were processed according to the routine program of a tissue processor, and paraffin blocks were prepared. Specimens were cut in 4 µm-thick sections by a rotary microtome, and mounted on gelatin-coated glass slides. Testis sections (4µm) were de-paraffinized in xylene, rehydrated in decreasing concentrations of alcohol in water, and stained with hematoxyline-eosin (HE) and DAPI reagent (Sigma chemicals Co, St. Louis, MO) and observed under the light and fluorescence microscopy, respectively, for evaluation of testicular cell density. The cell nuclei were stained with DAPI staining.

## Results

### *Biochemical analysis of type 1 diabetes induction in rats.*

Diabetes was induced in rats to evaluate whether high blood glucose affects morphological testis cell density. Rats with blood glucose of >250-300 mg/dL were defined as diabetic rats. Blood glucose levels on the day (4, 6, 8 and 20 after diabetes type 1 induction) of organ removal was <110 mg/dL for healthy groups and > 250 mg/dL for diabetic groups. STZ-treated rats showed typical features of diabetes, including significant hyperglycemia (blood glucose  $\geq$ 250mg/dl), insulin deficiency, and slow weight increase (BW<200gr), compared to normal controls (Figures 1,2,3).

### *Histological examination of testes obtained from STZ-induced type 1 diabetic rats:*

Histological sections of testis were taken from the rats at specific time points(4, 6, 8 and 20 weeks) post diabetes type 1 induction and compared to the untreated control group which revealed progressive reduction of density in various specific times after diabetes type 1

induction. These changes were not present in the non-diabetic rats. H & E staining of testes tissue sections from adult diabetic rats demonstrated general structure changes and gradual testicular cell density reduction at time point intervals (week 4, 6, 8 and 20) post diabetes induction. Also, DAPI staining of the same tissue sections confirmed the former data and exhibited that the number of nuclei in the testes from diabetic rats reduced significantly after week 4, 6, 8 and 20, post diabetes induction (Figure 4).

## Discussion

The present study was designed to analyze the effects of induced hyperglycemia (diabetes type 1) following STZ administration on morphological structure of testis tissues of male wistar rats. Type 1 diabetes (T1D) is an organ-specific autoimmune disease that results from T cell-mediated destruction of insulin-producing pancreatic beta cells in genetically predisposed individuals (18).

In order to induce hyperglycemia and diabetes type 1, we used STZ drug. The major effect of STZ is on pancreatic Langerhans  $\beta$  cells. Thus,

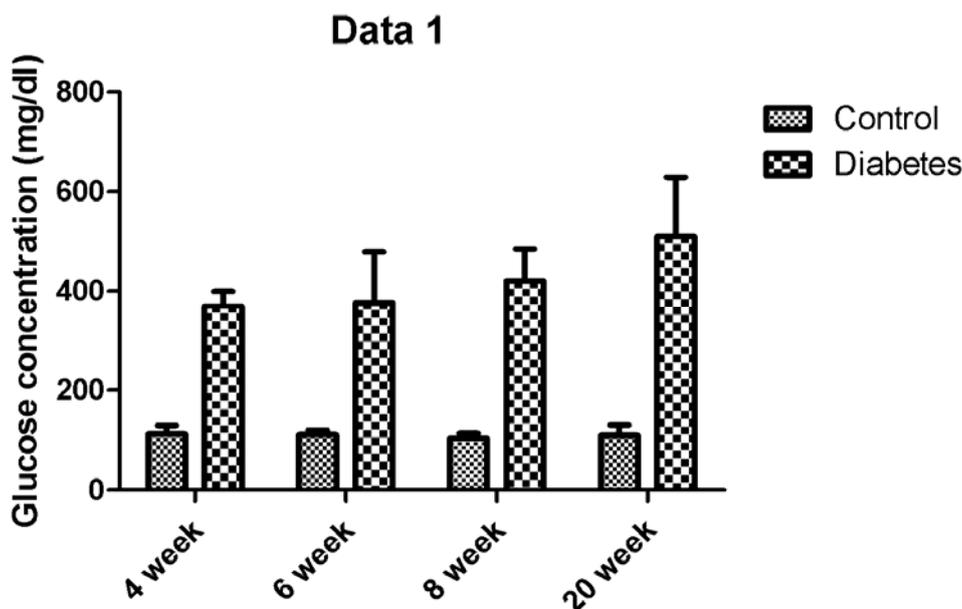


Figure 1. Blood glucose concentration in STZ-untreated control and STZ-treated diabetic groups at specific times (4, 6, 8 and 20 weeks) after diabetes type 1 induction.

Comparison (Mean $\pm$ SD) with the control group. \* $P$ <0.05, Sample size: 5 male rats, Statistical methods: ANOVA and student t-test.

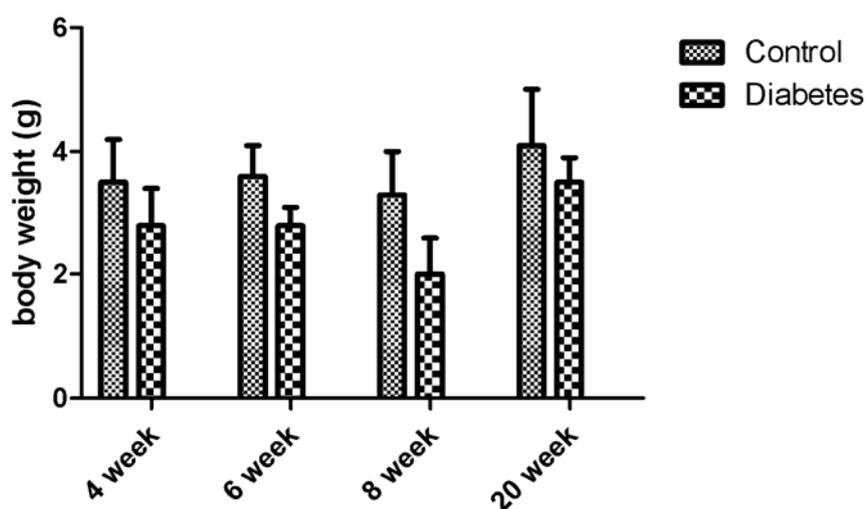


Figure 2. Changes in body weight in the study groups during the experiment. Comparison (Mean $\pm$ SD) of body weight between STZ-untreated control and STZ-treated diabetic group at specific time after diabetes type 1 induction, \* $P$ <0.05.

Sample size: 5 male rats

Statistical methods: ANOVA and student t-test

it seems that the changes appearing in other organs after STZ administration are related to diabetes and not to STZ. However, some of the changes may occur due to the acute toxic effect of STZ. Analysis of variance indicated that the groups which received STZ had less body weight than the other groups. The results

of blood glucose determination showed that blood glucose levels in diabetic groups increased more than non-diabetic groups. Elevated blood glucose is due to insulin-secreting beta cell destruction and reduced insulin levels.

### Data 1

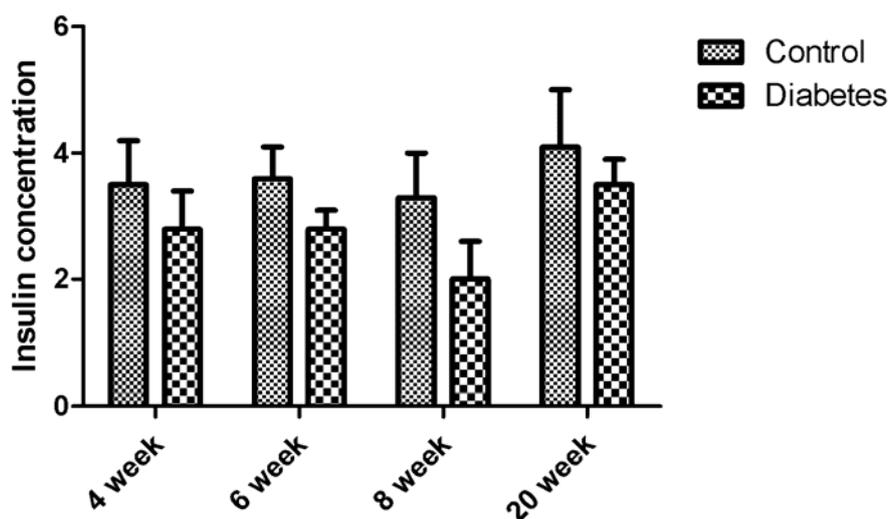
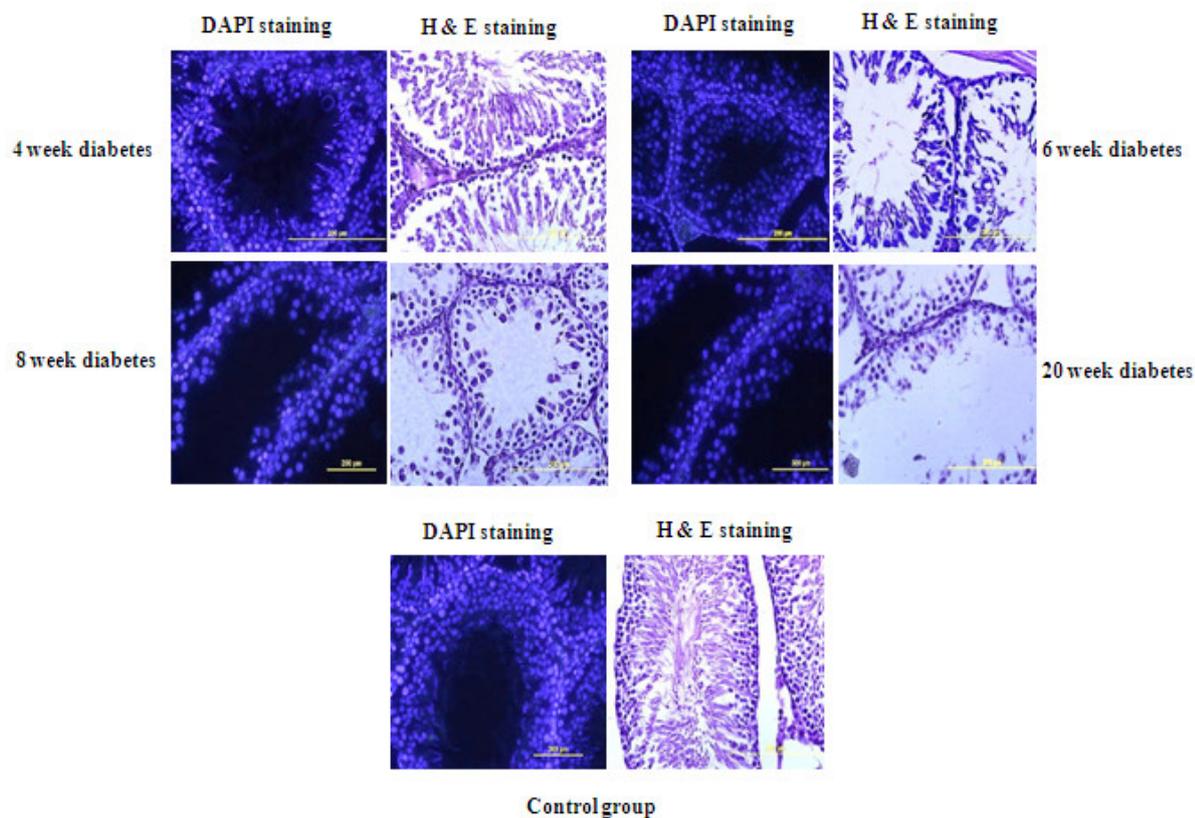


Figure 3. Blood insulin concentration in control and diabetic indicate STZ-untreated control and STZ-treated diabetic group at specific times after diabetes type 1 induction.

Comparison (Mean $\pm$ SD) with the control group. \* $P$ <0.05, Sample size: 5 male rats, Statistical methods: ANOVA and student t-test.



**Figure 4.** Histological sections of testis tissues from the STZ-untreated control group and the STZ-treated rats at the specific time points 4, 6, 8 (early phase) and 20 weeks (late phase) after diabetes type 1 induction. By subjective visual evaluation, the number of testicular cell density was reduced in all of the diabetic rats, especially after 20 weeks. The changes in seminiferous tubules from 4 weeks to 20 weeks are seen. The testicular histology of diabetic animals shows that the maximum reduction in cell density occurred after 20 weeks.

Histological studies in adult diabetic rats showed testicular cell density reduction. H & E staining demonstrated general structure of tissue sections and DAPI staining showed total number of nuclei in tissue sections. As the photomicrographs show (Figure 4), the nuclear cell density was declined in various times after diabetes type 1 induction, especially after 20 weeks. One of the proposed mechanisms of testicular cell density reduction is overexpression of apoptotic and pro-inflammatory mediators in the testis tissue, which can stimulate cell death (19). Therefore, the size and volume of testis tubules and hence testis volume are decreased in various diabetic groups. Diabetes changes cellular microenvironment, and leads to several unwanted effects (20).

A number of studies have shown that the inflammation induced by hyperglycemia is the main mechanism of the pathogenesis of cell

apoptosis and diabetic infertility (2,15,21,22). So, inflammation has a prominent role in apoptosis in various organs in hyperglycemic condition (23,24). It is said that the innate immune system plays a role in over-expression of pro-inflammatory cytokines in diabetic condition (25-28). Inflammatory cell-related apoptosis contributes to organ damage and micro and macro vascular complications (21). There are compelling evidences that the innate immune system plays a key role in early mechanisms triggering diabetes (26,29,30). A number of studies demonstrated that toll like receptors (TLRs) mediate innate immune responses and contribute to the induction of diabetes (30,31). Also, few studies reported that innate immunity and TLRs have a tight correlation with apoptosis and cell death (32-35). Also, it is demonstrated that in male infertile patients, oxidative damage has a critical role in cell apoptosis (2,36).

Several cellular and molecular mechanisms are proposed for cell apoptosis in diabetic subjects. Previous studies indicated that diabetes induces advanced glycation end products (AGEs). AGEs contribute to reactive oxygen species (ROS) production that lead to oxidative stress and cell death (19,20). Also, AGEs in diabetic conditions induce inflammation via over-expression of pro-inflammatory cytokines and chemokines such as TNF- $\alpha$  and IL-1 $\beta$  in various cells especially monocytes and macrophages (20,21). Pro-inflammatory cytokines increase endothelial permeability and induce leukocyte adhesion to vascular endothelium (20). Pro-inflammatory secretion by leukocytes leads to destruction of various tissues in diabetic subjects. The up-regulation of pro-inflammatory cytokines following hyperglycemia activates nuclear factor kappa B (NF- $\kappa$ B) which translocate from the cytoplasm to the nucleus, and regulates the over-expression of inflammatory response leading to cell impairment and apoptosis. Also, pro-inflammatory cytokines such as IL-1 $\beta$  can trigger the over-expression of Fas and induce Fas-mediated apoptosis (25). Other mechanisms are also proposed for cell apoptosis in diabetic conditions. Hyperglycemia causes to cell death through increase oxidative stress, release of intracellular Ca<sup>2+</sup>, activation of mitogenic agents and impairment of protein kinases cell signaling pathways (25,37,38). A study on human umbilical vein endothelial cells shown that hyperglycemia enhanced apoptosis and down-regulated vascular endothelial growth factor (VEGF). Elevated glucose in diabetic conditions up-regulated Bax protein and increased the Bax/Bcl2 ratio that can activate caspase-3 and initiate apoptosis (39,40). High glucose may initiate apoptosis by activating c-Jun NH2-terminal kinase/stress activated protein kinase (JNK/SAPK)(41). Another molecular mechanism proposed for apoptosis in diabetic condition is the over-expression of cyclooxygenase 2 (COX-2) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and next a caspase-3 activation that triggers apoptosis. Hyperglycemia can initiate

NF- $\kappa$ B activation and up-regulation of COX-2 that lead to PGE<sub>2</sub> production and cell death (42). Also, diabetic condition induce cell apoptosis through up-regulation of AGE receptors (RAGE) that caused to over-expression of pro-apoptotic genes including p38, c-Jun N-terminal kinase(JNK), caspase-8 and caspase3 (20). A main target organ affected after diabetes is the testis. Some previous studies reported that the diabetes can affect kidney (43,44), so, hyperglycemic conditions can induce apoptosis in renal cells and cause diabetic nephropathy (45-47). In addition to hyperglycemia, other factors such as fatty acids can contribute in pathological aspects of diabetes. For example, ectopic lipid accumulation can initiate cellular apoptosis and testicular dysfunction (48). Several studies reported that high concentration of glucose can induce ROS and pro-inflammatory mediators in diabetic conditions (20). Over-expression of these mediators can induce apoptotic cell signaling in various organs. Excessive accumulation of ROS in cells leads to oxidative stress (48). Probably, hyperglycemia initiates testis-cell apoptosis by intrinsic pathways including molecules Bcl-2, Mcl-1 and Bcl-xl. Also, pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 that produced by NLRP3 inflammasome activation may trigger testicular cell death (49,50). Recent studies reported that chronically-high glucose can initiate the over-expression of innate immune systems such as NLRP3 inflammasome (50). It seems that there is a major link between metabolic abnormalities and cell death pathways in diabetic conditions (20). Our results showed that diabetes type 1 leads to reduced cell density and increased apoptosis in testis tissue in various time points after diabetes type 1 induction. The highest reduction of cell density was observed after 20 weeks. Therefore, studies on the precise role of target genes in diabetes and cell death signaling pathways may yield potential molecular targets for developing novel therapeutics for control and prevention of

diabetic complications including diabetic infertility.

## Conclusion

Hyperglycemia and diabetes mellitus may adversely cause cell death and lead to cell apoptosis in testicular tissues through creating the AGEs, inducing pro-inflammatory responses, over-expression of ROS and initiating cell apoptosis pathway, all of which

lead to impairment of spermatogenesis and male infertility.

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