Cell Matrix Interaction in Decellularized Pancreatic Natural 3D Scaffold with Heparin Sulfate

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ABSTRACT

Arising technologies of regenerative medicine have made overwhelming the restraints of organs bioengineered in the laboratory possible. In order to simulate the biochemical, spatial and vascular relationships of the native extracellular matrix (ECM), a scaffold has been needed for bioengineered Pancreas. ECM scaffold can be created from all animal organs. Decellularization has been explained lately to generate native ECM scaffolds from compound organs such as the lung, the liver, the heart and more recently the pancreas. In the current study, generating the whole organ through three – dimensional pancreas scaffold using acellular bovine pancreas, has been explained. Previous studies have supported that the protocol used in the current study adequately eliminates cellular material while saving ECM proteins. In this research heparin sulfate was used for the impregnation of the scaffolds. Heparan sulfate proteoglycans (HSPGs) in the ECM bound to several signaling molecules and regulated ligand-receptor interaction, playing an essential role in embryonic development. It was shown that HSPG could arrange the stem cells in asinus and coated basal lamina and make a native morphological shape of acina cells in pancreas scaffold. In the current investigation, it was indicated that HSPG was intensively expressed in pancreatic islet β –cells after 1 week of age in a bovine pancreas.

Key Words: *Tissue Engineering, Heparin Sulfate, Bovine Pancreas, Pdx1*.

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INTRODUCTION

Nature's perfect biological scaffold material is ECM [1]. It is a complicated web of proteins and polysaccharides forming a complex meshwork inside the tissue that interacts with the resident cells to modulate cell behavior, such as migration, proliferation and different ions [2]. ECM contains variations of glycosaminoglycan's (GAGs), which includes chondroitin sulfate, hyaluronic, heparin sulfate (review 2009).

Heparan sulfate proteoglycans (HSPGs) are glycoproteins, with the conventional property containing one or more covalently attached heparin sulfate (HS) chains, a form of glycosaminoglycan [3]. Tissue_ specific alterations in the glycosaminoglycan structure provide a mechanism to FGF signaling [4]. For example, 2-0 and 6-0 sulphation are required for fibroblast growth factor (FGF) signaling activation by FGF_2 [5, 6]. It has been indicated that Pancreatic islets and specifically beta cells include high levels of HS, and HS in the islet microenvironment has been confirmed to play multiple roles [7, 8].

HS co-localizes with type 2 diabetes and after islet transplantation, and a role for HS in insulin secretion has been suggested [9, 10]. The purpose of the current examination was generating scaffold from decellularized pancreas tissue of bovine, and assessing how it influenced the behavior of mice mesenchymal stem cells in the presence of HSPGs.

MATERIALS AND METHODS

Preparation of decellularized and its evolution:

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The sacrificed animals' Bovine's pancreases were obtained immediately after their slaughter. Then, the pancreas samples were cut in the same shapes with a diameter of 2-3. Physical and chemical methods, in which the samples were incubated in 1% sodium dodecyl sulfate for 28h, were applied in order make the samples cell-free. Then, they were placed in phosphate buffer saline for 2 hours before being immersed in 75% ethanol. This procedure was repeated 8 times. After washing with sterile water, they were immersed in 1% Triton x-100 16h. Then, phosphate-buffered saline solution PH 7.4 (PBS) was rinsed through the organs for 4 hours. To avoid contamination, this complete procedure was performed under sterile conditions in a laminar airflow cabinet. To get the obtained scaffolds sterilized, they were put in 70% ethanol for 30 min, and in order to relieve the scaffolds from ethanol, distilled water was used to wash the samples, and then, the samples were soaked in a sterile PBS solution for 1 hr. Finally, the samples were placed in 6 well plates (Orange Scientific, Belgwme) which included 2 ml of culture medium DMEM (Dulbecco's Modified Eagle's Medium), and 10% fetal bovine serum (FBS, biosera), and then incubated at 37 ◦c with 5% co2. Eventually, the scaffolds were used for cell culture.

Impregnation of scaffolds with heparan sulfate:

Pancreatic scaffolds were incubated either without or with 0.1 U/ml heparinase in RPMI 1640 medium supplemented with 5.5 Mm glucose, 0.5 IU/ml streptomycin for 1 h at 37◦c. The scaffolds were washed, resuspended in medium above, supplemented with 10% fetal calf serum instead of BSA, and cultured for 24h at 37◦c in an atmosphere of 5% co^2 before cell seeding.

Cell Seeding: Stem cells were provided by the cell Blank of the Biotechnology Research Institute of Ferdowsi University of Medical Sciences, Mashhad, Iran. MSCs (Rat Mesenchymal stem cells) were used. Passage 2 – 3, were supplemented with 20% FBS and 100 v/ml penicillin and streptomycin (Merck, Germany) on 175 tissue culture flasks. The cells were cultured at 37˚c and in a 95% air 5% co2 atmosphere.

Recellularization and culture of Seeded – pancreatic construct:

In order to perform cell cultivation on sterile scaffolds, a cell suspension with a density of 60x105 cells in 50 was provided for each pancreatic scaffold. Scaffolds were divided into three groups: Control group 1 with no cells and no heparan sulfate (hs), control group 2 with cell and no hs, and the test group with cell and hs. Five pancreatic scaffolds were used in each group, and the experiments were repeated 3 times. One hour after seeding, 2ml culture medium was added to these cells. Their culture medium was changed every day or every other day.

Histological evolution:

Paraformal – Fixed and paraffin – embedded (Sigma-Aldrich) tissue sections were cut into 5 thick parts, and were stained with Hematoxylin and Eosin, Alcion Blue and DAPI.

Scanning electron microscopy (SEM):

Native and decellularized pancreases were fixed in 2.5% glutaraldehyde in 0.1M PBS (PH 7.4) for 60 minutes. Each sample was washed thoroughly 3 times with 0.1M PBS for 15 minutes. Next, the samples were fixed in 1% oso_4 in 0.1M PBS for 60 minutes. Then, 3 changes of PBS washing steps were applied for 15 minutes. Different concentrations of alcohol in a period of 15 minutes were used to dehydrate the samples. Furthermore, the obtained samples were dried at the critical point, and then coated with Au/Pd using a cressington coater 108A sputter coater. An SEM (LEO 1450VP, Germany) was used to take electron microscope images.

Quantitative RT – RCR:

At days (3, 5, 7, 9) of culture,3 groups consisting of the scaffold, scaffold seeded MSC (whit and without HS) and control were subjected to 0.25mg/ml collagenase for 30 min in order to release the islets into suspension. Islets from all groups were lysed in RLT lysis buffer supplemented with 1% bmercaptoethanol(Sigma) and frozen at -80˚ C until analysis. For assessing the specific gene expression in the cultured islets, reverse transcriptase polymerase chain reaction(RT-PCR) was used to measure mRNA production. Following RNA extraction, according to the manufacturer's protocol, cDNA was reversely transcribed using an Omniscript RT Kit (Bioneer). Custom primers (Invitrogen) for insulin and Pdx-1 genes were synthesized, and real–time PCR was performed using AccuPower PCR PreMix kit.

Table 1. Primers used for RT – PCR

	Primers for pdx-1(Rat)	Tm (DegC)	Salt (mM)	Primer (µM)	%GC	Length
Forward 5´-3´	CGGAGGAGAATAAGAGGA	60	50	200	50	18
Reverse5´-3´	CTCAAACAGCTCCCTTTA	59.4	50	200	44.4	18
						847
	Primers for Insulin gene (Rat)	Tm (DegC)	Salt (mM)	Primer(µM)	%GC	Length
Forward 5´-3´	TCCGCTACAATCAAAAACCAT	61.3	50	200	38.1	21
Reverse5´-3´	GCTGGGTAGTGGTGGGTCTA	66.1	50	200	60	20
						411

RESULTS

A brownish color could be observed gradually during decellularization until the organs appeared lucent. After the use of SDS, the organs became totally translucent. This did not change until the end of the protocol.

Histological evolution of Decellularized matrices.

The observance by the microscope was established by H/E staining (Fig-1): no cellular substantial was stained in the decellularized matrix, while the lobular structure of the pancreas could be saved. In addition, the connective tissue septa, and the ductal, as well as vascular network, were preserved in all the specimens. Alcion Blue staining was performed to visualize collagen fibers and acidic sulfated mucopolysaccharides as a plentiful constituent of the ECM; respectively (Fig-1). The decellularized ECM appeared exact to that of the controls in all cases. DAPI staining was used to confirm the complete removal of nuclei from the scaffold. The results showed that using 1% SDS for 28 hr. removed the cells from scaffolds completely (Fig- 2).

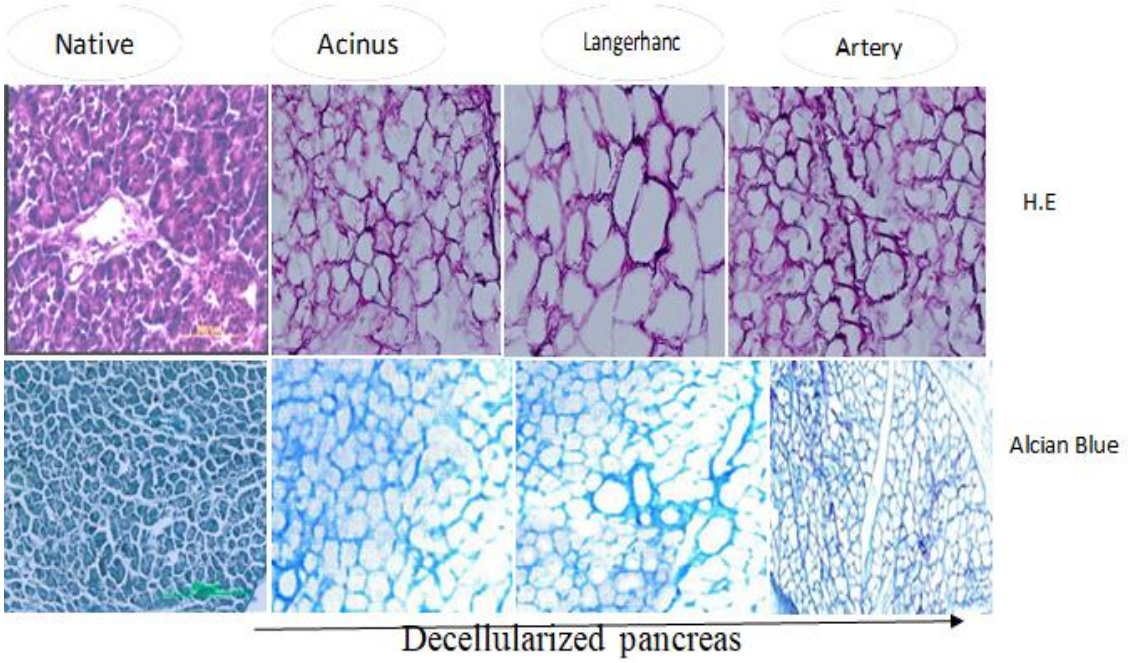

Fig. 1: Histology of the decellularized pancreata: Pancreata that were decelluraized via the asinus langerhuns or artery were stained with H.E and Alcian Blue to analyze the organ structure after decellularization and compare with native controls; no remaining cells were found inside the decellularized organs but the feature of the lobular microarchitecture of the organs was protected. Alcian blue staining qualitatively determined the retention of GAGs protein after decellularization.

Scanning electron microscopy (SEM) of decellularized matrices:

In comparison with native and decellularized pancreata, SEM images indicated that matrix Components were consecrated (Fig -3). In contrast with the native controls, empty spaces surrounded by networks consisting of fibers with different calibers could be found inside the ECM.

ECM characterization:

DAPI staining after decellularization, those scaffolds, which were fixed with paraformaldehyde fixator, were analyzed using fluorescence microscope, stained with DAPI, and then, the two pictures merged together. The findings of this study suggested the complete removal of the cellular and cytoskeletal elements while maintaining the ECM composition (Fig2c, d). Furthermore, the resulted ECM structure and fibril orientation were identical to that of the native organ (Fig2a, b).

ES Cell seeding and proliferation pattern:

To evaluate the potential of the decellularized pancreas as a scaffold for pancreatic tissue engineering, Mesenchymal stem cells were analyzed in no hs/hs impregnated scaffold in day 5,10,15,20.

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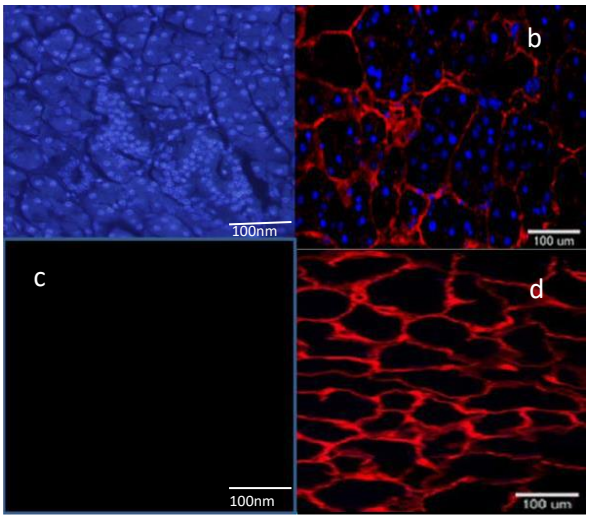

Fig. 2: DAPI Fluorescent staining of native pancreas(a,b) comparison with decellularized pancreas(c,d). Decellularized tissue with no sign of cells or cell fragments which proved the efficiency of the decellularization procedure but the preservation of important ECM proteins after decellularization (d) (×200 magnification).

Analysis of MSCs behavior in hs impregnated scaffold:

Histological studies (fig -4) in day five by using H.E staining and SEM images indicated that MSCs were found attached and spread on the surface of scaffolds (fig5-a, b). In day 5, dividing cells were obvious in the cell mass, which were being created. After 5 days, the scaffolds were analyzed for their survival and the shape of cells. From the histology, MSC cells in hs scaffold were mostly trapped in the asinus with the same arteriolar localization. The hs scaffold maintained noticeable integrity of the vascular basement membranes, because the cells stayed within the vessels and asinus and did not fill islet Langerhans space. The incubations after approximately 5 days showed typical immature MS-like cells completely filling asinus so that no lumen was present (Fig-4a). Histological studies of scaffolds showed a remarkable rise in the number of cells adhered to the scaffold and in some areas during day 10, in small cells. On day 15, slight cells' aggregations were migrated inside the islet Langerhans, and the proliferation in the vasculature showed a reticular appearance (Fig-ac), and the donor cells had nearly filled some islet Langerhans and asinus. On day 10, in vascular structures, a more mature morphologic appearance in asinar regions had changed into endothelial-like appearance (Fig-4f). Principally, cell death inside the central core-like domains of the larger vascular and tubular structure was shown with longer incubation periods which was nearly 5 days. The cells living in flow inside the scaffold from proximal to distal into the periphery of the organ were preserved. Some islets then dripped from the seeable vascular or acinar net, and came to the decellularized scaffold, while others remained inside the decellularized vessels. Totally, more islets were found inside the organ, when the scaffold was impregnated with hs, compared to the control.

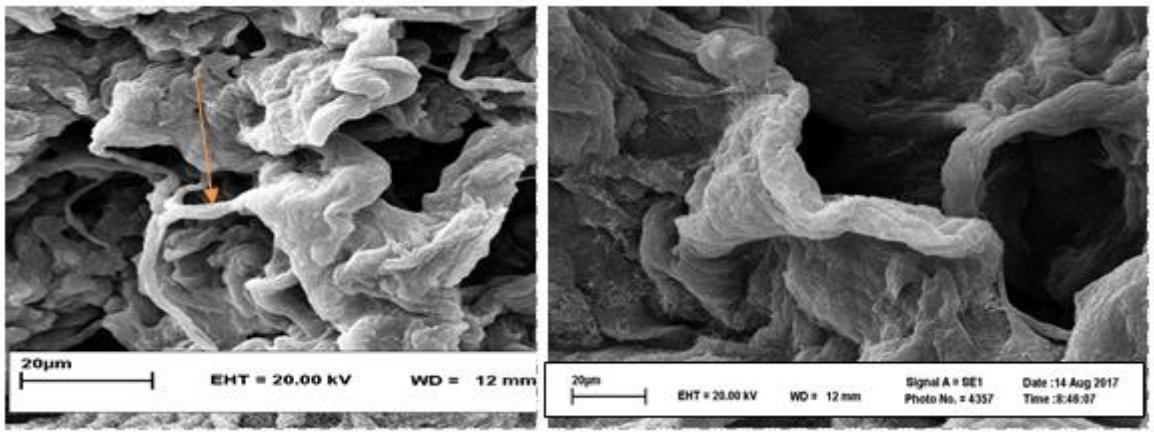

Fig. 3: Scanning electron microscopy (SEM) of decellularized pancreata.
SEM at a magnification of 1000 and 2000 was performed in order to analyze the matrix composition after decellularization. SEM of scaffold demonstrated the collagen fibers (arrows) in the decellularized scaffold.

Insulin secretion in heparin sulfate -treated islet

To investigate whether HS plays a role in β-cell function, the scaffolds were treated with heparin sulfate and analyzed for insulin secretion, and the islet insulin content was preserved in HS-treated islet. After 3 days of culture, insulin secretion was markedly increased in HS-treated scaffolds compared with the control scaffolds. These results indicated that the scaffold whit HS specifically impaired the stimulated insulin secretion. Many transcription factors were involved to activate the insulin

promoter; among them, pancreatic duodenal homeobox-1(pdx-1) was vital and was involved in pancreatic development, glucose metabolism, and a number of pancreatic functions [11]. In table 2, it was shown that HS increased the expression of pdx-1 gene in the HS treated scaffolds.

DISCUSSION

Decellularization is a tissue treatment process which eliminates cells. This process is often used in order to create bio-artificial organs. The decellularized tissue can be efficiently utilized to restore missing, damaged and unhealthy structures. Briefly, the first step is inefficient dissolution without affecting protein integrity in the tissue. Then recombinant endonuclease such as deoxyribonucleic (DNase) and ribonuclease (RNase) are used to degrade nucleic acids. Often, for completing decelluraziation, chemical (detergents and ionic solution) and physical (sonication, pressure, or freezing and thawing) methods are used. The final product is meant to be a scaffold that is capable of protecting and immersing decellularization [12]. In this study, a combination of chemical and physical methods was used for full decellularization. In the physical phase, the samples were transferred to -4° C. This induced the formation of ice crystals, in which changes of temperature caused minimal disruption in ECM. A dense meshwork of fibrous molecules was visible in the treated tissue [13]. In this study, SDS was used as a detergent for the chemical phase of decellularization. Previous studies have disclosed that SDS has been the best detergent for removing cells and cell debris from tissues [14]. The resulting decellularized pancreas had the capabilities to be considered as a successful decellularization with DAPI and H. E staining without nuclear materials. Decellularized pancreatic matrix was non-cytotoxic and illustrated the maintained essential ECM proteins in adult pancreas, including collagen I, collagen II (DAPI).

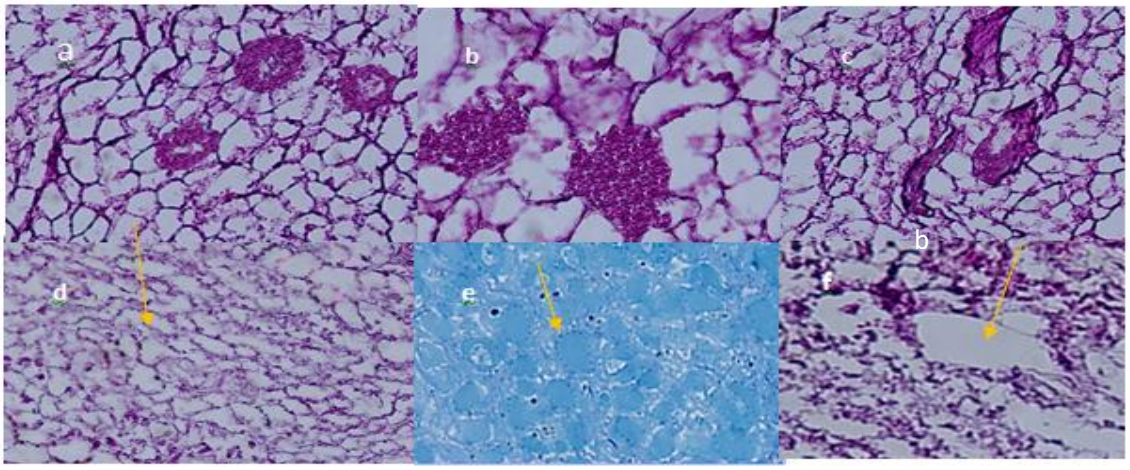

Fig. 4: H/E staining of the repopulated pancreata. MSC cells in HS scaffold were mostly trapped in the asinus, with the same arteriolar localization(day5) H.E (c), thin section (3µ) toluidine blue staining showed that cells migrated into the contiguous asinus over the ensuing incubation periods in HS.

At the cell surface, in the extracellular matrix and in basement membranes, heparan sulfate was examined and confirmed in histological staining and scanning electron microscopy [15]. This occurrence could be observed in somewhat flattened endothelial-like appearance in vascular structures of HS samples. In other studies, [16-19] integrin's mediate various interactions between basement membrane were noticed. Leukocyte migration through this barrier was supposed to involve the local degradation by matrix metalloproteinases and secreted heparanase, which might be needed for the dissolution of HSPG in the basement membrane [20, 21]. Stem cell populations that existed in the defined cellular microenvironments, which were known as stem cell niches, regulated the generation, maintenance and repair of different tissues during their development. In order to identify the stem cells' ability to maintain a self-perpetuating pluripotent state or to differentiate into the committed tissue-specific progenitor, these niches were taken into consideration [22]. Interestingly, most of the signaling molecules, for instance Wnts and FGFs, which involved in stem cell maintenance, were regulated by HSPGs [23, 24]. Regenerating agents (RGTAs) were engineered to large biopolymers in order to substitute HS specifically connected to matrix proteins, and the growth factors were eliminated after chorionic tissue injury [25]. These polymers preserve proteins connected to the extracellular matrix from proteolysis. RGTAs can interact with much heparin-binding growth –factor, such as FGF2 [26], transforming growth factor-β [27] and VEGF [28].

The role of HSPGs in stem cell differentiation at least ex vivo [29-31] was assessed exactly. Various research studies have indicated that biomechanical characteristics change after decellularization of tissues [32-34]. The research studies done before, stated that young modulus of decellularized pancreas was on average 3 times bigger than the native pancreas [35]. This was observed because there was a loss of GAG content from the decellularization protocol. Among the myriad of biological functions of GAG proteins, some have been related to the structural and biomechanical characteristics. GAGs had a steady negative charge that rendered them to hydrophilic, and attracting water into the tissue could cause an osmotic swelling, which in turn could contribute to tissues' physical properties. The elimination of GAG side chains by heparitinase/chondroitinase treatment was indicated to intensify the stiffness of the basement membrane of tissue [36]. The composition and expression pattern was important in the given anatomical location for directing or supporting the site-appropriate cell attachment and function [37].

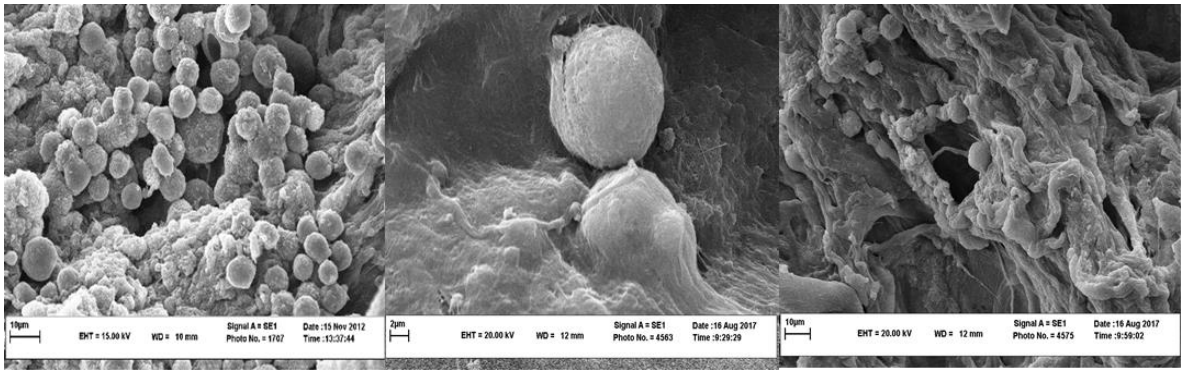

Fig .5: SEM images of the decellularized pancreatic scaffold after two weeks of culture with MSCs (a,b). On the surface of the scaffold (arrows), cell attachments can be observed.

In the native pancreas, the basic functional units of the pancreas were divided into two groups of the endocrine cells and exocrine cells. The endocrine cells were categorized as islets of Langerhans and secrete different polypeptides which were transferred to the other parts of the body through the vasculature. The ductal system was used to secrete the acinar exocrine cells. The activation of insulin gene transcription especially in pancreatic β-cells depended on multiple nuclear proteins that interacted with each other and with the sequences on the insulin gene promoter to build a transcriptional activation complex [38]. In the adult pancreas, pdx-1 up-regulated insulin secretion by binding and activating insulin promoter in β cells [39]. When the pdx -1 binding site was mutated on the promoter, it demonstrated a critical role of pdx-1 in the activation of the insulin promoter. The repopulation of decellularized pancreas with MSC that treated whit heparan sulfate showed the cytocompatibility of the scaffold, the decellularized constructs maintained the respective phenotypic expression (β-cells, acinar cells) after 7 days of the culture. Finally, it was shown that the decellularized pancreas matrix heparinized the supportive of β – cell function, as evidenced by the strong up – regulation of insulin gene expression. Lastly, it was shown that the decellularized pancreas matrix heparinized the supportive of β – cell function, as evidenced by the strong up- regulation of insulin gene expression.

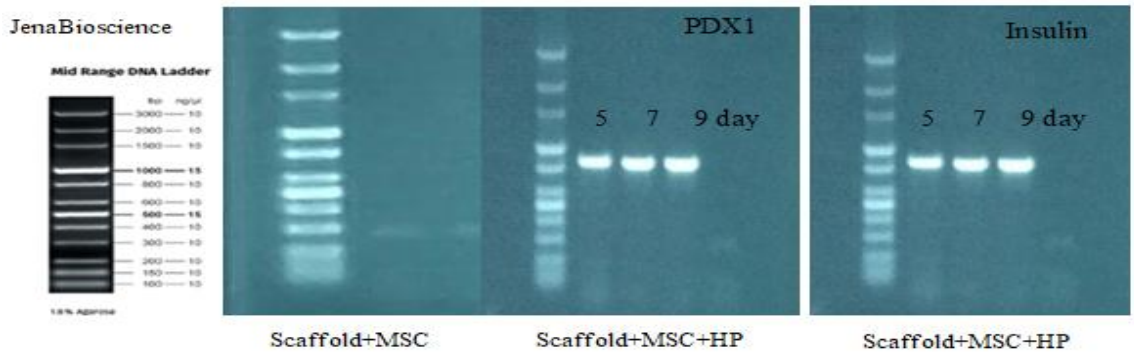

Fig. 6: Insulin secretion and gene expression scaffolds after heparintinase treatment.

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