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RESEARCH ARTICLE

Impaired sperma togenesis associated with changes in spatial arrangement of Sertoli and spermatogonial cells following induced diabetes

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Abstract

The current study was conducted to assess the relationship between testicular cells in spermatogenesis, through which the production of healthy and mature sperm is essential. However, it seems necessary to obtain more information about the three-dimensional pattern of the testis cells arrangement, which is directly related to the function of the testis after induction of diabetes.

Twelve adult mice (28-30 g) were assigned into two experimental groups: (1) control and (2) diabetic (40 mg/kg STZ). The epididymal sperm collected from the tail of the epididymis and testes samples were taken for stereology, immunocytochemistry and RNA extraction. Our data showed that diabetes could notably decrease the number of testicular cells, together with a reduction of total sperm count. In addition, the results from the second-order stereology indicated the significant changes in the spatial arrangement of Sertoli cells and spermatogonial cells in the diabetic groups, in comparison with the control ($P < .05$). Moreover, the immunohistochemistry results showed a significant reduction in Sex-determining Region Y (SRY) box 9 gene (*SOX9*), vimentin, occludin, and connexin-43 positive cells in the diabetic groups compared with the control ($P < .05$). Furthermore, our data showed that the expression of steroidogenic acute regulatory protein steroidogenic acute regulatory protein (StAR) and peripheral benzodiazepine receptor peripheral benzodiazepine receptor (PBR) was significantly reduced in the diabetic groups, in comparison with the control ($P < .05$). These findings suggest that structural and functional changes of testis cells after induction of diabetes cause the alterations in the spatial arrangement of Sertoli and spermatogonial cells, ultimately influencing the normal spermatogenesis in mice.

KEYWORDS

diabetes, mice, spatial arrangement, spermatogenesis, testis

Abbreviations: BTB, Blood testis barrier; cDNA, Complementary DNA; CE, Coefficient of error; DAPI, 4, 6-Diamidino-2-phenylindole; DM, Diabetes mellitus; DP, Dipoles; FITC, Fluorescein isothiocyanate; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; H&E, Hematoxylin and eosin; MuLV, Murine leukemia virus reverse transcriptase; Nv, Numerical density; qRT-PCR, Quantitative real-time polymerase chain reaction; SSCs, Spermatogonial stem cells; STZ, Streptozotocin.

1 | INTRODUCTION

Diabetes mellitus (DM) is accompanied by human reproductive impairment, and its impact on reproduction is manifested by a reduction in fertility and poor sperm quality.[1-3] DM is one of the metabolic diseases with abnormalities in protein, fat, and carbohydrate metabolism in target tissues.

The chronic hyperglycemia of diabetes also causes such complications and other dysfunctions in reproductive organs.[4,5] Several mechanisms are associated with reproductive dysfunction, such as oxidative stress, reduction in sperm motility, damage to the DNA in both nuclear and mitochondrial sperm, altered hormonal profiles, impaired blood-testis barrier (BTB) function, and abnormalities in spermatogenesis.[6,7] The effects of DM on the structure of testicular tissue have been reported. In this respect, disruption of the BTB, degeneration of spermatogonia cell and Sertoli cells, changes in interstitial tissue, reduction in the seminiferous tubules size, and poor quality of sperm and fertility were demonstrated.[8,9] The process of spermatogenesis is complex and coordinated, in which spermatogonial stem cells (SSCs) were transformed to specialized spermatozoa. This process involves an array of complex molecular, cellular, and biochemical events in the testis. Sertoli cells are the most vital components of testicular microenvironment and are located around male germ cells.[10,11] Sertoli cells are the essential somatic components in the seminiferous tubules and play a key role in the BTB.

In addition, they secrete important growth factors, hormones, and cytokines required for the development of germ cells. During the process of spermatogenesis, Sertoli cells produce several hormones and cytokines/growth factors, to determine properly the differentiating spermatogonia, spermatocyte meiosis, and progression of spermatids to spermatozoa.[12-14] Without the structural support, functional, and metabolic regulation of the Sertoli cells, differentiation of germ cell, and transformation into spermatozoa do not occur.[11] In fact, the anatomical relationship of Sertoli cells and spermatogonial cells in the spermatogenesis is essential for the production of healthy sperm.[11,15] Because the number of Sertoli cells specifies the size of the testis, it is speculated that a certain amount of spermatogonia is supported by each Sertoli cell.[16,17] Throughout the spermatogenesis process, germ cells are in direct contact with the Sertoli cells.

Many interactions between Sertoli and germ cells are regulated by autocrine and paracrine activity. However, attention has recently shifted to identifying the interaction between cells, because this information will be useful in clarifying the mechanism of cell-to-cell interaction.[11,16]

Since the underlying mechanisms effects of DM on male reproductive function remain largely unknown, the evaluation of the correlation between Sertoli cells and spermatogonial cells can help in understanding the mechanism of disorder such as diabetes in the process of spermatogenesis. Although some methods are available for data abstraction.

These methods include the quantitative estimation of histological particles such as volume, surface area, number, and length, which are known as the first order stereological methods. However, it seems necessary to obtain important information about the three-dimensional arrangement aspect of the histological samples, which directly affects theirs function.[18,19] Also, the second-order stereology method provides us with more information about the spatial arrangement of the cells. Researchers can study the spatial arrangements of the cells or even cellular organelles by a variety of techniques such as estimation of covariance "C(r)", pair-correlation functions "g(r)," and "cross-correlation function".[18] There are little or no data about the effects of spatial arrangement of Sertoli and spermatogonial cells in the normal functions of the testes during spermatogenesis in induced diabetic mice. In this study, we aim to find the relationships between the spatial arrangement alteration of testicular cells with the histological and molecular remarks of testis in diabetic mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Twelve adult male albino mice of similar body weights (25-30 g) were used. The experimental protocol was reviewed and approved by Ethical Committee at Shahid Beheshti University of Medical Sciences (IR. SBMU. RETECH.REC.1395.440). Adult healthy mice were allocated to control and diabetic groups (six animals per group) and were maintained under standard laboratory conditions.

2.2 | Induction of diabetes mellitus model

The streptozotocin (STZ) (Enzo Life Sciences, Inc., Farmingdale, NY) was dissolved in normal saline solution and was used for induction of DM at a single dose of 40 mg/kg (intraperitoneal injection). Seven days after the STZ injection, the blood glucose concentration more than 250 mg/dL was considered as a DM. All diabetic mice were maintained for 30 days after injection of STZ to establish DM.[20]

2.3 | Collecting a semen sample for sperm analysis

Thirty-five days after induction of diabetes, the semen samples were collected from the tail of the epididymis and then transferred to the 1 mL of Ham's F-10 media (Sigma-Aldrich Product Number N6635); after incubation at 37°C for 20 minutes, 10 μL of the sample was transferred to a slide and the sperm was observed by an inverted microscope and measured by counting chamber.

2.4 | Tissue preparation

Anesthesia induction was achieved in animals with a mixture of ketamine (dosage of 60 mg/kg body weight) and xylazine hydrochloride (dosage of 8 mg/kg body weight) and then the animals were killed. The testes samples were obtained and maintained in Bouin's for 1 day and embedded in paraffin blocks. Then, complete serial sections (10 μm thickness for volume and spatial arrangement estimation and 25 μm thickness for number estimation) were made using a microtome (Leica RM2125 RTS, Germany) according to the stereological methods. By a systematic uniform random sampling (SURS) manner, 10 sections of each samples were selected with a random number sampled starting between 1 and 10 and stained with hemotoxylin and eosin (H&E) staining (Sigma). It should be mentioned that the testis cells were distinguished from morphological aspect. The Leydig cells are polyhedral cells with eosinophilic cytoplasm and spherical nucleus and located in interstitial tissue of seminiferous tubules. Sertoli cells located at the base of the epithelium and they had large, basal, oval pale nucleus, but these cells were relatively few in numbers. Spermatogonia were located at the base of the epithelium, the spermatogonia were dome-shaped cells with dark or light round nuclei. Primary spermatocytes, at the middle zone of the germinal epithelium, there were largest cells of the seminiferous epithelium. The round spermatids were spherical or round cells towards the lumen.

2.5 | Volume of testis and interstitial tissue

The Cavalieri's principle was applied for the assessment of testis and interstitial tissue volume by the following equation[19]:

$$V_{total} = \sum P \times \frac{a}{p} \times t$$

The (ΣP) was sum of the falling points on the testis tissue sections. The (a/p) and (t) were area per each point and distance between the sampled sections, respectively.

2.6 | Number of testis cells

The optical dissector method was applied to estimate the testis cells number.[19] The numerical density (Nv) of testis cells was calculated with the following equation:

$$N_v = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{BA}$$

In this formula, (ΣQ) and (ΣP) are the cells number and the number of counting frame grid in all fields respectively. The (h) is the dissector height. The (a/f) is the area of frame. The (t) and (BA) are the real thickness of section and microtome section thickness, respectively. The total number of testis cells was calculated with the following equation:

$$N_{total} = N_v \times V$$

2.7 | Coefficient of error (CE)

The CE (V), was calculated by the following equation:

$$CE(V) = (\Sigma P^{-1}) \times \left[\frac{1}{240}(3\Sigma P_i^2 + \Sigma P_i P_{i+2} - 4\Sigma P_i P_{i+1}\right]^{1/2}$$

In this formula, (B) is the length of section boundary; (A) is the area of section. The CE for number of testis cells and volume of testis was calculated by the following equation[19]:

$$CE(N) = [CE^2(N_v) + CE^2(V)]^{1/2}$$

$$CE(N_v) = \left[\left(\frac{n}{n-1}\right) \times \left[\left(\frac{\Sigma(Q)^2}{(\Sigma Q)^2}\right) + \left(\frac{\Sigma(P)^2}{(\Sigma P)^2}\right) - \left(\frac{2\Sigma(QP)}{\Sigma Q \Sigma P}\right)\right]\right]^{1/2}$$

The CEs have been shown in Table 1.

TABLE 1 Coefficients of error (CE) for total volume and number of testis cells

Groups	Volume of testis	Number of spermatogonia	Number of primary spermatocyte	Number of spermatid	Number of sertoli cell	Number of Leydig cell
Control	0.04	0.03	0.04	0.05	0.05	0.03
Diabetic	0.05	0.04	0.03	0.03	0.03	0.04

2.8 | Covariance function

The covariance function was measured using the following equation[18,21,22]:

$$C(r)X = \frac{\sum \mathbf{DP(XY\ r)}}{\sum \mathbf{DP(ref\ r)}}$$

Both end points of dipoles (DP) of class size r = 1 (equivalent to 4.3 μm). To estimate "Vv", "C(r)," and "g (r)," the distance between the points (DP) ranged from r = 0 (equivalent to 0 μm) to r = 49; so, the total distance was 127.4 μm (49 × 4.3 = 210.7 μm).

2.9 | Pair correlation function

The pair-correlation function is the normalized covariance function obtained by dividing the covariance by the reference value (squared volume fraction)[18,23]:

$$g(r) = \frac{\mathbf{C(r)}}{\mathbf{Vv2}}$$

2.10 | Cross-covariance function

The function used to quantify spatial arrangement is cross-covariance (C(r)X, Y) and can be estimated using the following formula[18,23]:

$$C(r)X = \frac{\sum \mathbf{DP(XYr)}}{\sum \mathbf{DP(ref\ r)}}$$

2.11 | Cross-correlation function

The cross-covariance is normalized to eliminate volume fraction differences by the evaluation of cross-correlation function and the formula below[18,23]:

$$g(r)XY = \frac{\mathbf{C(r)XY}}{\mathbf{Vv(X\ ref)}\times\mathbf{Vv(Y\ ref)}}$$

2.12 | Immunohistochemical staining

After deparaffinized and rehydration, the sections were retrieval with 10 mM citrate buffer and then incubated with primary antibodies (Sigma-Aldrich Corporation, St. Louis, Missouri), (diluted 1 in 100). Then the appropriate secondary IgG antibody (diluted 1 in 200), the sections were incubated with 3′-diaminobenzidine (DAB) (Dako, Glostrup, Denmark). After immunohistochemical reaction, sections were counter-stained with hematoxylin (Merck, Whitehouse, NJ) mounted, and observed under a light microscope.

2.13 | TUNEL assay

Evaluation of apoptosis was done by terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL). TUNEL staining is an important technique for the analysis of DNA damage. After inducing diabetes by STZ, the tissues sections were stained according to the TUNEL protocols. Finally, percent of TUNEL-positive cells was quantified in three sections per testis.

2.14 | Immunocytochemistry

For immunocytochemical staining, anti-vimentin (Abcam), anti-occludin (Sigma), and anti- connexin-43 (Sigma), at a dilution of 1:100 overnight at 4°C were used. After washing the sections with phosphate-buffered saline (PBS) (30 minutes), they were incubated with the secondary antibody IgG (Sigma) conjugated with fluorescein isothiocyanate (FITC) at a 1:200 dilutions (1 hour) at room temperature. After staining with DAPI (4, 6-diamidino-2-phenylindole), cells nuclei were observed under a fluorescence microscope (Olympus).

2.15 | Analysis of StRA and PBR expression by real-time PCR

The total RNA samples were extracted and treated with DNase I (Roche, Basel, Switzerland) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized in a total volume of 20 μL using a commercial kit (Fermentas, Lithuania) at 42°C for 60 minutes according to the manufacturer's instructions. The Applied Real-time polymerase chain reaction (RT-PCR) (TaqMan) according to QuantiTect SYBR Green RT-PCR kit Takara Bio Inc, Japan) for quantification of relative gene expression was used. All studied forward and reverse primer pairs were designed according to the Primer 3 Plus software in exon-exon junction way to distinguish between cDNA and genomic DNA. Previously, the PCR primers were tested by Primer-Blast tool at the site, www.ncbi.nlm.nih.gov/tools/primer-blast (Table 2).

2.16 | Statistical analyses

The statistical analysis was performed by Mann-Whitney U-tests, Pfaffl method, and one-way analysis of variance (ANOVA) and all data was considered statistically significant as the $P \leq .05$.

TABLE 2 Primers design

Genes (accession number)	Primer sequences	Product size (base pair)	TM
GAPDH (GenBank: BC087743.1)	F = ATCACTGCCACTCAGAAGACTG R = TGGATGCAGGGATGATGTTCTG	91 bp	60
StAR (GenBank: AB001349.1)	F = GGTTCTCAACTGGAAGCAACAC R = TTTCCTTCTTCCAGCCTTCCTG	121 bp	60
PBR (GenBank: AH002238.2)	F = GTATGCTAGCTTGCAGAAACCC R = AATACAGTGTGCCCCAGATGG	74 bp	59

3 | RESULTS

3.1 | Body weight and blood glucose

At the beginning of the study and after 35 days, body weight alteration was assessed. Our results showed that diabetic mice had significant reduction in body weight in diabetes animals when compared with the normal control animals (Table 3). To confirm the diabetic model, blood glucose level was carried out. As shown in Table 3:, blood glucose levels were significantly increased in diabetic mice as compared with the normal mice ($P < .05$).

3.2 | Total sperm count

The results revealed that the total sperm count in animals induced with STZ has reduced compared to the normal control animals ($P < .05$). STZ produced an extreme significant reduction in sperm count in the diabetic groups in comparison with the control groups ($P < .05$) (Figure 1A-D). These findings demonstrated that the reduction of sperm count is induced by diabetes type 1.

3.3 | Testis and interstitial tissue volume

According to the data of the stereological test shown in Figure 2A-D, a significant decrease in the testis and interstitial tissue volume was observed in the diabetic group compared with the normal control group ($P < .05$). Statistical analysis of stereological assay demonstrated and results revealed that diabetic type 1 induced reduction in volume of testis and interstitial tissue.

3.4 | Total number of testis cells

Assessment of testis tissue revealed significant reduction in the numbers of testis cells in diabetic mice in comparison with the normal control mice ($P < .05$) (Figure 3A-E and Figure 4A-D). Total number of spermatogonia, primary spermatocyte, spermatid, Sertoli cells, and Leydig cells was investigated by stereology method and the data revealed that diabetic type 1 induced reduction in the number of testis cells.

3.5 | Convert a data matrix to image type using MATLAB software

DM altered the spatial distribution of the Sertoli cells and spermatogonial cells so that these cells were dissociated in some places. To better understand what happened, the data matrices of both the normal control and diabetic groups were converted to image type via MATLAB software (Figure 5A).

3.6 | Spatial arrangement and cross-correlation of Sertoli cells and spermatogonia cells

Assessments of g(r) for Sertoli cells and the dipole distances, r, were plotted against each other (Figure 6B and 6E). The estimated values from the start to the end of the curve (from r = 0 to 30.1 μm), (from r = 55.9 to 64.5 μm), (from r = 68.8 to 81.7 μm), (from r = 90.3 to 107.5 μm), (from r = 124.7 to 163.4 μm), (from r = 172 to 176.3 μm), and (from r = 202.1 to 210.7 μm) revealed a significant difference among the two groups. After the

TABLE 3 Body weight and blood glucose levels of the animal groups

Groups	First body weight (g)	Last body weight (g)	First diabetic blood glucose (mg dL-1)	Last diabetic blood glucose (mg dL-1)
Diabetic	25	20	89	375
Control	25	30	85	81

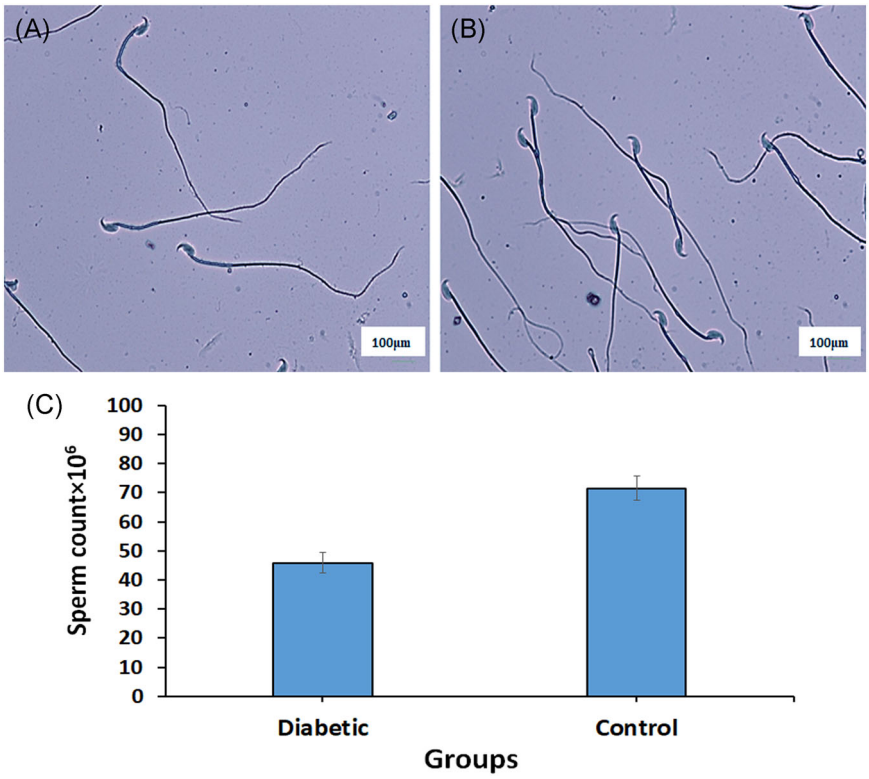

FIGURE 1 Photomicrograph of the sperm stained with Diff Quick, x100. A, Diabetic group; B, control group. C, The results showed that the total sperm count in diabetic groups decreased in comparison with the control groups ($P < .05$)

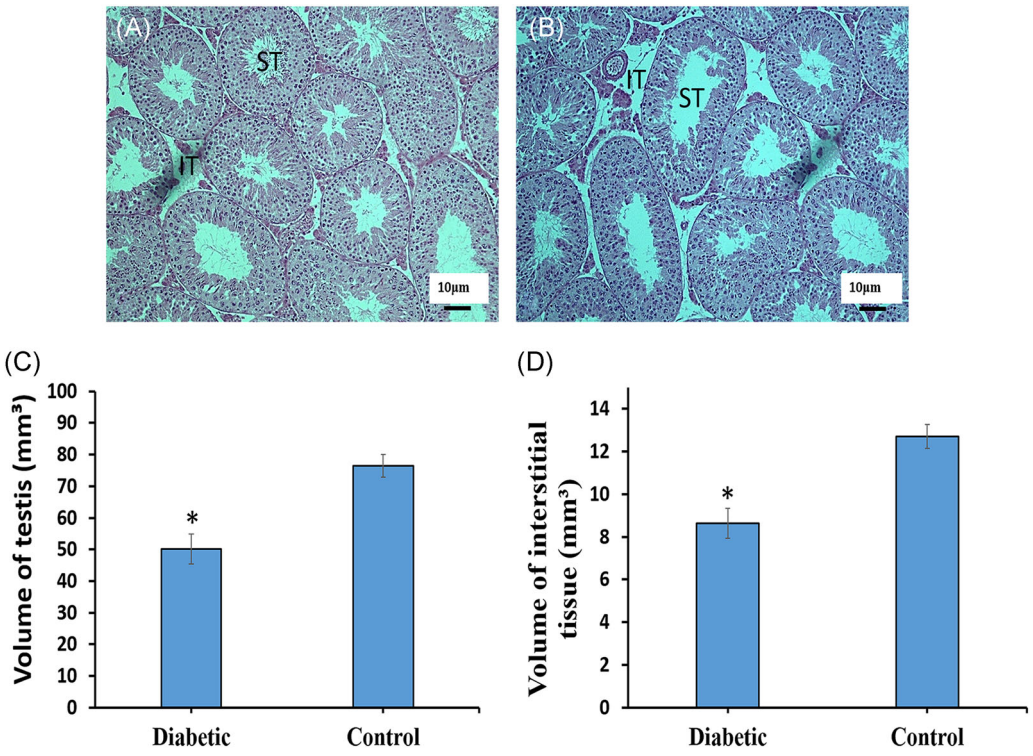

FIGURE 2 Photomicrograph of the testis stained with H&E, x10. A, Diabetic group; B, control group, interstitial tissue (IT), and seminiferous tubules (ST). C, Total volume of interstitial tissue in diabetic groups reduction in comparison with the control groups ($P < .05$). (D) Total volume of testis in diabetic groups decreased in comparison with the control groups ($P < .05$). H&E, hemotoxylin and eosin

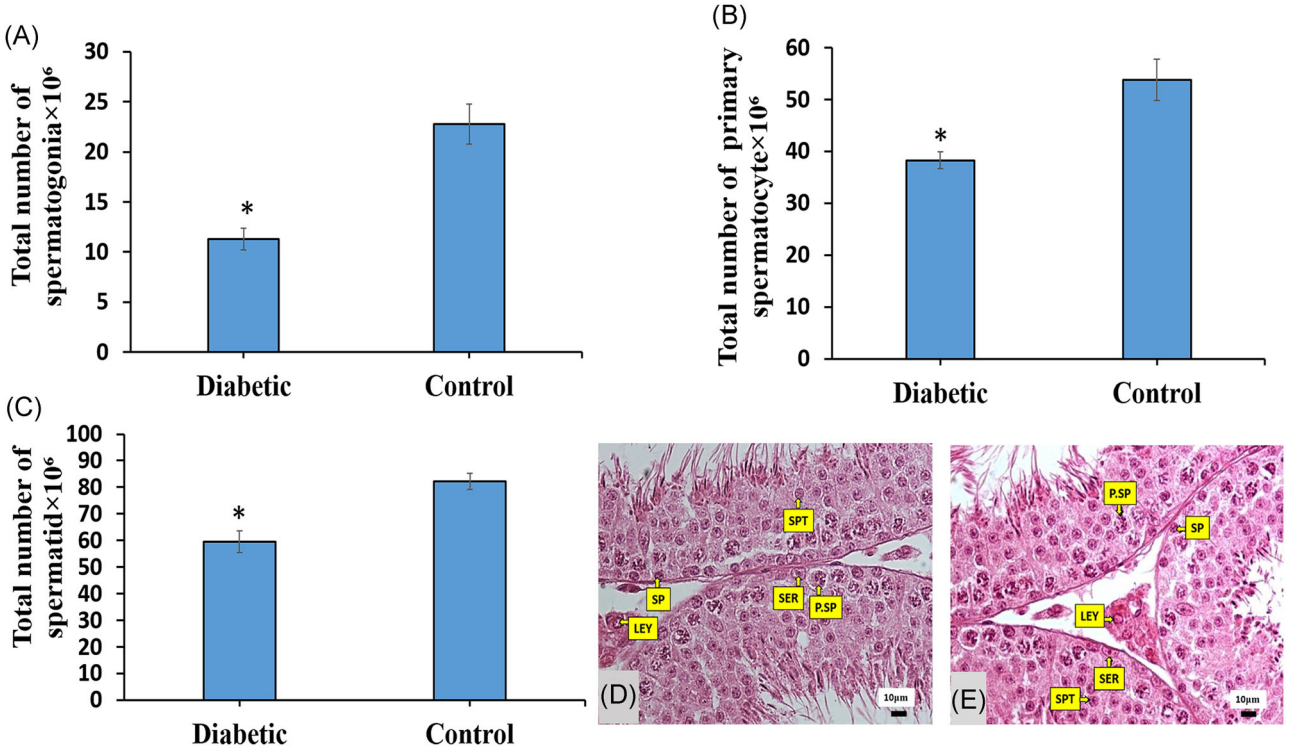

FIGURE 3 A, Total number of spermatogonia, primary spermatocytes, and spermatids in diabetic groups decreased in comparison with the control groups ($P < .05$). B, Total number of Sertoli and Leydig cells in diabetic groups decreased in comparison with the control groups ($P < .05$). C, Diabetic groups and D, control groups. Photomicrograph of the testis stained with H&E, x40. SP (spermatogonia), P. SP (primary spermatocyte), SPT (round spermatid), SER (Sertoli cell), LEY (Leydig cell)

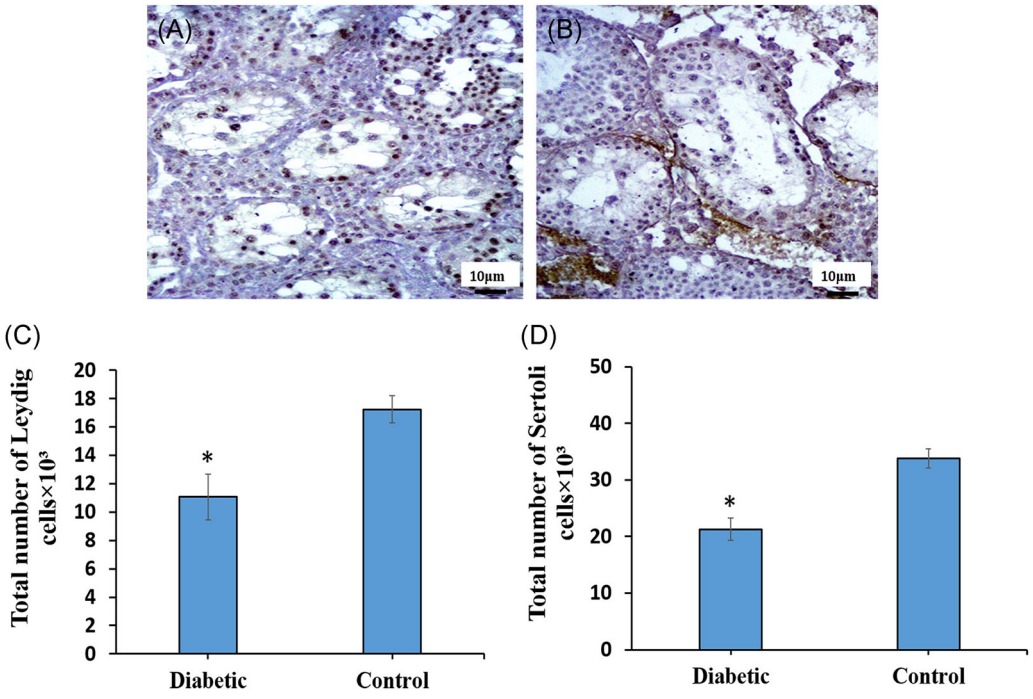

FIGURE 4 Photomicrograph of the testis immunohistochemistry staining for SOX9, x10. A, Diabetic groups and B, control groups. C, Total number of of Sertoli cells in diabetic groups decreased in comparison with the control groups ($P < .05$). D, Total number of Leydig cells in diabetic groups decreased in comparison with the control groups ($P < .05$)

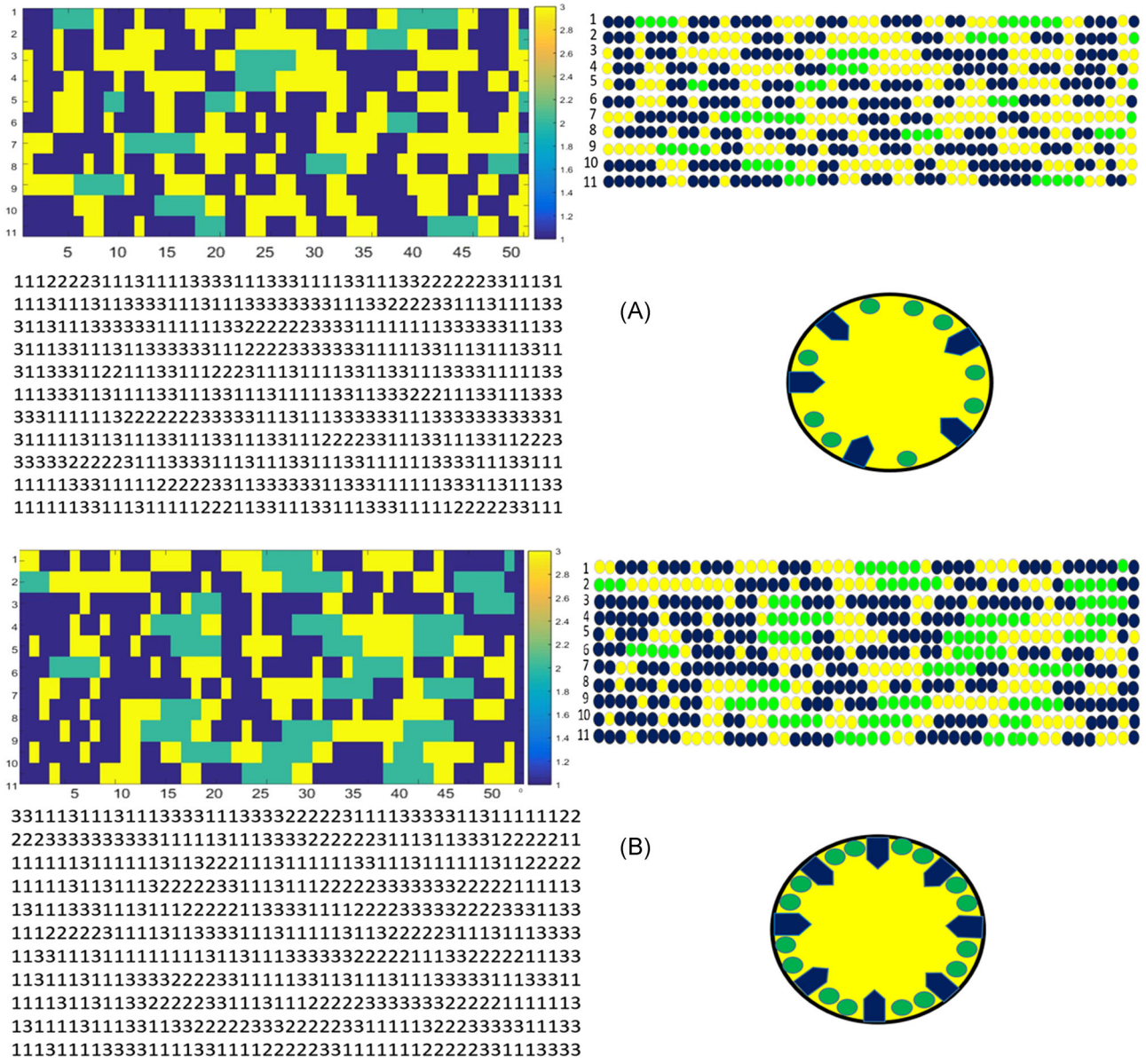

FIGURE 5 Convert a data matrix to image type and display of Sertoli cells (1) dark blue, spermatogonial cells (2) green and connective tissue (3) yellow. A, Diabetic groups; B, control groups. A and B are cartoons that illustrate the differing morphologies of testicular tubules in diabetic and control groups

gap, there are the data points for both the normal control and diabetic groups that were arranged randomly in longer distances ($P < .05$).

Assessments of g(r) for spermatogonial cells and the dipole distances, r, were plotted against each other (Figure 6C and 6E). The estimated values from the start to the end of the curve (from r = 0 to 12.9 μm), (from r = 38.7 to 68.8 μm), (from r = 81.7 to 116.1 μm), (from r = 129 to 141.9 μm), (from r = 159.1 to 176.3 μm), and (from r = 206.4 to 210.7 μm) showed a significant difference between the study groups. After the gap, there are the data points for both the normal control and diabetic groups that were arranged randomly in longer distances ($P < .05$).

Based on the cross-correlation curve, a negative correlation can be seen between Sertoli cells and spermatogonial cells (from r = 0 to 38.7 μm), (from r = 43 to 81.7 μm), (from r = 86 to 124.7 μm), (r = 129 to 154 μm), (r = 159.1 to 172 μm), and (r = 193 to 210.7 μm) of the diabetic mice in comparison with the normal control mice ($P < .05$). When the cross-covariance was located at a higher position than the reference line, it shows a positive correlation among components. But if the cross-covariance is located below the line, it means there is a negative correlation. Based on the findings in the present work, there is a positive correlation among the cells of the Sertoli cells and spermatogonial cells in the normal control

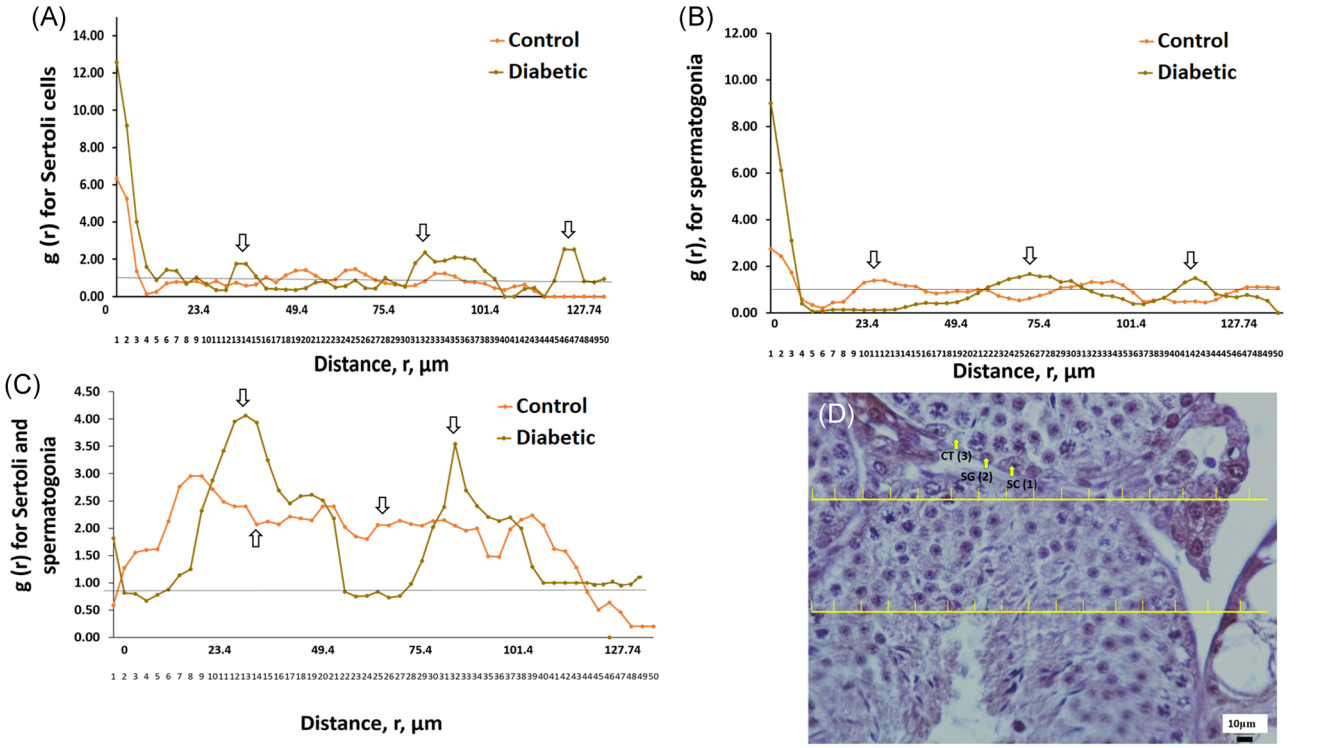

FIGURE 6 A, Relationship between pair-correlation function and dipole distance for Sertoli cells in diabetic and control groups. B, Relationship between pair-correlation function and dipole distance for spermatogonial cells in diabetic and control groups. C, The cross-correlation function, g(r), between the Sertoli cells and the spermatogonial cells plotted versus distance, r, in micrometers. The dots are the mean g(r) across the five animals in the diabetic control and groups. The horizontal reference line corresponds to values expected for a random spatial arrangement [g(r) = 1]. Arrows indicate significant differences. (↓) indicate gmax and gmin for both groups. The arrow (↓) indicates the value of the distance for which there was a significant difference of cross-correlation function. the micrograph illustration that Coding of the cells for generating a matrix. D, A transparent lattice of points was constructed to serve as a set of dipole probes. Each row composed of 50 points and 49 equidistant intervals. For each testis, 11 trials (a total of $P = 550$ test points) were conducted. For every trial, the nature of the tissue component underlying each test point was noted and all the information was recorded on a 50×11 matrix. The point interval (r) corresponded to a distance of 4.3 µm had the chance of being included within the same profile of the spermatogonia. Each test point was coded as 1, 2, and 3 if the point was laid on the Sertoli cells, spermatogonial cells, and connective tissue, respectively

mice, whereas in the diabetic mice, the cells exhibit a negative correlation (Figure 6D and 6E).

3.7 | Percent of apoptotic cells in testis

The study results in diabetic groups showed a significant increase ~50% in TUNEL-positive cells in comparison with the control group ~27% in TUNEL-positive cells ($P < .05$) (Figure 7A-E). Quantification of apoptotic cells percent in the testis showed that diabetes induced cell death in STZ-induced diabetic mice.

3.8 | Vimentin immunohistochemistry

Figure 8A shows the vimentin immunohistochemistry in the control and diabetic groups. Positive vimentin cells reduction was observed in the diabetic groups. However, our results which were obtained from the Sertoli cells showed that a significant disruption in vimentin could be observed ($P < .05$). Immunohistochemical staining showed that the distribution pattern of vimentin filaments in Sertoli cells changes in diabetic rats.

3.9 | Immunohistochemistry of occludin and connexin-43

Figure 8B and 8C shows the expression of occludin and connexin-43 in testicular tissues after induction of type 1 diabetes. The expression of connexin-43 in testis showed a significant decrease in the diabetic mice when compared with the normal control animals ($P < .05$). Nonetheless, no significant differences existed in the expression of occludin in the study groups. Immunohistochemical staining showed that diabetic type 1 induced disrupts BTB by decreasing BTB junction protein Occludin and Connexin-43 expression.

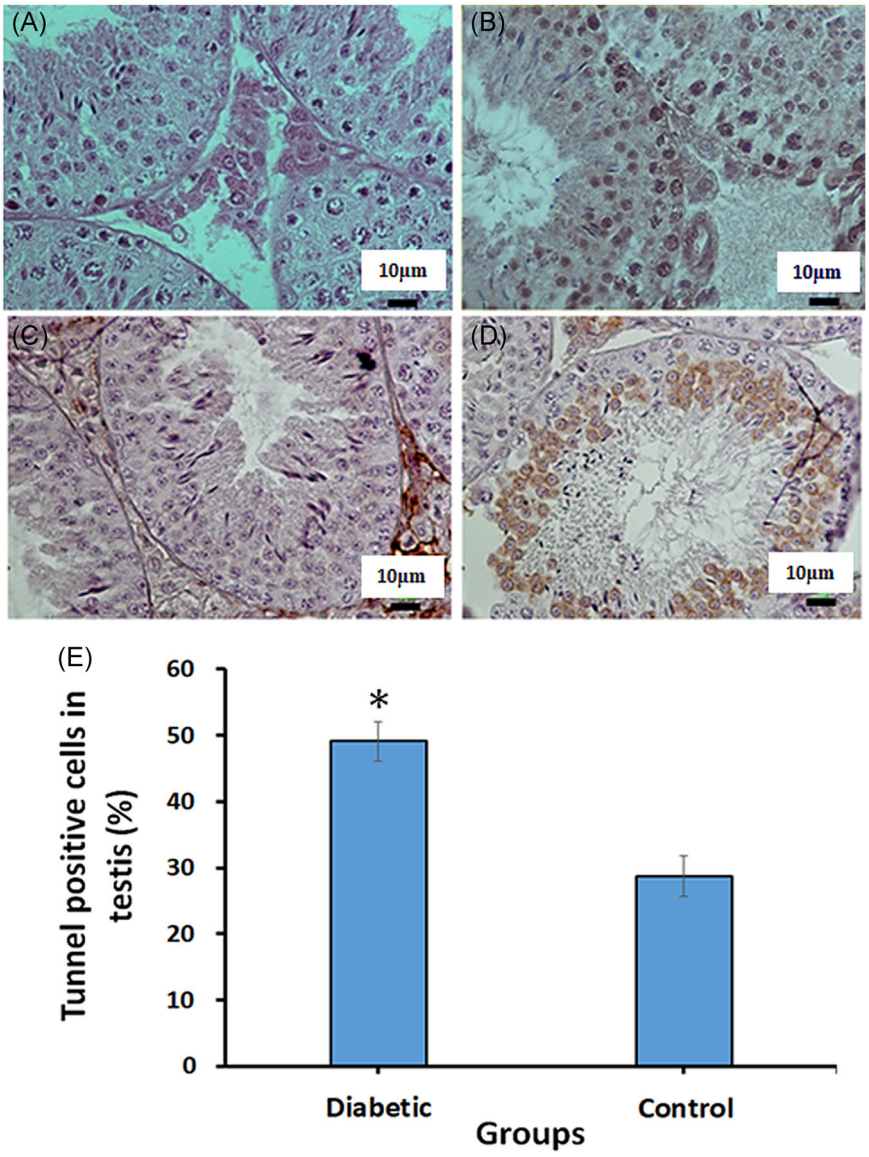

FIGURE 7 A-D, TUNEL detection of apoptotic cells in the testes of the (A) negative control, (B) positive control, (C) control groups, and (D) diabetic groups and apoptotic cells (brown). E, Representation of the estimated parameters of the testis. Percent of TUNEL positive cells in testis in the different groups are shown. The significant difference between diabetic with the control groups is indicated. *$P < .05$. TUNEL, terminal-deoxynucleotidyl transferase mediated nick end labeling

3.10 | Expression levels of StAR and PBR

The relative mRNA expression levels of StAR and PBR between the different groups were normalized by control and quantified. As shown in the figure below, the levels of StAR and PBR expression in testis tissue of mice were significantly reduced in the diabetic mice when compared with the normal control animals ($P < .005$) (Figure 9). Our results demonstrated that the levels of StAR and PBR which are involved in steroidogenesis and cholesterol transport were significantly reduced in diabetic mice.

4 | DISCUSSION

Dysfunction of the spermatogenesis is a major complication that occurs in DM, indicating the urgency of creating novel approaches for a modification. The aim of the current study was to examine the role of spatial arrangement in the dysfunction of testicular cell in diabetic mice.

Our results clearly confirmed the decrease in the testis and interstitial tissue volume, with loss of testis cells and spermatozoa, and an increase in TUNEL-positive cells in STZ-induced diabetic mice. Furthermore, our immunostaining data showed a decrease in vimentin, occludin, and connexin-43 protein levels in the testis and also the reduction of *StAR* and *PBR* genes expression in the diabetic mice group.

These findings validate the results of a previous study in which it was shown that the DM causes a reduction of the cellular population of spermatogenesis, depletion of germ cells, and interruption of spermatogenesis in the testis of mice, following induced DM.[24,25] Casado et al reported that diabetes causes frequent abnormal histology and atrophy of

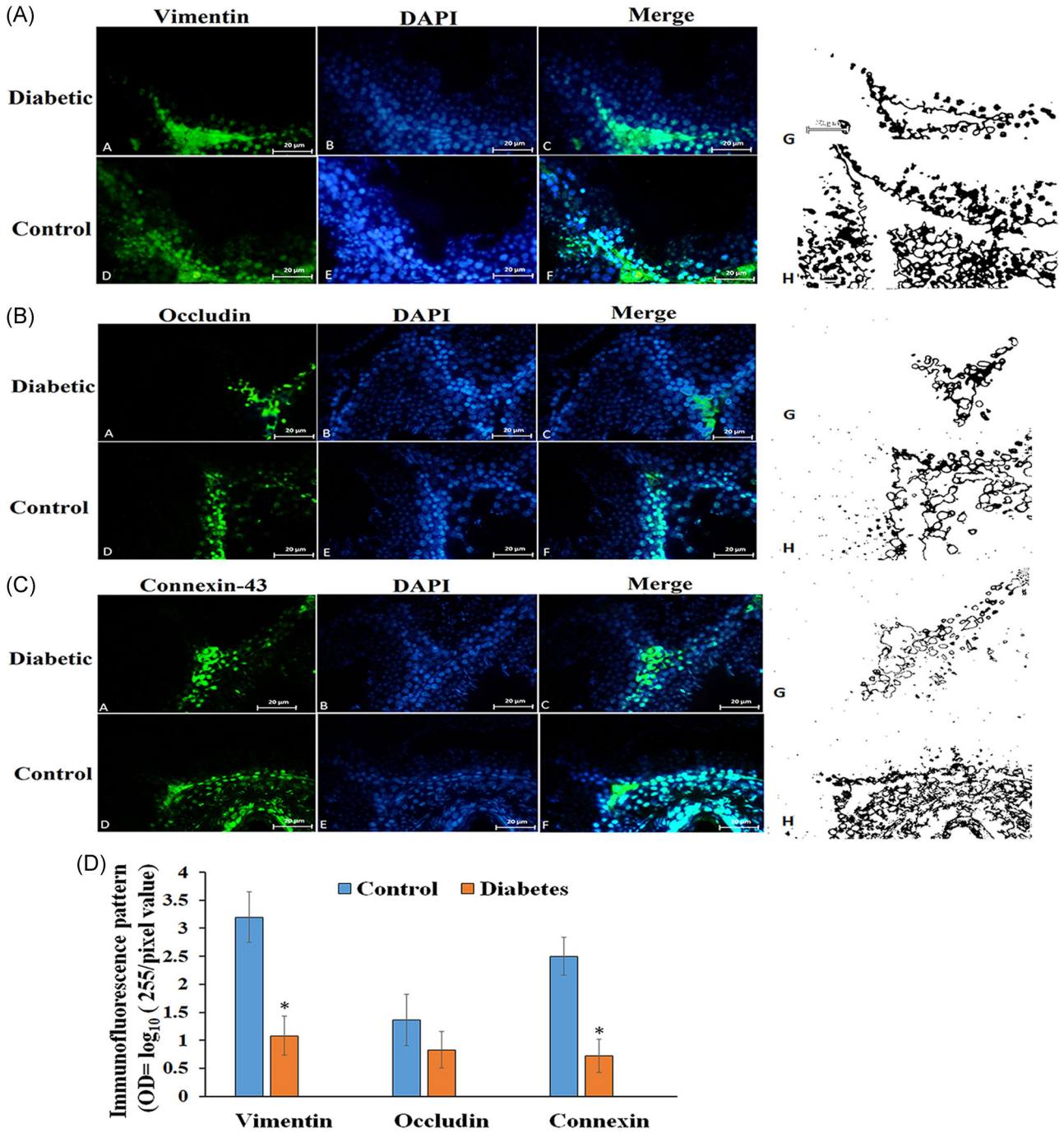

FIGURE 8 A-C, The patterns of immunofluorescence of vimentin, occluding, and connexin in the diabetic and control groups. A-H, Immunofluorescence pattern (OD = $\log_{10}$ (255/pixel value) of vimentin, occluding, and connexin-43 in testis in the different groups. D, The significant difference between diabetic with the control groups is indicated. *$P < .05$. Scale bar = 20 μm

the seminiferous tubules, decreases the tubules' diameter, and reduces spermatogenetic cell series.[5] These results suggest an alteration of spatial distributions of the testis cells in this study, and suggests that there is a relationship between the disruption of the spatial arrangement of the testicular cell and diabetes. The plots of pair-correlation function of Sertoli and spermatogonia cells indicated a wide range of gap in the spatial distribution of Sertoli and spermatogonial cells in the diabetic mice. Of course, these gaps can be filled with connective tissue. In this regard, the alteration of cellular nutrition and destruction of diabetic testicular cells can change glucose transport and lead to impaired spermatogenesis.[5,7]

The spatial arrangement of the cell is highly associated with correlation between cells and cell-to-cell interactions. Thus, these events lead to a

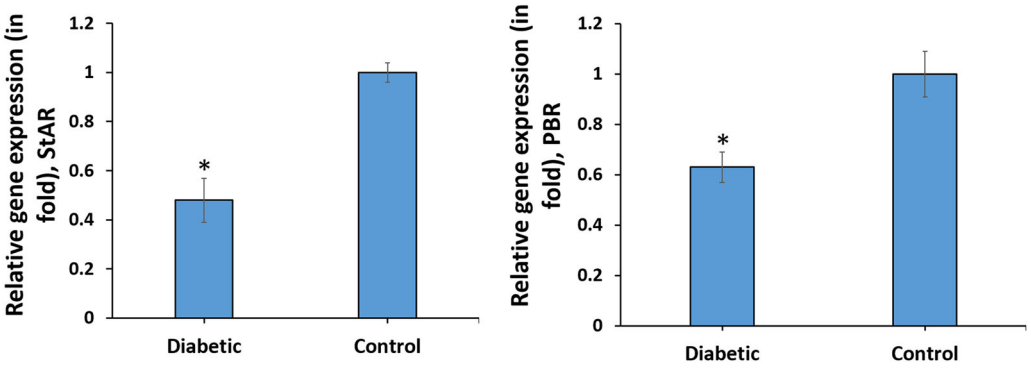

FIGURE 9 Real-time-PCR analyses of testes. mRNA expression levels of StAR and PBR from control- and diabetic mice. Asterisks indicate a statistically significant difference between diabetic groups compare to the control groups, $*P < .05$. PCR, polymerase chain reaction

spermatogenic cells' loss in the testes and will undoubtedly change the spatial pattern of the Sertoli and spermatogonial cells, and indicate relationship between them. The number of Sertoli cells determines an efficient spermatogenesis and direct correlation of the number of germ cells with the number of functional Sertoli cells.[6,9] A decrease in testosterone level and altered BTB in diabetes cause disruption of structure and function of Sertoli cells, leading to apoptosis of germ cells.[6] Probably, the decrease in the number of sex cells counted may also be due to cell death that is affected by the failure of their production process. In this study, TUNEL-positive cells significantly increased in diabetic group (50%), compared with the control group (27%).

Diabetes induces apoptosis, causing the loss of Sertoli and spermatogonial cells in the testicles and disturbing the relationship between them, without undoubtedly altering the structure of Sertoli cells and spermatogonial cells, and ultimately lead to disruption in spermatogenesis. In this study a good justification for this Sertoli cell loss is that the testis tissue has low level of protein expression of SOX9. Immunohistochemistry results for vimentin, occludin, and connexin-43 show a decrease in the levels of protein expression in the diabetic group. In other words, two mechanisms exist that may directly or indirectly affect the incidence of testis cell apoptosis. First, the distribution of cell junction between germ cells and adjacent Sertoli cells affects the structure of testicular tubules and the process of spermatogenesis. Second, increase in Sertoli cell death and apoptosis can lead to rapid and massive germ cell death.[5,6,9]

Vimentin constitutes the cytoskeleton of Sertoli cells and plays a special role in sperm formation, maintaining Sertoli cells structure, and spermiation.[26,27] Previous studies reported that alteration in intracellular vimentin may induce TJ changes among Sertoli cells and lead to changes in BTB permeability.[12,28]

It has been shown that within the testes, gap Junction (GJ) protein, connexin-43, and tight junction (TJ) protein, occludin play a vital role in mammalian spermatogenesis and provide cross-talks between Sertoli cells and also between Sertoli cells and germ cells.[26,27] Moreover, some studies showed that connexin-43 in Sertoli cells are important for blood-testis barrier (BTB) formation and BTB homeostasis.[28,29] It is well known that TJ and GJ proteins can support spermatogenesis and both junctional proteins represent an important regulator for cells-cells communication and BTB formation and function.[12,26,27]

It has also been shown that the alterations in the levels of *StAR* and *PBR* genes expression and testosterone synthesis may be related to testicular response to endocrine disruptors.[29,30] This reduction in the expression of *StAR* and *PBR* genes is involved in cholesterol transport into the mitochondria and steroidogenesis and may result in corresponding reduction in testosterone synthesis and impaired spermatogenesis in testes following DM.

Therefore, in this study we observed the quantitative alterations in the spatial distributions of the Sertoli and spermatogonial cells, within the testis of diabetic mice, using second-order stereology. In this study, we proposed a new insight for the spatial arrangement of cells that describes the cell-to-cell interactions related to the normal function, uncontrollable cell function, and pathological condition in diseases.

5 | CONCLUSION

Therefore, it can be concluded that the spatial arrangement of testis cells and the role of Sertoli cells in spermatogenesis will be significantly important for the

control of male fertility. Overall, this study demonstrates that the structural and functional changes of testis cells brought about by streptozotocin induced diabetes alter the spatial arrangement of Sertoli and spermatogonial cells, and these changes alter the spermatogenesis process in mice.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The authors declare that all experiments protocols were approved by the Ethics Committee, deputy of research, Shahid Beheshti University of Medical Sciences, Tehran, Iran. All methods were carried out in accordance with relevant guidelines and regulations.

AUTHOR CONTRIBUTIONS

Mohammad-Amin Abdollahifar designed this study conducted the stereological study and performed and writing the drafted the manuscript. Ensieh Sajadi provided the clinical data and sample. Sara Dadras and Mohammad Bayat helped to draft the manuscript. SB and HZ carried out the animal model. Sanaz Ziaeipour and Fatemeh Mazini performed the statistical analysis. Mahsa Kazemi, Mohammad Bagheri, and Aida Valizadeh carried out the real-time PCR and carried out the immunohistochemistry. All authors read and approved the final manuscript.

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