Fabrication of Bioartificial Pancreas Using Decellularized Rat Testicular Tissue

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Abstract

I. Background: Diabetes is a chronic disease that is associated with a decrease or disfunction of β-cell. In the present study, fabrication of bioartificial pancreas using MIN-6 β-cell line seeded in decellularized rat testicles (testis-ECM) was investigated.

II. Methods and Results: In this experimental study, the whole body of testes were decellularized and after characterization, were seeded by MIN-6 cell line. The expression of insulin-related genes and proteins including Pdx-1, Glut2, Insulin, and Neurogenin-3 were evaluated. By using the radioimmunoassay method; insulin secretion was assessed under different concentrations of glucose. Seeded scaffolds with or without MIN-6 cells were transplanted to the rat's mesentery and their blood sugar and body weight were evaluated every three days for 28 days and analyzed with H&E staining. Histological assessments indicated the cells were completely removed after decellularization. The scaffold had no toxic impacts on the MIN-6 cells.

Insulin release in response to different concentrations of glucose in 3D culture (testis-ECM) was significantly more than the traditional 2D monolayer culture. Moreover, the relative genes and proteins expression were significantly higher in the 3D culture, compared to the 2D control group. In vivo transplantation of the testis-ECM scaffolds showed appropriate positions for transplantation with angiogenesis and low infiltration of inflammatory cells. The recellularized scaffolds could drop blood sugar levels and increase the body-weight of STZ-diabetic rats.

III. Conclusions: Our study clearly confirmed that ECM valuable organ scaffolds prepared by decellularization of the testicular tissue is suitable for the fabrication of bioartificial pancreas for transplantation.

1. Introduction

Diabetes is a long term metabolic disorder that is prevalent around the world and associated with many complications. There are two types of diabetes: type I diabetes is an autoimmune illness in which the insulin-secreting pancreatic cells are destroyed [1]. Type II diabetes is caused by dysfunction and disorder in insulin production. In both types of diabetes, the number of pancreatic β-cells decrease [2]. Replacing β cells or transplanting Langerhans islands are the only long-term stable treatments for diabetes [3]. However, the clinical application of islet transplantation is hampered by the following limitations: lack of donors and the need to suppress the immune system throughout life [4]. In recent years, the generation of insulin cells secreted from isogenous cells has proven to be the most common problems explained above. However, a gradual degradation in cell function and post-transplant activity has prevented the use of insulin-secreting cell transplants in the improvement of type I diabetes [5].

The MIN-6 cells are very similar to the islands isolated from the pancreas, which is proposed functionality suitable to learn more about the processes of glucose-stimulated insulin production in pancreatic β-cells [6, 7].

The development of a scaffold has resulted from advances in tissue engineering with the same microenvironment to seed cells before replacing tissue or organs. Success in the extra-cellular matrix (ECM) and recellularized scaffold has been reported in many organs, including the liver, respiratory tract, nerve, tendon, bladder, and mammary gland [8-10]. Natural scaffolds can be obtained from natural tissues through decellularized tissue by physical, enzymatic and chemical methods [11]. Matrix proteins are one of the most evolutionarily protected proteins. Therefore, by removing xenogenic and allogeneic cells from tissue, scaffolds can be made the least immune response and also creates an intact three-dimensional (3D) structure for regeneration of tissues and used in regenerative medicine [12]. The testes have different tissue structures such as interstitial tissues, blood vessels and mucosa rich in fibronectin, collagen and proteoglycan [13, 14]. The existence of multiple ECM in testicular tissue, has become a suitable model for using in tissue engineering [15].

In the present study, the effect of Testis-ECM on MIN-6 cells insulin secretion was investigated, as well as the effectiveness of MIN-6 seeded scaffolding on the body weight and blood sugar concentration of STZ-induced diabetes in rats were assessed.

2. Materials And Methods

2.1. Decellularization of the rat's testes

For preparing scaffold and in vivo study, normal Wistar rats (6-8 weeks old, 160-180 g) were used. This study was carried out in accordance with the Animal Ethics Committee's guidelines (approval number:IR.ABADANUMS.REC.1397.019). After euthanizing the animals, the testes were collected, several holes were made on the specimens with a needle and immersed with phosphate-buffered saline (PBS) for 5 h, eluted in deionized distilled water (DDW) for 2 h and reimmersed in PBS for one overnight, put them in NaCl for 72 h each 12 h the specimens were eluted in DDW. Then, immersed with 1% Triton X-100 (Sigma, USA); after 12 h specimens were removed and placed in DDW for 4 h and followed by sodium dodecyl sulfate (SDS)1% (Sigma, USA) for 48 h at room temperature. In order to reduce the remaining DNA, 1% DNase (Sigma, USA) in PBS (pH 7.2) was perfused for 2 h. The collected samples were washed in DDW for 4 h and sterilized with 70% ethanol, washed with PBS, and finally lyophilized by using freeze–drier at -50°C for 24 h (Christ Alpha 2-4 LD-plus, Osterode am Harz, Germany). In order to characterization the decellularized testes (Testis-ECM), hematoxylin-eosin (H&E) and 4',6-diamidino-2-phenylindole (DAPI) staining techniques were used.

2.2. DNA quantitative analysis

In order to extract DNA of native and Testis-ECM, dsDNA Assay Kit (QIAGEN, Germany) was performed in accordance with the manufacturer’s recommendations. A spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA) was used to measure the amount and concentration of DNA at 260/280 nm.

2.3. Scanning electron microscope for examination the 3D structure of the Testis-ECM

For the analysis of micro-construction of the Testis-ECM, scanning electron microscopy (SEM) was used. Fixation of specimens was performed in 2.5% glutaraldehyde for 24 h at 4°C. Respectively, they were dehydrated and dried with gradually more concentration of ethanol and hexamethyldisilazane. Eventually, the specimens were covered with gold using a Q150R- ES sputter coater (Quorum Technologies, UK) and imaged using a VEGA3 microscope (TESCAN, Czech Republic).

2.4. Cell culture

MIN-6 cells were supplied by the Iranian Biological Resource Center. Dulbecco's modified Eagle's medium was used to culture the cells (DMEM, GIBCO) supplemented with L-glutamin 2mM, 50 μM Mercapatoethanol, 20 mM Hepes and 15% (FBS) fetal bovine serum (all supplied by GIBCO). The cells were kept at 37°C in a humidified atmosphere with 5% CO_2 incubator, and sub-cultured when they reached 80% confluency.

2.5. Recellularization of the Testis-ECM scaffolds

For experimental (in vitro and in vivo) studies, sterilization of Testis-ECM was performed with 3% peracetic acid in 4% ethanol, lyophilized by freeze-drier at -50°C for 24 h after elution with PBS and finally Testis-ECM transversely cut into 5 mm pieces and re-sterilized with UV rays for 2 h. They transferred to culture dishes with FBS overnight at 37°C and 5% CO_2. MIN-6 cells (2×10^6) were trypsinized and suspended in one ml culture medium and seeded on each Testis-ECM, After 30 minutes, DMEM was added to the cell-seeded Testis-ECM and incubated at 37°C and 5% CO_2 for 3 days. Experimental design groups for in-vitro assessments include:

I: Traditional monolayer culture of MIN-6 cell line (2D)

II: Recellularized scaffolds (3D): The Testis-ECM were seeded by MIN-6 cells.

2.6. Evaluation of proliferation and viability of cells

After the preparation of the Testis-ECM, its toxicity was evaluated to not contain any toxic substance during the decellularization protocols. For insurance of proliferation and viability, MIN-6 cells were harvested and loaded at a density of 5×10^4 per each Testis-ECM (3D) and traditional monolayer culture (2D) in each well of 96-well plate for 1, 3, and 7 days. After removing the supernatant, the cells were eluted with PBS and incubated for 4 hours with 30μl of 3-(4, 5-dimethylthiazolyl2)-2, 5-diphenyltetrazolium bromide (MTT, M5655; Sigma-Aldrich). 120μl of dimethyl sulfoxide (Sigma-Aldrich) for 15 min was added to the wells. Finally, the amount of absorption was performed at 570 nm.

2.7. Evaluation of glucose-stimulated insulin secretion

The cells were seeded in 12-well plates. They were first eluted with PBS three times. The cells were then pre-incubated for 1 hour at 37°C with (KRBH) Krebs–Ringer HEPES buffer (pH 7.4) supplemented together with 0.5 percent BSA and 2.8 mM glucose (Sigma, USA, G7528). After this period, for the GSIS assay, the MIN-6 cells were incubated in KRBH for 1 hour with various concentrations of glucose (containing 5, 16.7,

and 25 mmol/L glucose). The supernatant was removed and kept at -80°C until use. The insulin concentration were determined using an insulin ELISA kit (Millipore).

2.8. Real Time RT-PCR

The expression of Pdx-1 (pancreatic and duodenal homeobox 1), Glut-2 (glucose transporter 2) and Insulin genes were evaluated in the recellularized scaffold. The GAPDH was considered a housekeeping gene. RNA extraction was done by using RNeasy Mini kit (Qiagen, Germany). Then isolated the RNAs were converted to cDNA by cDNA synthesis kit (Qiagen, Germany). For each gene, the reaction mixture contained 10 µL master mix Syber Green (Biofact, Korea), 7 µL nuclease-free water, one µL of each sense and antisense primers and one µl cDNA (Table 1). Over 40 cycles of PCR was done according to this program: 95˚C for 10 min, 95˚C for 15 seconds, 60˚C for 1 min. Finally, the $2^{-\Delta\Delta CT}$ method was used for data analysis.

Table 1
Primer sequences

Genes	Forward	Reverse
PDX-1	AAA CGGCACACACAAGGAGAA	AGACCTGGCGCTTCACATG
GLUT-2	CAGCTGTCTTGTGCTCTGCTTGT	GCCGTCATGCTCACATAACTCA
Insulin	TCTTCTACACACCCATGTCCC	GGTGCAGCACTGATCCAG
GAPDH	ACCCAGAAGACTGTGGATGG	TTCTAGACGGCAGGTCAGGT

2.9. Western blotting analysis

The supernatant fraction of the homogenized rat's testis was subjected to Western blot analysis. Total protein concentration in the supernatant were determined using the Bradford method. To perform this laboratory method, an equal amount of proteins were isolated by 12 percent SDS-PAGE polyacrylamide gel method. Following the electrophoresis, the gel proteins were transferred to PVDF paper, which was then immersed in the blocking solution for 90 minutes. The paper was then immersed in the primary antibody; Insulin (sc-52034; Santa Cruz, USA), and Neurogenin-3 (sc-136002; Santa Cruz, USA) antibodies overnight and on the second day it was washed 3 times with Tris-buffered saline-Tween (TBST) 0.1% solution and the paper was incubated with the secondary antibody; antirabbit pzs610 and antimouse (sc-516102; Santa Cruz, USA) for 90 min. After this stage, the blots were identified by using enhanced chemi luminescence (ECL).

2.10. In Vivo study

Thirty Wistar rats were randomly allocated to four groups (6 animals in every group):

Group I: Control group (healthy rats)

Group II: received 65 mg/kg STZ without interference (diabetic rats)

Group III: was administrated first by 65 mg/kg STZ and then the unseeded Testis-ECM scaffold was implanted into their mesentery

Group IV: was administrated first by 65 mg/kg STZ and then the Testis-ECM scaffold implanted into their mesentery

The scaffolds were made of 0.5× 0.5 cm^2 pieces and cocultured in the 4-well plate with MIN-6 cell suspension (2×10^6). STZ was administrated to cause type I diabetes in rats. STZ was injected intra-peritoneally after dissolving in citrate buffer (PH: 4.5). Three days after STZ injection, blood sampling was collected by cutting off the tip of their tail, and animals with a blood sugar higher than 300 mg/dl were considered as successful diabetic models. The blood sugar and body weight of all groups were evaluated for 28 days.

The animals were anaesthetized before transplantation by intramuscular injection of 10 mg/kg xylazine and 100 mg/kg ketamine; In III and IV groups, the scaffolds with and without MIN-6 cells were grafted on mesentery by using 8-0 nylon sutures. For closing the wall of the abdomen and flank 3-0 nylon sutures were used. After surgery, the animals received analgesics, antibiotics and their fasting blood sugar was measured for three consecutive days and then every five days. The animals were retained under the standard situation for 28 days. The animals were immolated and the grafted tissues from each animal were dissected and maintained in 10% formalin. Paraffin sections (5μm) were prepared and stained with hematoxylin and eosin (H&E) for histological evaluations.

2.11. Statistical Processing

The data were analyzed using one-way ANOVA, LSD, and Mann–Whitney U test, and they were presented as the mean ± SD. $P<0.05$ was investigated significant. Data of each group was repeated four times. Data has been analyzed by SPSS 21.0 (Chicago, IL, USA)

3. Result

3.1. Characterization of Testis-ECM scaffold

At the end of the decellularized protocol, the scaffold had a translucent white appearance in comparison with native testis. H&E staining showed removal cellular elements and also DAPI staining demonstrate a few nuclei in some Testis-ECM scaffolds (Fig. 1). Assessment of quantification test on the dry weight of decellularized scaffold showed a significant decrease in the amount of DNA compared with native tissue (Fig. 2). SEM analysis showed a three-dimensional micro construction of the Testis-ECM scaffold (Fig. 3).

3.2. MTT assay

The MIN-6 cells were cultured into the Testis-ECM and the cell viability, survival and proliferation were evaluated with that in 2D condition after 1, 3 and 7 days. On the first day, the percentage of the surviving cells seeded on the scaffold was considerably lesser than the 2D condition ($P < 0.001$). However, on day

3rd, the viability percentage in both 3D and 2D conditions was similar. On 7th day, the viability percentage of the 2D group was considerably diminished compared to the 3D condition (Fig. 4). MTT assay was demonstrated that the Testis-ECM as a scaffold was non-toxic.

3.3. Insulin secretion in response to glucose stimulation

In the 3D condition, insulin release in response to different concentrations of glucose showed a significant increase as compared to the 2D condition. In the 3D conditions, insulin secretion at low glucose concentration was almost 2.3-fold and at higher glucose concentration was 4.4-fold greater than the 2D condition ($P < 0.001$) (Fig. 5).

3.4. Gene expression assessment

To study the effects of Testis-ECM on the β-cell function, gene expression of the critical transcription factors; including pancreatic PDX1, Glut2 and insulin were analyzed. As illustrated in Fig. 6, the 3D condition showed a significant increase in expression of Pdx-1 (2.83-fold), Glut-2 (9.8-fold), and insulin (2.75-fold) genes compared to the 2D control group.

3.5. Western blotting

As described in Fig. 7 at the end of the experimental phase, a significant increase in PDX1, and NgN3 protein levels were observed in MIN-6 cells seeded in Testis-ECM scaffold compared to the 2D conditions as a control group ($P < 0.001$) in western blot analysis. The 3D conditions showed a significant increase in the expression of NgN3 (3.4-fold), PDX1 (3.1-fold) protein levels compared to the 2D control group.

3.6. Evaluation of Fasting Blood Sugar test and body weight

The unseeded scaffolds were implanted into the mesentery group, the results were the same as diabetic group and also the animals lost their weight. In the cell-seeded scaffolds implanted into the mesentery group observed that the blood sugar levels begin to decline gradually and blood sugar levels effectively from day 8 until day 28. Animal weight data confirm these results (Fig. 8).

3.7. Histology study

The obtained evidence of the grafts after 28 days showed that there was no mark and indication of adhesion in those implanted in all groups. The implanted scaffold with or without cell seeding; in the mesentery became small like connective tissue. In addition, at the implantation site, active angiogenesis perspicuously was seen (Fig. 9). Weak infiltration of inflammatory cells into the grafted scaffold was also observed (Fig. 10).

4. Discussion

In the present study, decellularized testicular tissue (Testis-ECM) as a scaffold was used for MIN-6 cell culture in order to generate an artificial pancreas which is an ideal sample for integrity microenvironment, rich in ECM and important biomaterial for tissue engineering [16]. Analysis of images related to H&E,

DAPI staining and SEM showed that the decellurized method could successfully obliterate many elements of the cells at the same time that still remains the structure of the tissue. Quantitative evaluation of nuclear material DNA confirmed very little residue. The residual DNA removal and cell remnant from the tissue leads to better relocation to the scaffold, reduces the immune response and as well as the viability of cells [17]. The whole body of testes were decellurized using the triton X-100 and detergent ionic (SDS) and finally physical process. Detergents such as SDS has an effective role on the ECM and retain the 3D structure of the matrix [18].

Ultimately one of the most important for normal cellular behavior of decellularized scaffold is retaining the construction and combination of ECM after recellularization [19, 20].

According to the SEM images, seeded MIN-6 cells on the testis-ECM scaffold were formed cluster to multifaceted which were similar to other reports. Beta cell aggregation is natural and results in increased insulin secretion when compared to single cells [21, 22].

Recellularized scaffold with MIN-6 cells not only produced insulin but was also able to release insulin in response to different concentrations of glucose. This finding indicates that the testis-ECM can preserve MIN-6 function as insulin secretion. The insulin secretion in response to glucose by the MIN-6 cells in 3D culture was significantly more than the traditional 2D monolayer culture. In line with our results, INS1E cells growing in a 3D manner improved endocrine function in comparison to their 2D counterparts [23]. Wan, J., et al. (2017). discovered that mouse stem cell derived β-cells seeded on rat pancreas-ECM had a 2-fold increase in insulin gene expression compared to those that were not seeded on pancreas-ECM [24]. Furthermore, Chaimov, D., et al. (2017). discovered that hepatocytes transdifferentiated to β-cells on pancreas-ECM increased insulin secretion by a factor of four [25]. When compared to 2D culture as a control group, the seeded testis-ECM had a 4.4-fold increase in insulin secretion.

While MIN-6 increased insulin secretion, it can as well as induce glucose sensitivity when seeded on softer scaffolds than harder [12]. These findings showed that 3D condition wherein cells were seeded to a more physiologically related microenvironment would be more ideal than the 2D on the plastic plates.

As shown in the results, expression of insulin-relate genes (including Glut2, PDX1, and Ngn3) in the 3D culture was obviously higher than the 2D culture. This finding indicates that testis-ECM can stimulate the MIN-6 cells to produce insulin.

Glut2 regulates glucose uptake in β-cells and is required for insulin release in response to glucose [26].In a previous study, MIN-6 cells in the presence of glucose can express Glut-2 and glucokinase [6, 7].

PDX1's ability to activate insulin gene transcription is dependent on its ability to interact with other transcription factors such as Glut-2, Ngn3, and insulin [27].Ngn3 expression is a critical factor for development of pancreatic endocrine cells and functional indicator of Langerhans islet cell precursor populations during pancreatic evolution [28]. In vivo, ectopic co-expression of Pdx-1 and Ngn3 has been

shown to transform acinar exocrine cells into insulin-secreting cells [29]. Also, findings showed that co-expressing PDX1 with Ngn3 had a synergistic effect on insulin expression [29-31].

As shown in the MTT results, the testis-ECM did not decrease the viability of the MIN-6 cells. This finding indicates non-toxic impact of the decellular-testis scaffold on the MIN-6 cells. Other studies showed that human and rat testes-ECM did not toxic impacts [32, 33].Toxicity tests based on cytocompatibility revealed that the pancreas-ECM was not have toxic to cells [34, 35]. Goh et al. investigated cell survival and insulin gene expression when the Min-6 cell line was perfused into 3D mouse pancreas-ECM [35].

Laminins and collagens are examples of chemicals that are thought to help islets survive. Pdx-1, insulin 1 and insulin 2, glucagon, somatostatin, and Glut-2 may all benefit islets survival by modulating the expression of particular transcription factors and hormones [36]. The testis has different tissue structures such as interstitial tissue, blood vessels, and mucosal lining rich in fibronectin, collagens (I, IV and VI), laminin and proteoglycan [37, 38]. Interestingly, collagens I, III, IV, V and VI, as well as laminin and fibronectin, have been found in islet ECM [39, 40].Proteoglycans are associated with collagen and fibronectin that affects fibrillogenesis, cell attachment and cell migration [14].Both fibronectin and laminin affect β-cell differentiation, proliferation, and even their insulin secretion. Lin et al. showed that ECM proteins such as fibronectin or laminin boosts pancreatic differentiation with enhance in insulin and Glut-2 gene expressions, proinsulin and insulin protein levels, as well as insulin secretion in response to increased glucose concentration [41].In another study, artificial hydrogels containing laminin, collagen IV, and nidogens provide an optimal environment for MIN-6 cell growth and insulin secretion in vitro [42, 43].

The effect of exogenous collagen VI on islet cell survival and function was investigated by Llacua, et al. (2018). Their results have demonstrated that incorporation of collagen type VI inclusion in immune-isolated human islets protects viability and survival of human pancreatic islets in vitro, according to their finding [44].

Successful results from in vitro experiments led us to in vivo experiments to transplant the testis-ECM scaffold. The results of our in vivo study showed that MIN-6 cells could survive properly in scaffolding and the penetration of immune cell was low. Evaluation C-reactive protein (CRP, a convenient marker of inflammation) level confirm the low immunoreactivity of the mice to the testis-SEM scaffold (Supplemental file).

The data also showed that angiogenesis was successfully performed on scaffolds transplanted to the mesentery. The results corroborate that the decellularized pancreatic scaffold is a biocompatible scaffold.

In our study, recellularized scaffold grafts showed the potential to control blood sugar and body weights. Consistent with this finding, subcutaneous transplanted MIN-6 cells induced low blood sugars in diabetic and non-diabetic mice [45-48]. In another study, HyStem-C matrix promoted MIN-6 cell growth in vivo with a reduction of blood sugars in mice [49].

De Carlo et al. found that diabetic rats with islets with pancreas-ECM transplanted subcutaneously within a PEG hydrogel kept hyperglycemia for up to 41 days, whereas islets with Pancreas-ECM but no PEG only retained hyperglycemia for a few days before replacing to a diabetic condition [50]. In diabetic mice, Chaimov et al. transplanted islets and pancreas-ECM hydrogel subcutaneously within alginate capsules into diabetic mice [25]. They investigated a transient reduction in blood glucose but no long-term diabetes reversal.

5. Conclusion

Present study demonstrated a simple protocol for preparing a testicular scaffold with minimal damage to ECM and its components as a novel 3D cell culture platform. Our in-vitro and in-vivo results indicated that the decellularized testis can be considered as an ideal substrate for the β-cell transplantation in diabetic subjects. Therefore, we assume that Testis-ECM can serve as an ideal scaffold for generation bioartificial pancreas.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Samaneh Karimi], [Layasadat Khorsandi]. The first draft of the manuscript was written by [Samaneh Karimi] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Ethics Committee of Abadan University of Medical Sciences (approval number:IR.ABADANUMS.REC.1397.019).

Consent to publish

Not applicable.

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Supplemental File

The Supplemental File is not available with this version.

Figures

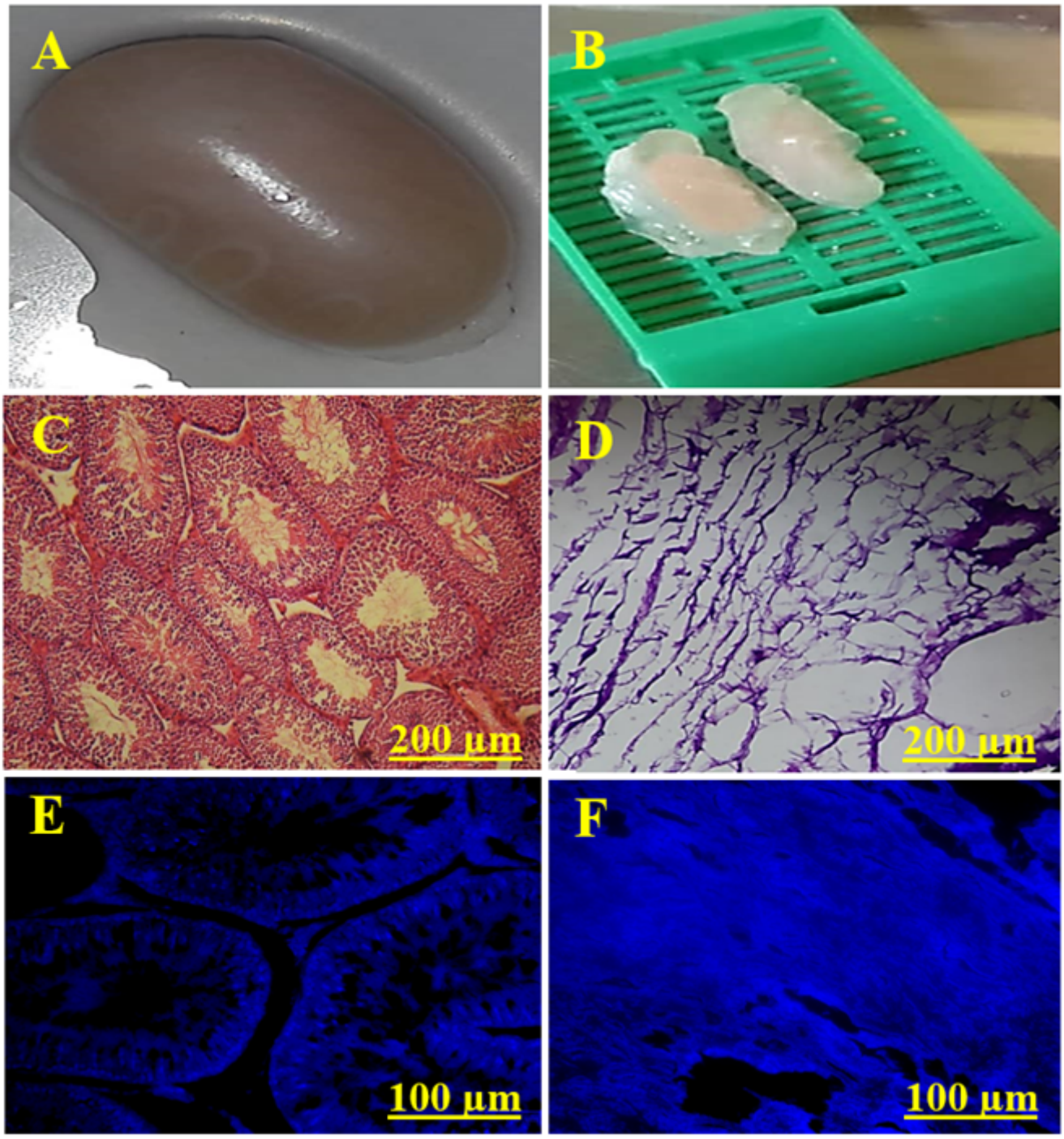

Figure 1

Characterization of the Testis-ECM scaffold. a and b: Gross picture of native testis, and decellularized testis; H&E staining of native testis (c), and decellularized testis (d). DAPI staining of native testis (e), and decellularized testis (f).

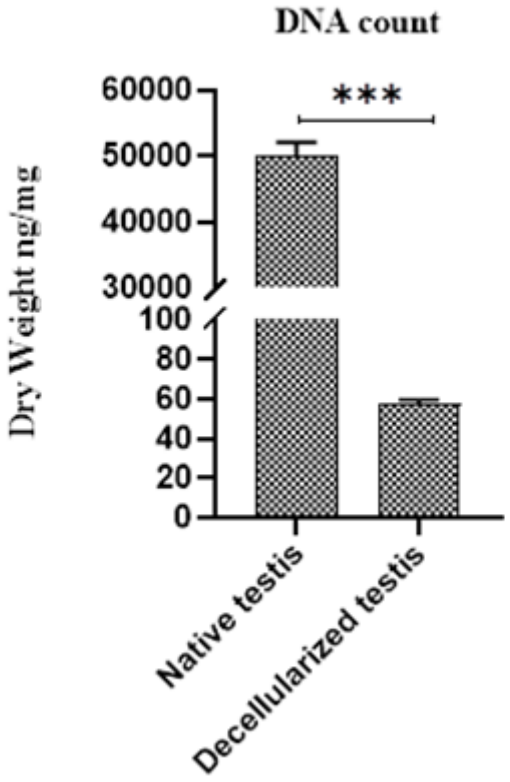

Figure 2

DNA quantification revealed significant cell removal in decellularized testis in comparison to the native testis (n=6). *** $p < 0.001$

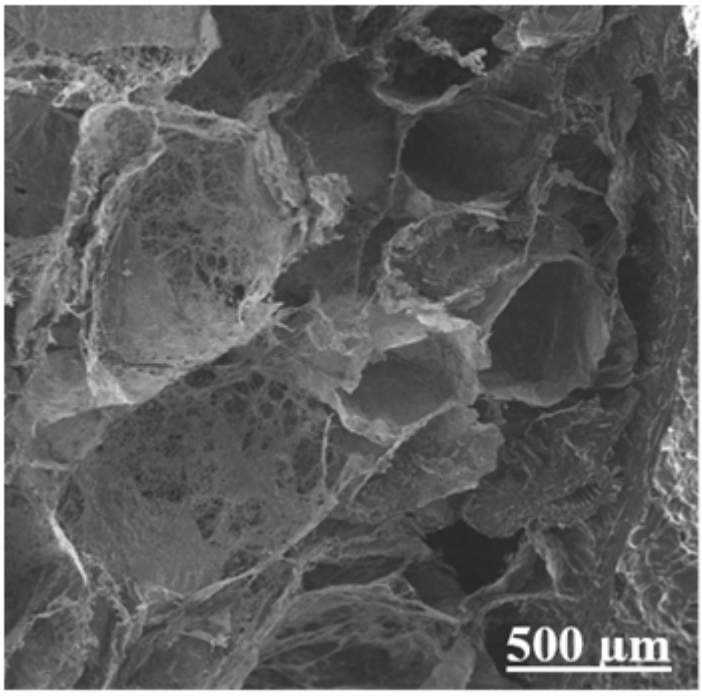

Figure 3

Scanning electron microscopy (SEM) of testicular tissue after decellularization showing ultrastructure of the seminiferous tubules in a transverse section.

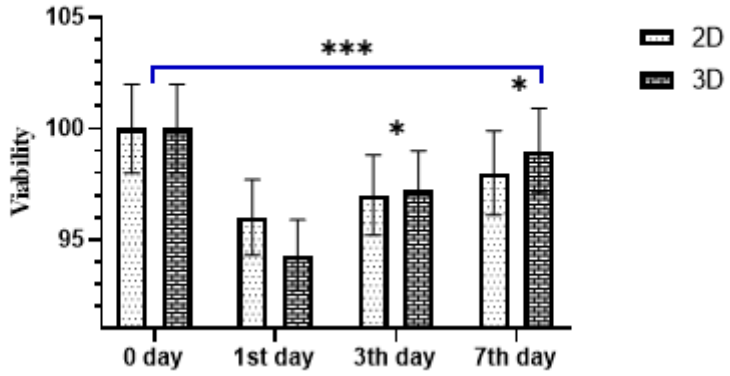

Figure 4

Cell viability percentage in both 2D and 3D groups in different days. Values are presented as mean ± SD (n=6). *P < 0.05 (comparison with 2D group as control)

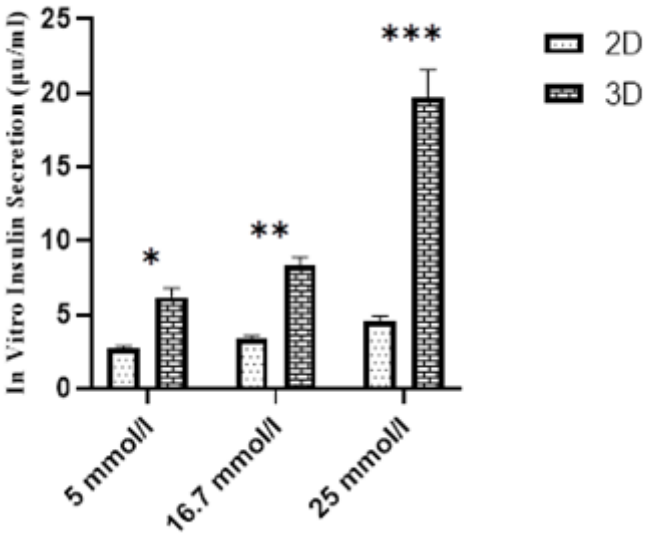

Figure 5

In Vitro insulin secretion in response to different concentrations of glucose. Values are presented as mean ± SD (n=5).

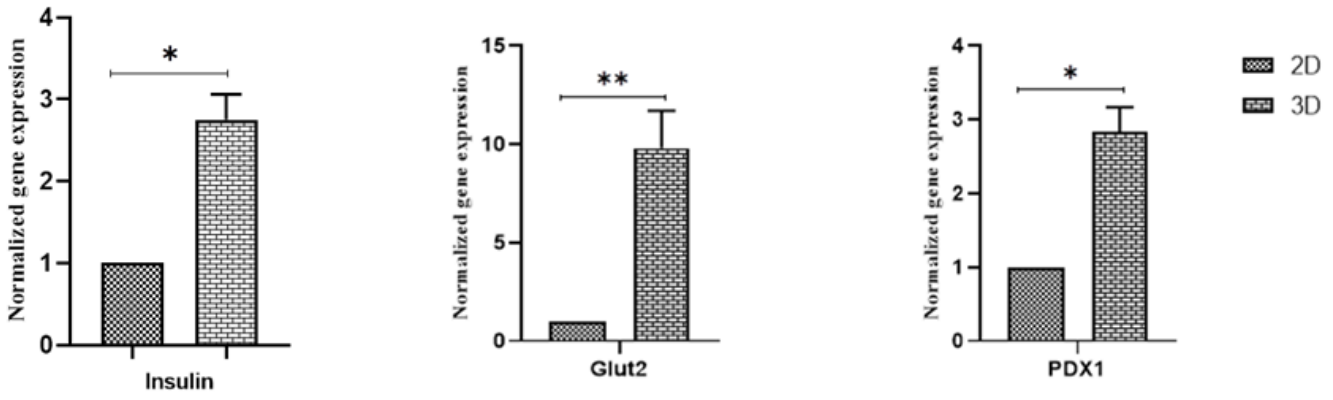

Figure 6

Gene expression of the transcription factors PDX1, Glut2, and Insulin in MIN6 cells cultured on cell-culture plate as 2D group and decellularized testis as scaffold after 7 days. The relative genes expression were calculated after normalization to the GAPDH. Values are mean ± SD, *P < 0.05, **P < 0.01 compare to Control group.

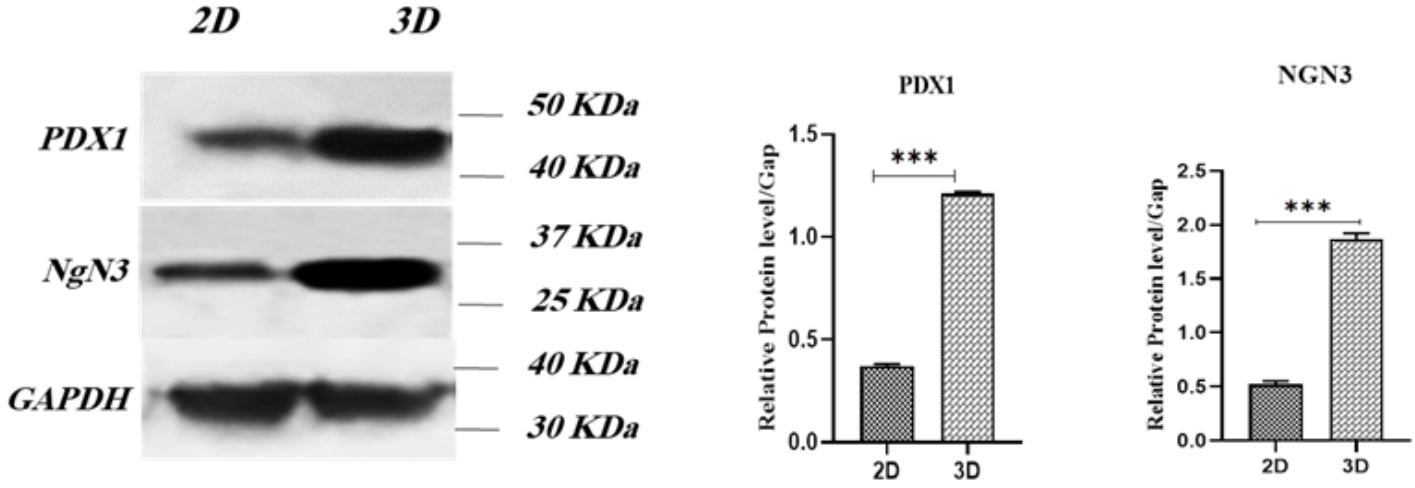

Figure 7

Expression of PDX1and Ngn3 in MIN-6 cells cultured on cell-culture plate as 2D group and testis-ECM as scaffold after 7 days. The relative genes expression were calculated after normalization to the GAPDH. Values are mean ± SD, ***P < 0.001 compare to Control group.

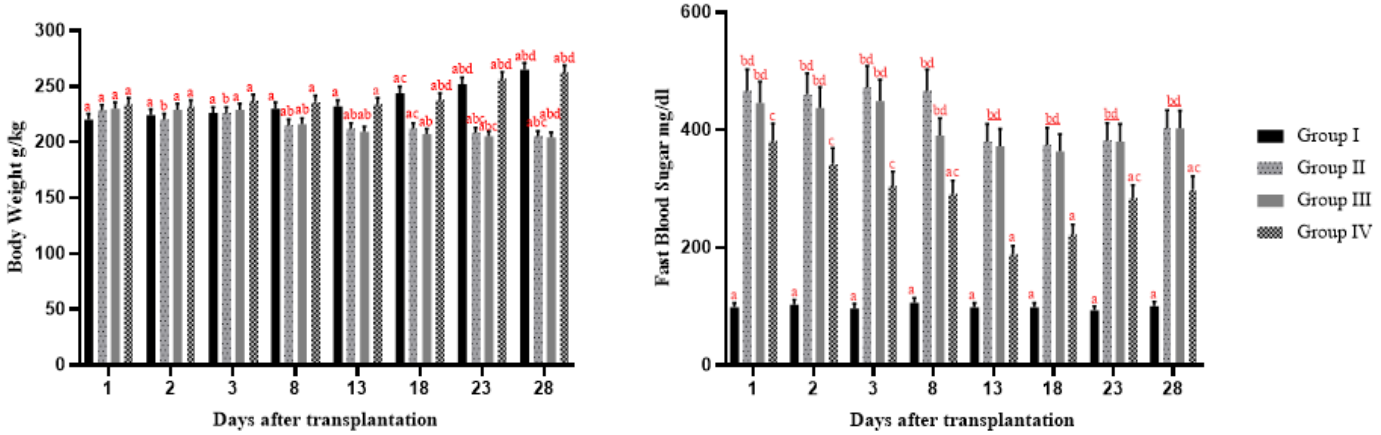

Figure 8

Body weight (left graph) and fast blood sugar (right graph) after 28 days in vivo transplantation. I: healthy rat; II: diabetic rat without interference; III: diabetic rat with scaffold transplantation; V: diabetic rat with recellularized scaffold transplantation group. Data have been presented as mean ± SEM (n=6). There were no significant differences between the columns, containing at least one similar letter. However, different letters reveal a significant difference ($p < 0.05$).

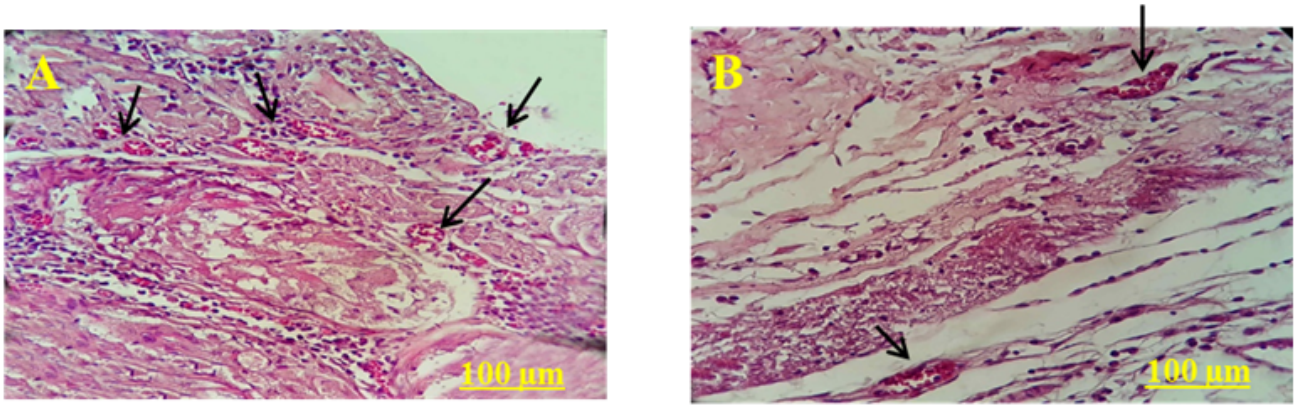

Figure 9

Certain angiogenesis can be seen perspicuously among its surrounding at the implantation site, after 28 days in vivo transplantation in both recellularized scaffold (a) and scaffold (b) (black arrows), Bar = 100μm.

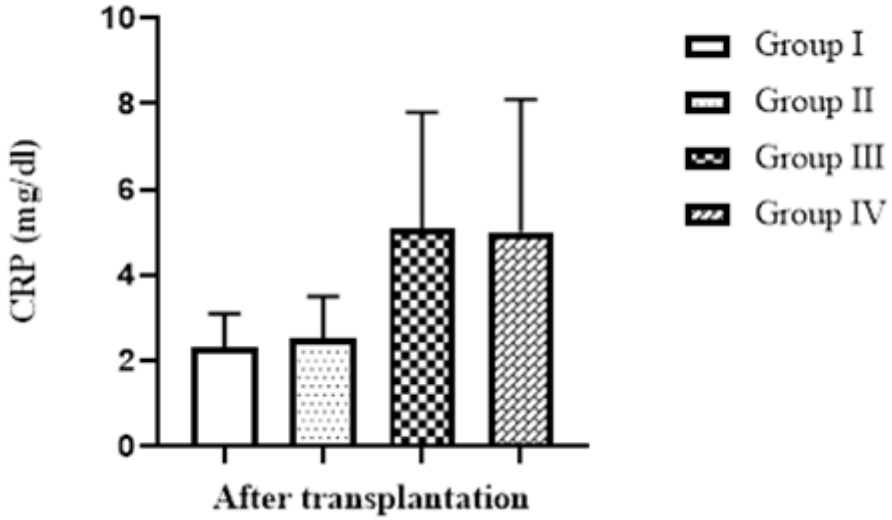

Figure 10

The CRP level of rat's blood serum was calculated after 28 days in vivo transplantation. I: healthy rat; II: diabetic rat without interference; III: diabetic rat with scaffold transplantation; V: diabetic rat with recellularized scaffold transplantation group. Data have been presented as mean ± SEM (n=6). There were no significant differences between the groups.