

The potential of the incorporated collagen microspheres in alginate hydrogel as an engineered three-dimensional microenvironment to attenuate apoptosis in human pancreatic islets

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Abstract

Background: Tissue engineering is considered as a promising tool for remodeling the native cells microenvironment. In the present study, the effect of alginate hydrogel and collagen microspheres integrated with extracellular matrix components were evaluated in the decrement of apoptosis in human pancreatic islets.

Methods: For three dimensional culture, the islets were encapsulated in collagen microspheres, containing laminin and collagen IV and embedded in alginate scaffold for one week. After that the islets were examined in terms of viability, apoptosis, genes and proteins expression including BAX, BCL2, active caspase-3, and insulin. Moreover, the islets function was evaluated through glucose-induced insulin and C-peptide secretion assay. In order to evaluate the structure of the scaffolds and the morphology of the pancreatic islets in three-dimensional microenvironments, we performed scanning electron microscopy.

Results: Our findings showed that the designed hydrogel scaffolds significantly improved the islets viability using the reduction of activated caspase-3 and TUNEL positive cells.

Conclusion: The reconstruction of the destructed matrix with alginate hydrogels and collagen microspheres might be an effective step to promote the culture of the islets.

Background

The isolation process of the human pancreatic islets from their niche leads to loss of biological and mechanical support of the cells. In this stressful situation, the apoptosis is activated in the islets; hence, eliminating this process becomes critical in order to reduce the number of tissues needed for pancreatic islet transplantation (1-4). Tissue engineering is an attractive strategy for remodeling extracellular matrix (ECM) and improving the culture and transplantation methods.

In order to design an efficient engineered tissue, we need to pay attention to the native tissue niche. In the pancreas, the islets are surrounded by a capsule of collagen I, collagen IV, laminin, and fibronectin (5). Hydrogels are useful tools to encapsulate the pancreatic islets (6). Cell encapsulation in hydrogels provides opportunities to eliminate immunological responses and ameliorate the cells viability and function during *in vitro* culture and transplantation. In many studies, alginate was used for pancreatic islets encapsulation. Alginate is a biocompatible, inexpensive and available bioactive molecule (7). It was reported that this polysaccharide dose not interact with the pancreatic islets function (8, 9). In addition, the alginate is one of the few substances that can be processed in the form of hydrogel to encapsulate the cells in physiological conditions (10). In this regard, Lee et al. evaluated the effect of alginate and alginate-collagen composite on the pancreatic islets isolated from rat. They showed superiority of collagen-alginate composites on alginate. Moreover, xenotransplantation of these composites in BALB/c mice indicated that the cells had a high viability and appropriate function to control the glucose level (11). On the other hand, previous findings showed that the application of ECM components including laminin and collagen IV facilitated the survival of mice pancreatic islets (12).

In 2009, researchers used collagen-microspheres in alginate gel to improve the performance of glucose derived neurotrophic factor (GDNF)-secreting HEK293 cells (13). They found out that collagen microspheres in alginate gel increase the secretion level of GDNF over time. Therefore, this engineered structure was also potentiated to control the delivery of GDNF.

According to the importance of the three dimensional (3D) microenvironment and ECM in the maintenance of cell integrity, viability, and function, reconstruction of the cells microenvironment can be a step forward in improving the implant outcome of the cells. This study was conducted to examine alginate hydrogel and collagen microspheres-containing the essential elements of ECM including collagen IV and laminin-embedded in alginate hydrogel scaffolds for attenuating apoptosis in human pancreatic islets.

Methods

Human pancreatic islets isolation and culture

Human pancreases were harvested from Shiraz organ transplant center under ethical protocols approved by Shiraz University of Medical Sciences, and in accordance with the Code of Ethics from Declaration of Helsinki and its later amendments for experiments involving humans. The pancreas was digested in Richordi chamber containing several marbles and collagenase/neutral protease (Serva, Germany). After confirming the isolated islets by DTZ staining, purification step was done in a COBE 2991 cell processor, using Biocoll (Biochrom, Germany) density gradient. The samples with more than 70% purity- the ratio of DTZ-stained part to the whole digested tissue- were plated in non-adherent cell culture flasks (Corning, Canada) in the presence of CMRL 1066 (Gibco, UK) supplemented with 1% FBS (Gibco, UK), 1% L-glutamine, 1% antibiotic-antimycotic (Sigma, Germany), and 6.25 µg/ml ITS (Sigma, Germany). The islets were cultured overnight in 5% CO₂ at 37°C (14-16).

Scaffold preparation

One thousand islet equivalents (IEQ) were assigned to each group, including the control group without scaffold (Con.), Alginate hydrogel group (Alg.), collagen I microspheres incorporated in the alginate hydrogel group (Coll. I & Alg.), collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group (Coll. I, IV & Alg.), collagen I microspheres containing laminin incorporated in the alginate hydrogel group (Coll. I, Lam & Alg.), and collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group (Coll. I, IV, Lam & Alg.).

The pancreatic islets were encapsulated in the scaffolds according to a previous protocol with some modifications (13). In order to produce the microspheres, eight part 2mg/ml collagen I (Sigma, Germany) was mixed with one part 10X RPMI 1640. In the groups containing collagen IV or/and laminin, these ECM components were added with the final concentration of 1 mg/ml. The pH of the mixture was adjusted to 7.4 with 1M NaOH, if needed. Then, the pancreatic islets of each group were mixed with the 100 µl neutralized combination and dispensed as 2µl droplets onto a sterile 60 mm petri dish coated by UV-

irradiated parafilm. For gel formation to occur, the droplets were incubated at 37°C for 1h. After that, the microspheres were cultured overnight in completed CMRL medium. The next day, the microspheres were transferred to a 15ml tube and settled for 15 min to separate the microspheres from the media.

In order to prepare that alginate gel, 1000 IEQ pancreatic islets or 50 microspheres were suspended in 250 µl sodium alginate (Sigma, Germany) with a final concentration of 2%. The mixture was then added to a 2cm² mold and incubated in 100 mM CaCl₂ solution for 15 min at room temperature (Fig. 1). Then, the gel was detached from the well and washed with normal saline. Finally, the scaffolds were placed in 6 cm culture plate in complete CMRL medium for one week.

Inverted Microscope and Scanning Electron Microscope (SEM) analysis

After one-week incubation in different groups, the morphological changes of the islets were evaluated under inverted microscope. To analyze the engineered structures and human pancreatic islets using SEM, we used a previous method with some modifications (11). We washed the scaffolds in PBS and fixed them in 2.5% glutaraldehyde for 1h (Sigma, Germany). The structures were dehydrated in serial dilutions of 25%, 50%, 75%, and 95% ethanol for 30 min each. The scaffolds were dried and analyzed under SEM. For observing the structures with the SEM (Tescan Vega3,) the samples were mounted on stubs and coated with gold.

Viability of the pancreatic islets

To examine the effect of the scaffolds on the pancreatic islets viability after one week, we applied fluorescent dyes (Sigma, Germany)(17). In this regard, 5mg/ml fluorescein diacetate (FDA) and 2mg/ml propidium iodide (PI) were used to detect the live and dead cells, respectively. The stained islets were evaluated under fluorescent microscope (CKX53, Olympus, Japan). The viability rate was estimated by the ratio of FDA positive area to the whole of each islet.

Gene expression in the pancreatic islets

The expression of BAX, BCL2, and BAX/BCL2 ratio genes were evaluated by real time RT- PCR. RNA of the free and encapsulated islets were extracted by RNA-Sol isolation kit (Alphabio, Canada). Then, cDNA was synthesized using PrimeScript™ RT Reagent Kit (Takara, Japan). The primer design was done by NCBI tool Primer BLAST and GAPDH was considered as the housekeeping gene. The primer pairs were: GAPDH, F: GCTCATTTCCTGGTATGACAACG and R: CTCTCTTCCTCTTGTGCTCTTG; BCL2, F: GATGGGATCGTTGCCTTATGC and R: CAGTCTACTTCCTCTGTGATGTTGT; and BAX, F: TTCTGACGGCAACTTCAACT and R: GGAGGAAGTCCAATG TCCAG. The amplification of the target was done by SYBR® Premix Ex Taq™ II (Takara, Japan), using Applied Biosystems StepOnePlus™ Real-Time PCR system (ABI, USA) in 40 cycles. The fold changes of each gene was calculated by $2^{-\Delta\Delta CT}$.

Protein expression in the pancreatic islets

The assessment of BAX, BCL2, and active caspase-3 proteins expression was performed by immunocytochemistry method. The free and embedded islets in the scaffolds were fixed in 4% paraformaldehyde for 15 min. The islets and the scaffolds were both embedded in low melting agar (Sigma, Germany) and paraffin (18). The cell blocks were sectioned at 5µm thickness. Immunocytochemistry was performed using primary antibodies against BAX, BCL2, and active caspase-3 (abcam, USA). Incubation in the primary antibody was done overnight. After incubation in the secondary antibody (abcam, USA) for 1h, the positive cells were detected by 3,3'-Diaminobenzidine (DAB). Counterstain was carried out with Hematoxylin staining. The samples were evaluated and the H-score was calculated as follows: H score=1 × (% light staining) + 2 × (% moderate staining) + 3 × (% strong staining) (19).

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay

To determine the TUNEL positive cells, sections of the cell blocks were assessed by Click-iT® Plus TUNEL Assay (life technology, France). In this regard, the sections were deparaffinized in xylene and serial dilutions of ethanol. The TdT reaction was conducted for 60 min. After that, Click-iT® Plus reaction was performed for 30 min. DAPI (sigma, Germany) staining was used for counterstaining. The stained samples were analyzed under fluorescence microscope (CKX53, Olympus, Japan).

Insulin and C-peptide stimulation indexes

For insulin and C-peptide stimulation indexes assays, the pancreatic islets in the control and scaffold groups were exposed to 2.8 mM glucose in RPMI 1640 supplemented with 0.5% BSA. After 1h, the medium was collected. Next, the islets were incubated in 20 mM glucose in RPMI 1640 supplemented with 0.5% BSA for 1h, and the medium was collected. The concentrations of Insulin and C-peptide in the collected culture media was determined *via* ELISA kits (Monobind, USA) and microplate spectrophotometer (BioTek Epoch™, USA) at 450 nm. The ratio of the secreted insulin or C-peptide concentration in 20mM to 2.8mM was calculated as the stimulation index (20).

Statistical analysis

Statistical analysis was performed *via* Kruskal-Wallis and Post-hoc test in SPSS software and the charts were drawn in Graph Pad Prism 6 Program. The data are presented as mean ± S.E. P < 0.05 was considered to be statistically significant.

Results

Morphological features of the human pancreatic islets were maintained in different scaffolds

Subsequent to the culture of the islets in the presence and absence of hydrogels for one week, they were evaluated by inverted microscope. The pancreatic islets in different scaffolds preserved their native spherical structure. In the control group- free of scaffold- the integrity of the peripheral cells in the islets seemed to have somewhat distorted (Fig. 2).

The evaluation of the islets and hydrogel structures by SEM revealed that the islets were visible as spherical structures with high integrity. However, the islets integrity was altered in the control group. SEM images demonstrated that the alginate hydrogel and microspheres produced a porous network. Yellow arrow in Fig.3B indicates that the alginate has a rough surface. Moreover, Fig. 3D, E, and F show that the islets have been implanted in the microspheres.

The produced scaffolds maintained the pancreatic islets viability

The assessment of the islets using live-dead staining showed that in the absence of 3D microenvironment, the islets died. However, in the presence of alginate hydrogels and microspheres, the islets viability was significantly maintained ($P < 0.001$). There were no differences between the scaffold groups for cellular viability (Fig. 4).

The gene expression of BAX and BCL2 and the ratio of BAX/BCL2 were not altered in different scaffold groups

The study of apoptotic (BAX) and anti-apoptotic (BCL2) genes expression and the BAX/BCL2 ratio showed that there was no significant difference between different groups of hydrogel scaffold ($P = 0.285$ for BAX gene, $P = 0.152$ for BCL2 gene and $P = 0.744$ for BAX / BCL2 ratio).

The results indicated that the expression of BAX in all groups containing microspheres was approximately lower than (Alg.) group. On the other hand, the expression of BCL2 had increased in all microsphere groups. In the Coll. I, IV, Lam & Alg. group, the reduction of BAX gene expression and the increase of BCL2 gene expression was slightly higher than the other groups. A comparison of BAX / BCL2 ratios showed that this ratio was lower in all microsphere groups especially in the Coll. I, IV, Lam & Alg. group. All in all, there were no significant differences in genes expression between the groups (Fig. 5).

The presence of 3D microenvironment inhibited the activation of caspase-3 in human pancreatic islets

Immunocytochemistry studies after one week of incubation showed that there were significant differences in the level of proteins expression (Fig. 6, 7, and 8). Evaluation of the BAX protein indicated that the expression of this apoptotic protein in the control group was more than the other groups. The reduction of this protein expression was significant in the hydrogel scaffold groups the Coll. I & Alg. ($P < 0.001$), the Coll. I, IV & Alg. ($P < 0.001$) and the Coll. I, IV, Lam & Alg. ($P = 0.019$).

AS the expression of anti-apoptotic protein, BCL2, a slight decrease in the expression was observed in the hydrogel scaffold groups compared to the Con. group, but this decrease was not significant ($P = 0.563$).

Investigating the expression of active form of caspase-3 protein showed that the expression of this protein in the Con. group was significantly higher than all hydrogel scaffold groups ($P < 0.001$), and this decrease in expression was significant in all groups, except for the Alg. group.

The hydrogel scaffolds drastically reduced the TUNEL positive cells in human pancreatic islets

The application of TUNEL technique on the pancreatic islets after one-week of incubation in different groups indicated that there was a significant difference between the number of TUNEL positive cells ($p < 0.001$). A large number of cells in the Con. group were TUNEL positive while in the hydrogel scaffold groups ($P < 0.001$ for the Alg., Coll. I & Alg., Coll. I, IV & Alg., and Coll. I, Lam & Alg. groups and $P = 0.032$ for the Coll. I, IV, Lam & Alg. group) they were negative or limited (Fig. 9).

There was no statistically significant difference in insulin and C-peptide stimulation indexes between the different incubation conditions

Subsequent to one-week incubation of the pancreatic islets, cells performance was investigated in the presence or absence of scaffolds. In this regard, after treating the cells with the concentrations of 2.8 and 20mM glucose, their insulin and C-peptide stimulation indexes were examined (Fig. 10A and B). The findings showed that there was no significant difference in insulin secretion ($P = 0.791$) and C-peptide ($P = 0.190$) indexes between different groups.

Discussion

The mechanical and enzymatic digestion during pancreatic islet isolation leads to destruction of ECM, resulting in loss of biological and mechanical protection. In this regard, tissue engineering is a promising way for restoring ECM by maintaining the structure and function of the cells. Recent studies have shown that the culture of human islets in 3D scaffolds improves the morphology, viability, and performance of the islets during pre-transplant stage.

In the present study, the effect of 3D hydrogel microenvironment with different compounds was used to evaluate the viability and functionality of human pancreatic islets of Langerhans. For this purpose, the islets in different groups the Con., Alg., Coll. I & Alg., Coll. I, IV & Alg., Coll. I, Lam & Alg., and Coll. I, IV, Lam & Alg. were incubated for one week and then examined for morphology, viability and functionality. In order to culture the islets in alginate hydrogel scaffolds, in four groups of; the Coll. I & Alg., Coll. I, IV & Alg., Coll. I, Lam & Alg., and Coll. I, IV, Lam & Alg., first, the islets were encapsulated in the collagen I microspheres in the presence or absence of collagen IV and laminin, and finally placed in the alginate hydrogel platform.

Evaluation of the islets structure with inverted optical microscopy showed the maintenance of the cells native morphology in the hydrogel scaffolds. The obtained results were confirmed by SEM. Our findings showed that spherical-3D morphology and integrated pancreatic islet structures were preserved in all hydrogel scaffolds groups. On the other hand, it seemed that the structural integrity of the islets in the control group was relatively disturbed.

According to the Nomenclature Committee on Cell Death (NCCD), using fluorescent dyes as one of the criteria to confirm the lack of membrane integrity and cell death, in this study, cell viability was examined with PI. It was clearly observed that the viability of the pancreatic islets in the course of one week in the control group was significantly reduced, while in hydrogel groups, it was completely preserved.

Previous studies showed that hydrogels, by simulating hydrophilic elements of the ECM, were desired tools for the encapsulation of the pancreatic islets. Recently, researchers have used various biomaterials and methods to achieve an optimal and efficient system for the reconstruction of the human pancreatic islets ECM. Cellular scaffolds design should be done in a way that they can mimic the inherent ECM, by maintaining cellular viability.

In this study, the main substrate of the hydrogels was alginate. Studies have shown that the use of alginate polysaccharide has many advantages. Alginate is the most common biomaterial for islets encapsulation. Alginate capsule processing is possible in physiological conditions, including body temperature, physiological osmolarity and pH (21, 22). Moreover, alginate is generally biocompatible and biodegradable. It has been shown that alginate is suitable for encapsulating the cells and controlled delivery of bioactive molecules. In addition, this biomaterial can affect cell growth, metabolism and insulin production (8, 23, 24). Investigation of the intrinsic microenvironment of human pancreatic islets showed that their ECM consisted of collagen I, III, IV, V and VI, laminin and fibronectin (25, 26).

Zhang et al. studied the survival of pancreatic islets in a collagen matrix of type I for 7 days. They found that cellular viability was significantly higher than that of the control group. In addition, the islets enclosed in the collagen matrix had a much lower number of active caspase-expressing cells than the control group (27). Lee et al. also made a comparison between the rat pancreatic islets cultured in the alginate and the alginate-collagen type I composite, and observed that the used composite remarkably improved the cells viability (11). On the application of ECM factors, researchers examined the survival rate of mice islets in silk hydrogel containing laminin and collagen IV. This study showed that Silk alone could help to increase the islets survival, and adding laminin and IV collagen cannot promote survival in this hydrogel (12). In a previous study, perfluorodecalin-enriched fibrin matrix led to the reduction of caspase-3 activation in the cultured human pancreatic islets. This structure provided a favorable chemical and physical environment for these cells, but the study was only performed within 24 hours (28). In general, studies on the evaluation of apoptosis following the use of 3D scaffolds in human pancreatic islets are very limited.

In the present study, we observed that in all groups with hydrogel scaffolds the viability of human pancreatic islets was significantly higher than the control group, and the presence of collagen and laminin did not have any superiority over pure alginate hydrogel. In total, all scaffolds designed in this study were able to fully maintain the viability of the islets (Fig. 11).

Given the importance of cellular life in the prognosis of pancreatic islet transplantation, it seems essential to investigate the cause of islets' death in order to prevent this destructive process. In this regard, the present study focused on the internal pathway of apoptosis - as the main cause of pancreatic islets death. Therefore, the expression of genes and proteins involved in the process of apoptosis and the number of positive TUNEL cells was evaluated.

Although the analysis of BAX and BCL2 genes expression and the BAX/BCL2 ratio revealed that there was no significant difference between different groups of scaffolds, we observed an increase in the BCL2

gene and a decrease in the BAX gene and BAX/BCL2 ratio. On the other hand, the evaluation of BAX, BCL2 and active caspase-3 proteins indicated significant differences between the hydrogel scaffold groups and non-scaffold group. BAX expression decreased in all scaffold groups, but contrary to the expectation, the expression of BCL2 showed a non-significant reduction. Furthermore, we found out that the expression of active caspase-3 was reduced in all hydrogel scaffold groups. This reduction was significant in the surrounded microspheres by alginate hydrogel. The contradiction between significant differences at the level of genes and proteins expression might have been due to the effect of scaffolds on post-translational proteins modifications.

In order to further clarify the cell death pathway, after the evaluation of active caspase-3 protein expression, we measured the number of positive TUNEL cells. In the control group, a large number of cells were TUNEL positive, as the high expression of active caspase-3 and enormous cell death was evident. However, the findings showed that although caspase-3 expression was slightly expressed in 3D-scaffold groups, very limited number of cells were TUNEL positive. This discrepancy may suggest that caspase-3 activation is not necessarily indicative of apoptosis (29). On the other hand, it seems that there is a threshold for the destructive activity of active caspase-3. Geske et al. showed that apoptotic cells induced by p53 could be released from apoptosis during the removal of apoptotic stimulus. Their study suggested that DNA repair could be activated in this process and in some cases results in a return from the cell death pathway (30). All in all, studies suggest that the mechanisms involved in apoptosis are complex and a cascade of molecular events guides intrinsic and extrinsic apoptosis. Previous evidence is based on the fact that these two pathways are interconnected and the molecules involved in one path can affect the other (31).

The performance of pancreatic islets is an indicator of the islets transplantation prognosis. In this study, the evaluation of insulin and C-peptide secretion indexes suggested that hydrogel scaffolds did not have an adverse effect on the secretory function of these islets.

With respect to the application of collagen microspheres, Wang et al. in their study suggested that collagen microspheres, compared with single-layer cultures, increased the production and secretion of GDNF from HEK293 cells (13). Subsequently, Lee et al. embedded these cells in collagen microspheres, incorporated in alginate hydrogel and compared GDNF secretion in these conditions with cells embedded in collagen I and alginate composites (13). They found that in the composite, stable release of GDNF occurred throughout the entire culture period, and its release level was controlled by various concentrations of alginate. On the other hand, due to proliferation of HEK293 cells in collagen microspheres enclosed in alginate scaffolds, GDNF release was increased steadily. In the present study, there was no significant differences in the index of insulin and C-peptide secretion between the groups containing microspheres and the control group. Overall, the 3D scaffolds designed in this study had no adverse effect on the activity of pancreatic islands.

In the present project, our main problem was shortage of human pancreas for research. Obtaining appropriate pancreas for such studies is so difficult and costly. Because there is an inclusion and

exclusion criteria for selecting the suitable pancreases. Moreover, we processed the pancreases which gained informed consent for research. Actually, the superiority of the present study was using human pancreas. The previous studies were mainly on animal sources and cell lines. On the other hand, we had to performed different analysis techniques on the cells in different groups, including viability, gene expression, immunohistochemistry, TUNEL assay, and insulin and C-peptide secretion. Therefore, we limited our target groups to what was mentioned in the manuscript. Moreover, we applied immunocytochemistry in order to examine the protein expression. It would be worthwhile to evaluate the protein levels quantitatively using western blotting if we get access to enough islets to isolate a pool of proteins.

In terms of the lack of significant improvement in the stimulation indexes, it seems that focusing on the scaffold structure is useful in further studies. Previously, researchers explained that high concentration of alginate can increase the scaffold stiffness and cause substances diffusion problems (32). Moreover, Hart et al. reported that densely encapsulated islets led to postponed and restricted insulin secretion (33). Importantly, although we did not observe negative effects of the mentioned scaffolds on the stimulation indexes, we need to design an effective encapsulation system that does not compromise the diffusion of secreted insulin from the islets. In this regard, it is important to examine alginate hydrogel enriched itself with the extracellular matrix components, without encapsulating in microspheres, to improve the results in future studies.

Conclusion

Due to limited survival and efficiency of the pancreatic islets, following the destruction of ECM during the isolation process, rebuilding appropriate matrix can be an effective step to promote their cultivation and transplantation. Based on the obtained results and the apparent effect of the designed scaffolds on the viability of human pancreatic islets, there is a possibility to consider them as cell carriers in regenerative medicine.

Declarations

- Ethics approval and consent to participate

Human pancreases were harvested from Shiraz organ transplant center under ethical protocols approved by Shiraz University of Medical Sciences, and in accordance with the Code of Ethics from Declaration of Helsinki and its later amendments for experiments involving humans

- Consent for publication

“Not applicable”

- Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

- Competing interests

"The authors declare that they have no competing interests"

- Funding

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- Authors' contributions

M.K.: Design of the work, acquisition, analysis, and interpretation of the data; drafting the work.

1. K.: Performed the pancreatic islets isolation procedure, data gathering, and the article revision.
2. S. S.: Performed the pancreatic islets isolation procedure, and the article revision.

N.A.: Design of the work, coordinate the study, interpretation of the data, and the article revision.

M.HA.: Performed the pancreatic islets isolation procedure, and the article revision.

B.G.: Interpretation of the data, and the article revision.

MH.K. and R.Y.: Interpretation of the data, and the article revision.

E.E.: Performed the pancreatic islets isolation procedure, and the article revision.

A.SH. and N.M.: Coordinated the study, interpretation of the data, and the article revision.

S.N.: Interpretation of the data, and the article revision.

IH.AA.: Interpretation of the data, and the article revision.

All authors approved the manuscript for the submission. They agree to be accountable for all aspects of the work.

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Abbreviations

Extracellular Matrix: ECM

Glucose Derived Neurotrophic Factor: GDNF

Islet Equivalent (IEQ)

Control group without scaffold: Con.

Alginate hydrogel group: Alg.

Collagen I microspheres incorporated in alginate hydrogel group: Coll. I & Alg.

Collagen I microspheres containing collagen IV incorporated in alginate hydrogel group: Coll. I, IV & Alg.

Collagen I microspheres containing laminin incorporated in alginate hydrogel group: Coll. I, Lam & Alg.

Collagen I microspheres containing collagen IV and laminin incorporated in alginate hydrogel group: (Coll. I, IV, Lam & Alg.).

Scanning Electron Microscope: SEM

Fluorescein Diacetate: FDA

Propidium Iodide: PI

Terminal Deoxynucleotidyl Transferase DUTP Nick-End Labeling: TUNEL

Nomenclature Committee on Cell Death: NCCD

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Figures

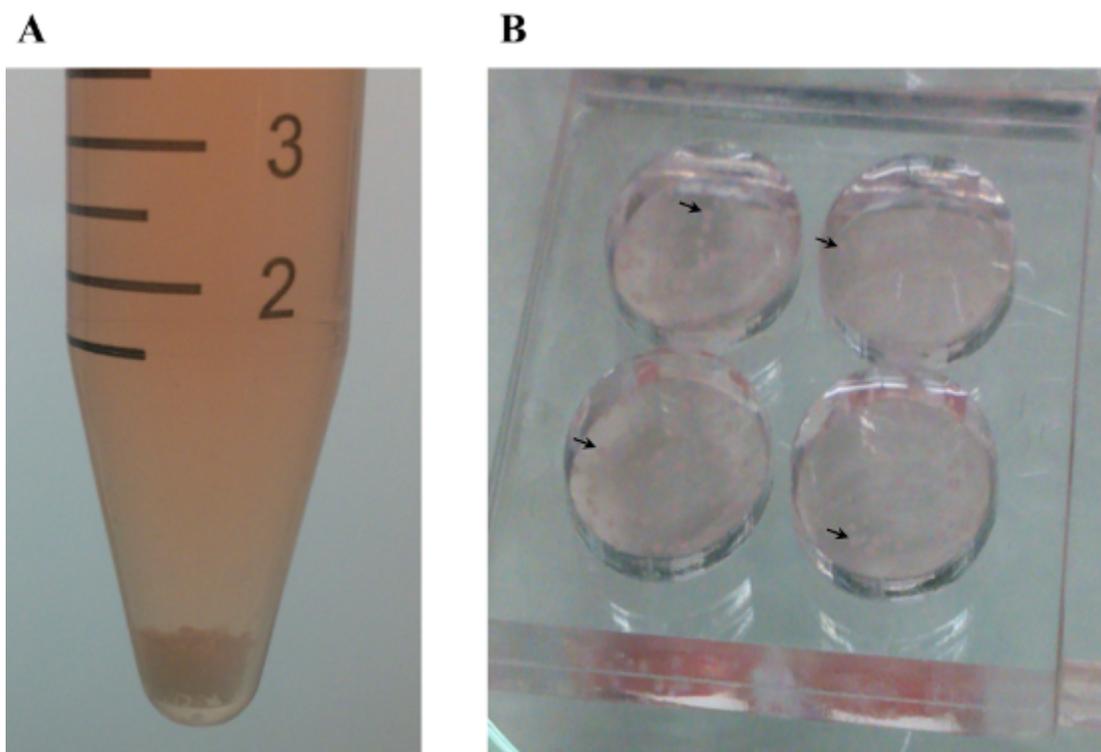


Figure 1

Scaffold preparation. Sedimentation of the microspheres after 15 minutes (A) and molding of the sodium alginate solution (B). The arrows indicate the microspheres in alginate gel.

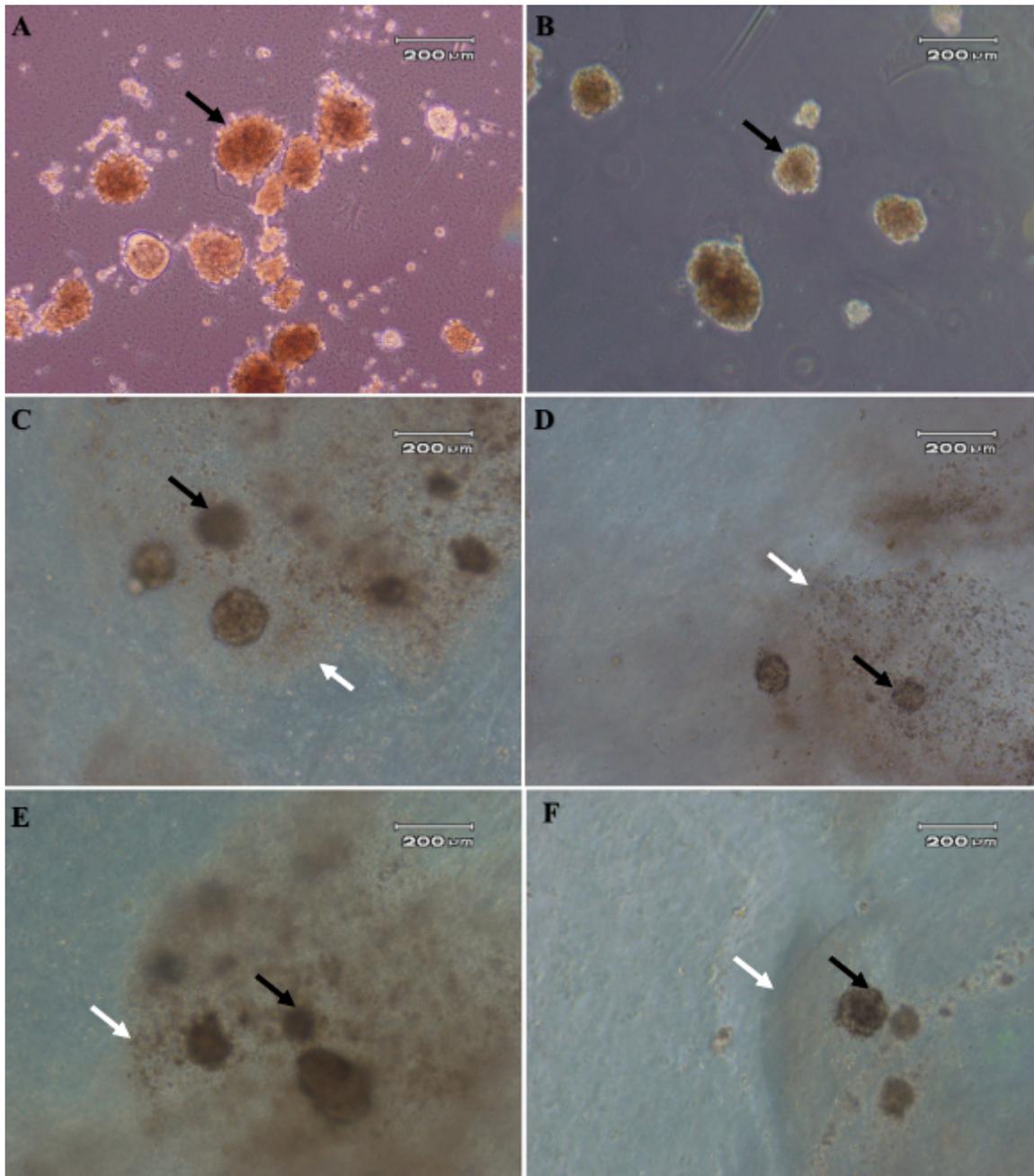


Figure 2

Microscopic view of the pancreatic islets in different groups. The islets maintained their spherical morphology in different hydrogel groups. The black arrows indicate the pancreatic islets and the white arrows show the microspheres. A: Control group, B: Alginate hydrogel group, C: Collagen I microspheres incorporated in the alginate hydrogel group, D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group, E: Collagen I microspheres containing laminin incorporated in the alginate hydrogel group, and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group.

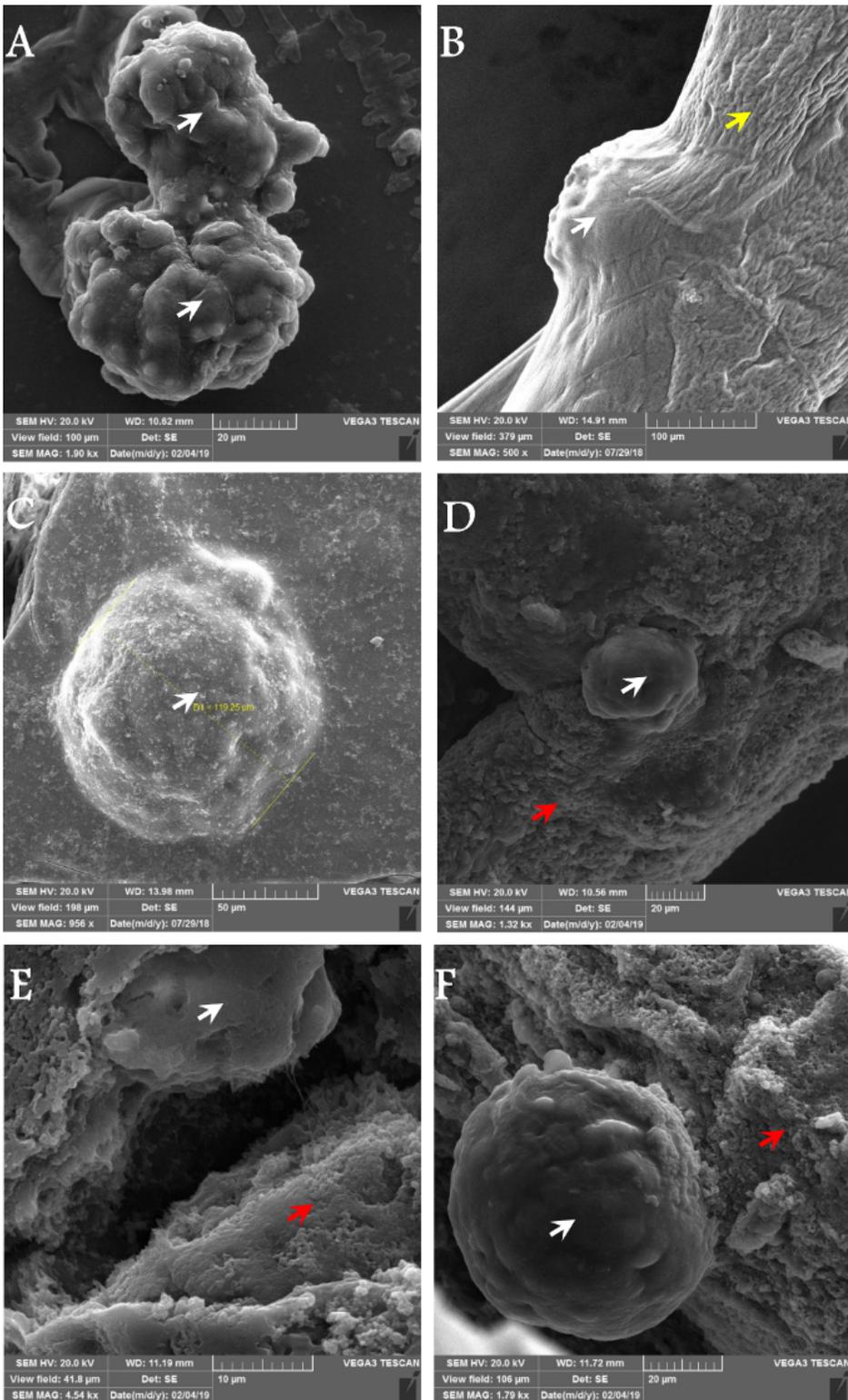


Figure 3

SEM view of the islets in different groups. The islets maintained their spherical morphology in different hydrogel groups. The yellow arrow indicates the rough surface of alginate hydrogel. The white arrows show the pancreatic islets and the red arrows depict the microspheres structures. A: Control group, B: Alginate hydrogel group, C: Collagen I microspheres incorporated in the alginate hydrogel group, D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group, E: Collagen I

microspheres containing laminin incorporated in the alginate hydrogel group, and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group.

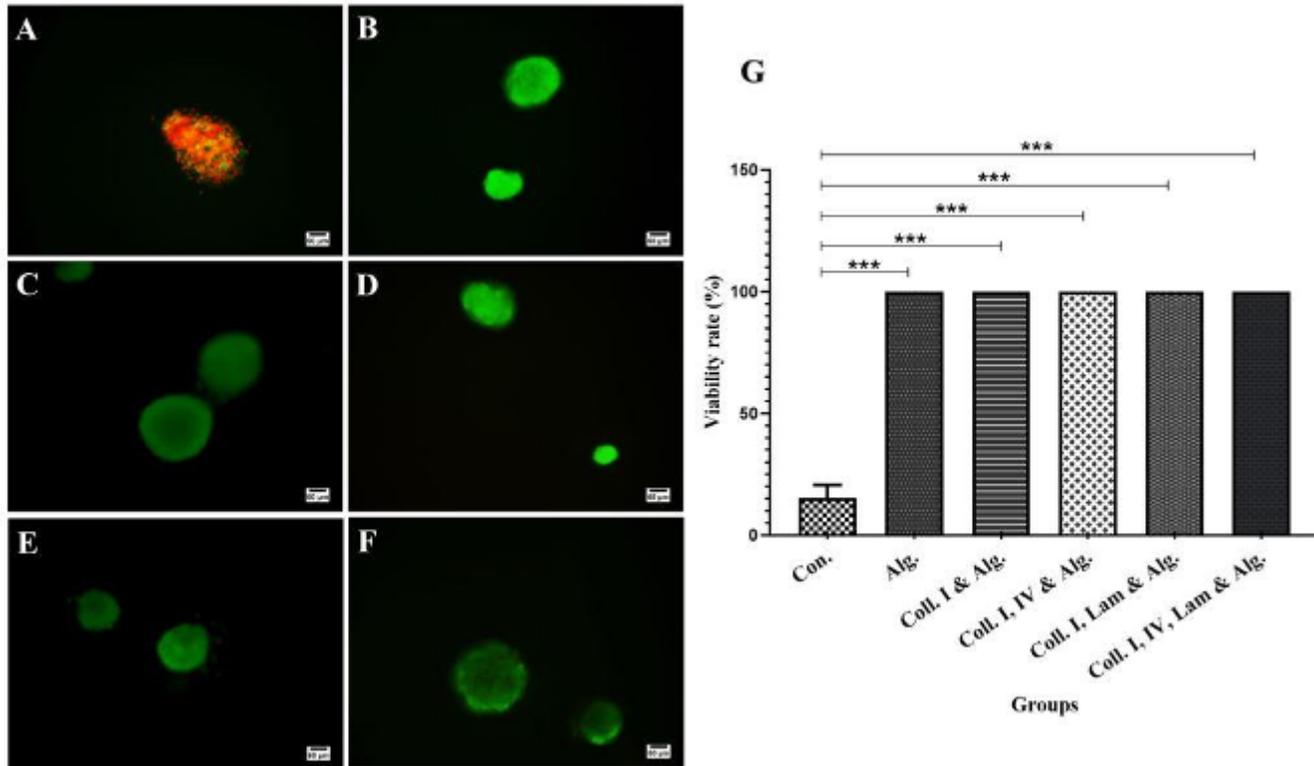


Figure 4

The viability of human pancreatic islets in different groups. Live and dead cells were stained by FDA and PI, respectively. Fluorescent microscopy images were taken separately by the appropriate filter and then were mixed with Image J software (A-F). As shown in the illustrations, in the control group (A), a large number of cells died, but in the presence of hydrogel scaffolds in alginate hydrogel (B), collagen I microspheres incorporated in alginate hydrogel (C), collagen I microspheres containing collagen IV incorporated in alginate hydrogel (D), collagen I microspheres containing laminin incorporated in alginate hydrogel (E), and collagen I microspheres containing collagen IV and laminin incorporated in alginate hydrogel (F) the cells survived. The viability rate histogram shows significant differences between the islets viability in 3D microenvironments compared to the control group (G). Data are reported as Mean \pm SEM. ***: P<0.001). A: Control group (Con.), B: Alginate hydrogel group (Alg.), C: Collagen I microspheres incorporated in the alginate hydrogel group (Coll. I & Alg.), D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group (Coll. I, IV & Alg.), E: Collagen I microspheres containing laminin incorporated in the alginate hydrogel group (Coll. I, Lam & Alg.), and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group (Coll. I, IV, Lam & Alg.).

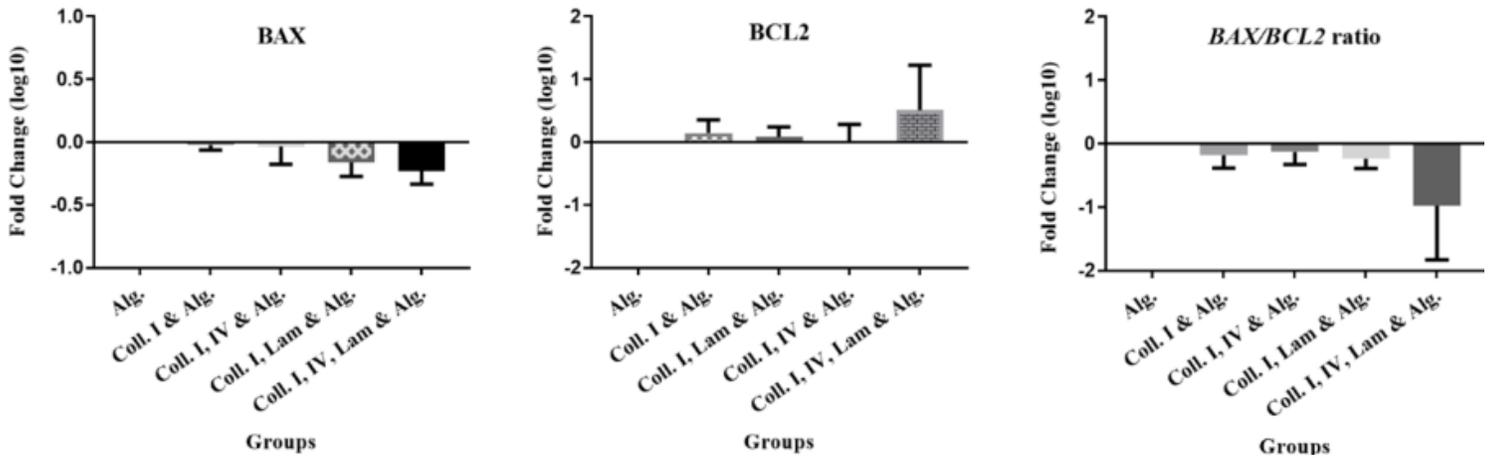


Figure 5

The expression of apoptotic (BAX) and anti-apoptotic (BCL2) genes and their ratio between different scaffold groups. (Alg.): Alginate hydrogel group, (Coll. I & Alg.): Collagen I microspheres incorporated in the alginate hydrogel group, (Coll. I, IV & Alg.): Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group, (Coll. I, Lam & Alg.): Collagen I microspheres containing laminin incorporated in the alginate hydrogel group, and (Coll. I, IV, Lam & Alg.): Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group.

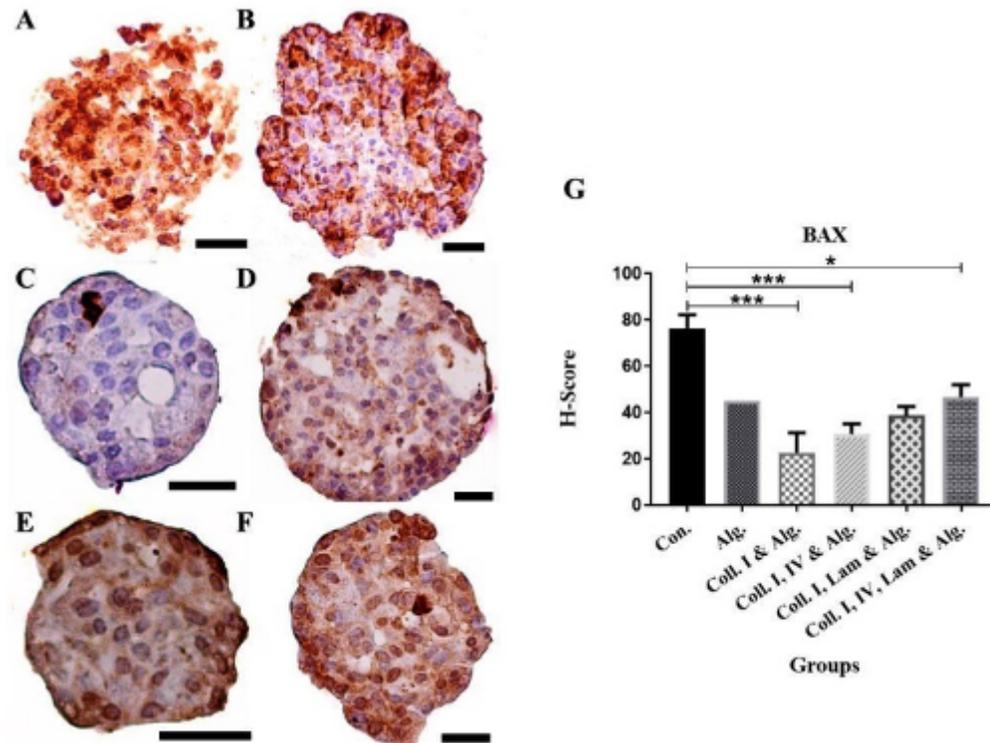


Figure 6

Immunocytochemistry analysis of BAX protein expression in different groups. The brown spots are the positive areas and the nuclei are visible in blue (A-F). The histogram shows the H-score in each group (G). Data are reported as Mean \pm SEM. (Measurement index of images = 20 μ m. *: P<0.05 and ***: P<0.001).

A: Control group (Con.), B: Alginate hydrogel group (Alg.), C: Collagen I microspheres incorporated in the alginate hydrogel group (Coll. I & Alg.), D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group (Coll. I, IV & Alg.), E: Collagen I microspheres containing laminin incorporated in the alginate hydrogel group (Coll. I, Lam & Alg.), and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group (Coll. I, IV, Lam & Alg.).

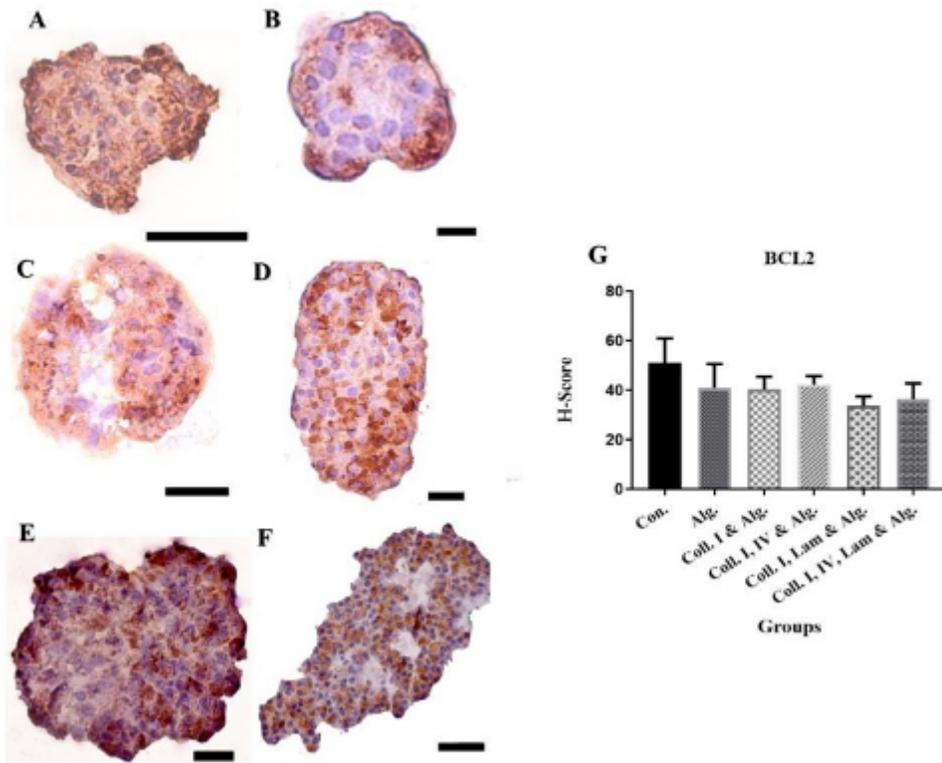


Figure 7

Immunocytochemistry analysis of BCL2 protein expression in different groups. The brown spots are the positive areas and the nuclei are visible in blue (A-F). The histogram shows the H-score in each group (G). Data are reported as Mean \pm SEM. (Measurement index of images = 20 μ m) A: Control group (Con.), B: Alginate hydrogel group (Alg.), C: Collagen I microspheres incorporated in the alginate hydrogel group (Coll. I & Alg.), D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group (Coll. I, IV & Alg.), E: Collagen I microspheres containing laminin incorporated in the alginate hydrogel group (Coll. I, Lam & Alg.), and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group (Coll. I, IV, Lam & Alg.).

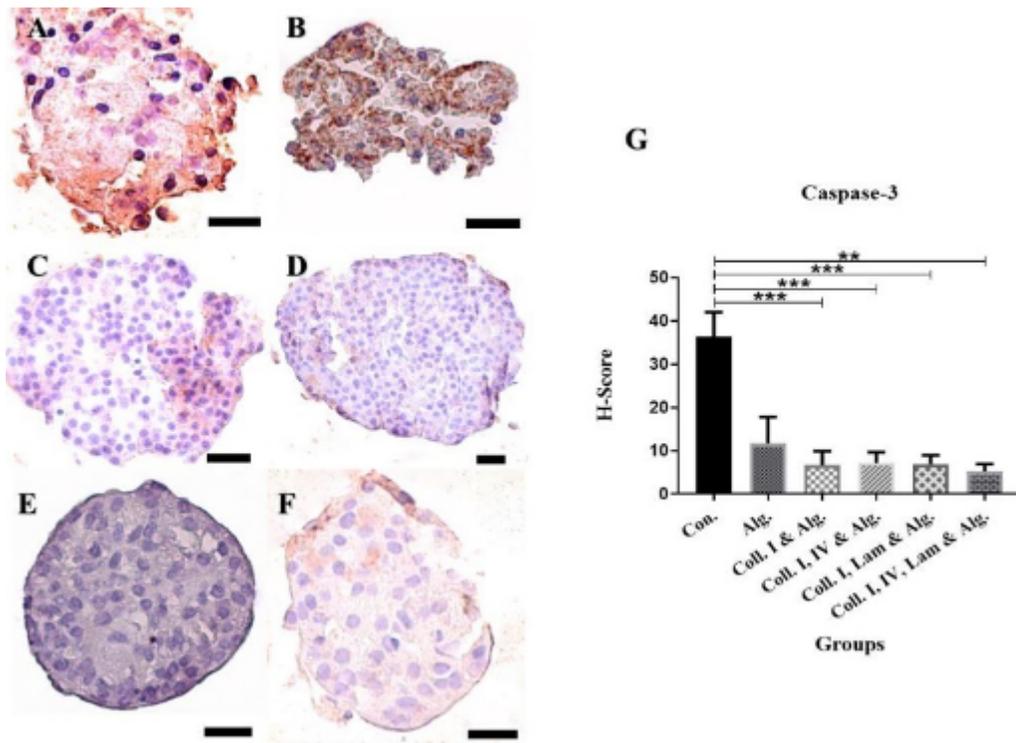


Figure 8

Immunocytochemistry analysis of active caspase-3 protein expression in different groups. The brown spots are the positive areas and the nuclei are visible in blue (A-F). The histogram shows the H-score in each group (G). Data are reported as Mean \pm SEM. (Measurement index of images = 20 μ m. **: P>0.01 and ***: P<0.001) A: Control group (Con.), B: Alginate hydrogel group (Alg.), C: Collagen I microspheres incorporated in the alginate hydrogel group (Coll. I & Alg.), D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group (Coll. I, IV & Alg.), E: Collagen I microspheres containing laminin incorporated in the alginate hydrogel group (Coll. I, Lam & Alg.), and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group (Coll. I, IV, Lam & Alg.).

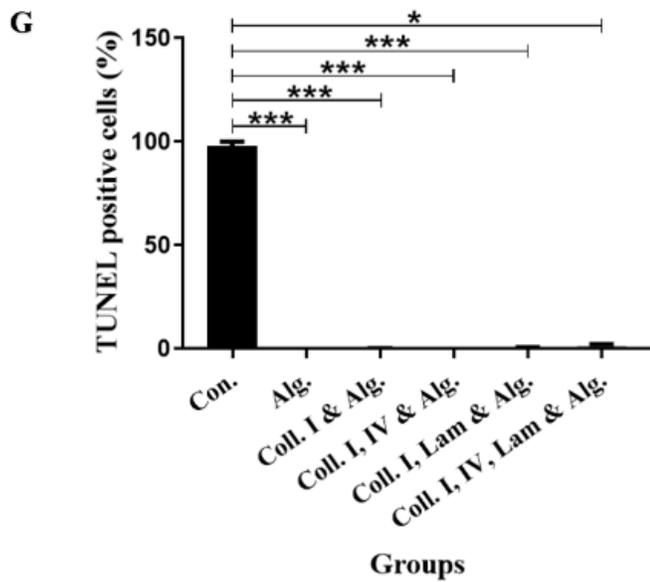
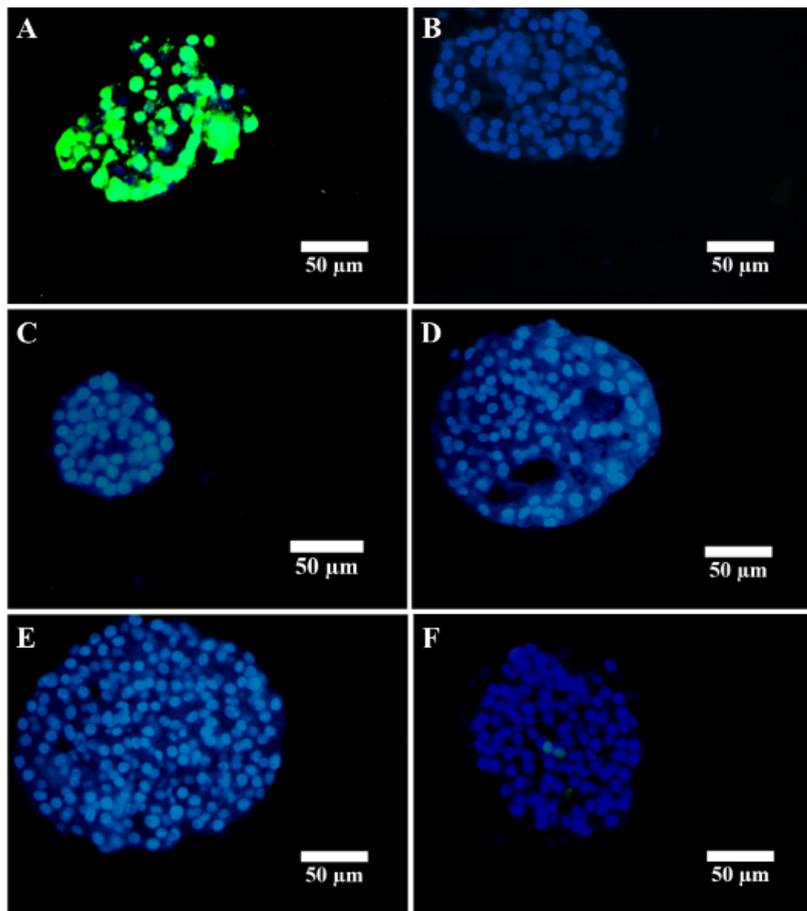


Figure 9

TUNEL staining in different groups. The green spots as the TUNEL positive cells and the nuclei are visible in blue (A-F). The histogram shows the percentage of TUNEL positive cells in each group (G). Data are reported as Mean \pm SEM. (*: $P < 0.05$ and ***: $P < 0.001$) A: Control group (Con.), B: Alginate hydrogel group (Alg.), C: Collagen I microspheres incorporated in the alginate hydrogel group (Coll. I & Alg.), D: Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group (Coll. I, IV & Alg.), E:

Collagen I microspheres containing laminin incorporated in the alginate hydrogel group (Coll. I, Lam & Alg.), and F: Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group (Coll. I, IV, Lam & Alg.).

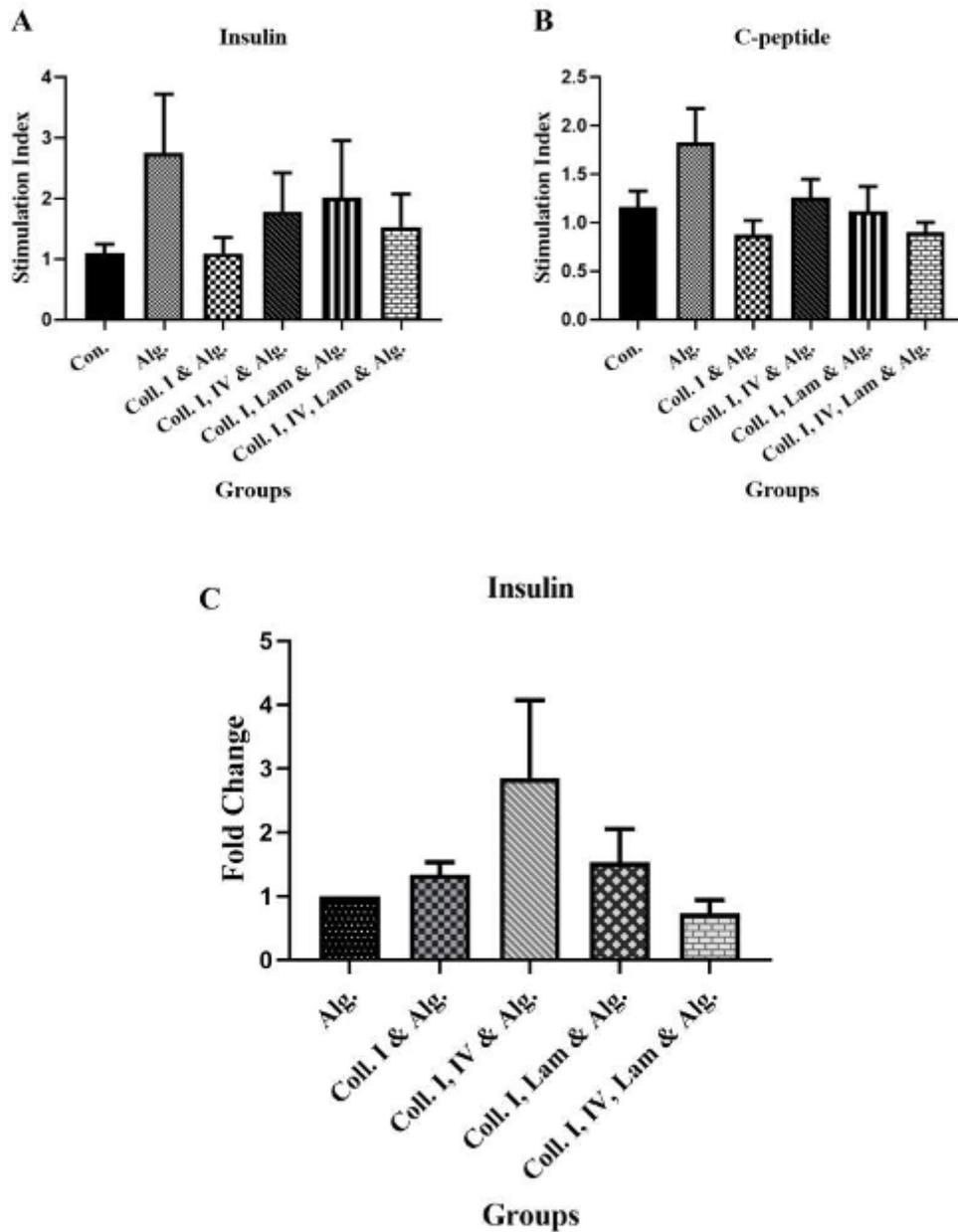


Figure 10

The evaluation of insulin (A) and C-peptide (B) stimulation indexes in the presence and absence of hydrogel scaffolds. Data are reported as Mean \pm SEM. (Alg.): Alginate hydrogel group, (Coll. I & Alg.): Collagen I microspheres incorporated in the alginate hydrogel group, (Coll. I, IV & Alg.): Collagen I microspheres containing collagen IV incorporated in the alginate hydrogel group, (Coll. I, Lam & Alg.): Collagen I microspheres containing laminin incorporated in the alginate hydrogel group, and (Coll. I, IV, Lam & Alg.): Collagen I microspheres containing collagen IV and laminin incorporated in the alginate hydrogel group.

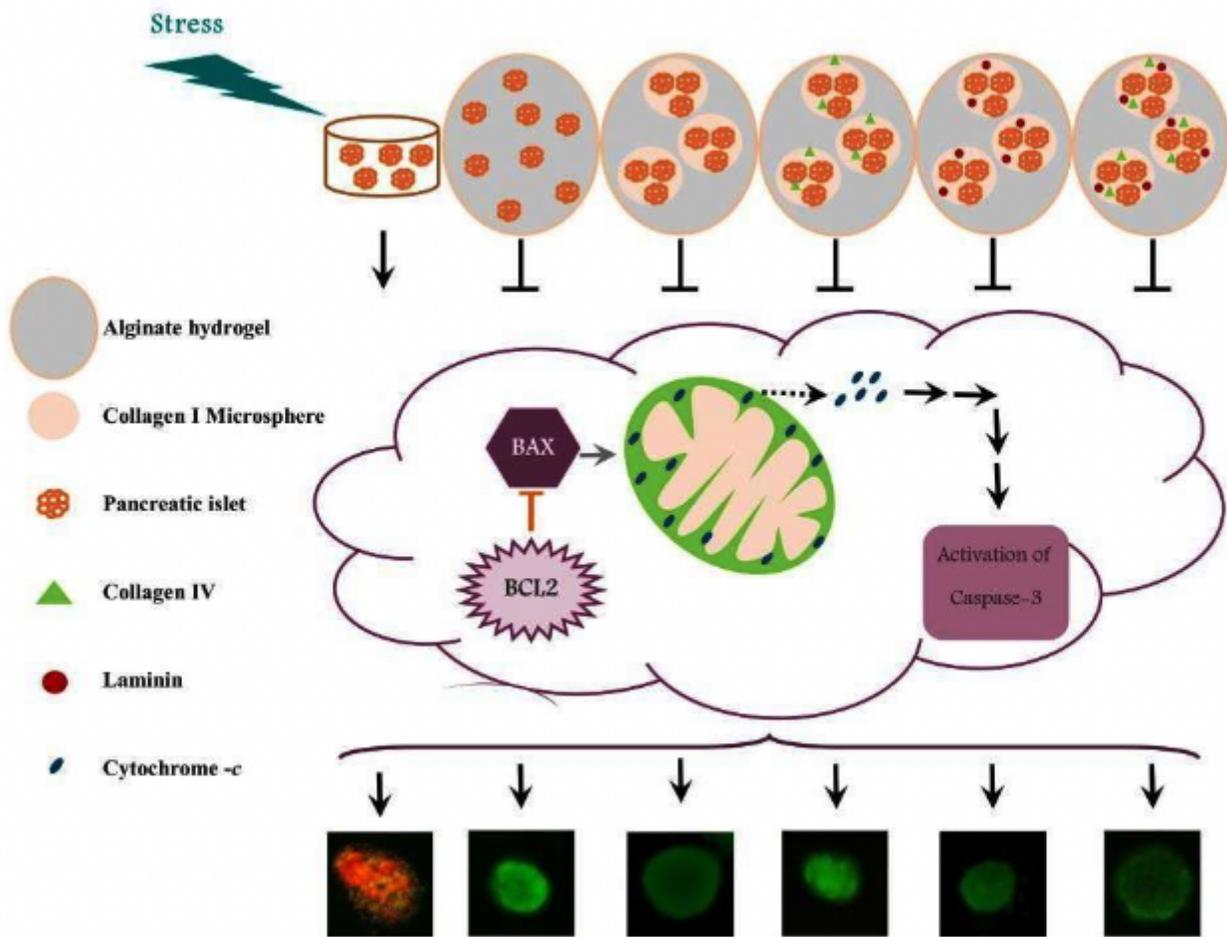


Figure 11

A schematic illustration of hydrogel scaffolds effect on the islets apoptosis and viability. The enclosed area represents the internal pathway of apoptosis.