comparative Study on Surface Geometric Properties of Decellularized and Glutaraldehyde fixed Bovine Pericardium and Possible Effects on Cell Seeding

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Original Article

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ABSTRACT

Background: Collagen fibers arrangement and density play a pivotal role in cell migration and proliferation. Bovine Pericardium (BP) is a collagen – rich tissue which is currently used as a biological scaffold.

Aim of the work: The aim of this study was to examine the effects of the decellulrazation processes on the surface architecture and geometry of the BP and consequently cell seeding and epitheliliazation onto it.

Methods and Results: To achieve these goals bovine pericardium (BP) was decellularized with 1 % TritonX-100 and 0.1 % sodium dodecyl sulfate . Control group was only treated by PBS and post fixed in glutaraldehyed (GAD) 1%. The buccal cell suspensions were then overlaid on the serous side of the BP scaffolds at concentrations2×10^5 Cells/mL. 10 days following seeding the samples were fixed and SEM results were analyzed by Matlab software. The pore surfaces were measured 182.05±11.61µm2 and 132.44±12.35 µm2 in acellular and GAD BP respectively ($P<0.05$). The con surfaces were measured 93.54±13.41 µm2and 114.78±11.67 μm^2 acellular and GAD PB respectively ($P<0.05$).The thickness of collagen bundles in decellularized BP and GAD BP (10 random fields) were obtained 19.5±6.3 µm and 16.8±.99 µm respectively ($p<0.05$).

Conclusion: The results of acellular group showed more attached cells onto scaffold, epithelialization growth, and spreading on the scaffold. While in contrast GAD-fixed group only scattered cell clumping were seen. Our findings showed that topographical changes after decellularization could provide a suitable growth pool or microenvironment, that can influence cellular attachment and subsequently cell-ECM integration in biological scaffold.

Key Words: Bovine pericardium, collagen, decellularization.

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INTRODUCTION

Evolving new scaffolds from biodegradable materials has been considered as the primary aim of the bioengineering field[1,2]. Bovine pericardium (BP) is a collagen-rich biological tissue which is widely used in reconstructive surgeries. Advantages such as superior biocompatibility, easy handling, low cost and possibly reduced rates of infection have made its use more common in comparison with other scaffolds[3]. Currently BP is being used in cardiovascular(valve prosthesis), ophthalmic and urologic (laboratory experiments) surgeries[4,6],however there are reports that BP use is associated with long term issues including restenosis by cell clumping, calcification, thrombosis and hyperplasia[3].Although the underlying responsible mechanisms for such side-effects have not been thoroughly understood[7], it is known that the method of preparation play a critical role in occurrence of progressive tissue degeneration . In order to achieve the ideal results and reduce mentioned long term side effects, many studies have turned their attention to the methods of the scaffold preparation and biomechanical analysis. Studies have reported that use of BP- fixed in glutaraldehyde (GAD) , as cross-linker agent, is associated with activation of the immune system[8,9], triggering an inflammatory reaction[10,11] that leads to infiltration of the collagen matrix, rupture of the collagen-elastin network , calcification and eventually failure of treatment in clinical cases[12,13]. In order to overcome the limitations of GAD fixation, several treatments have been examined. Currently decellularization of BP has been proposed as an alternative method, gold standard, to eliminate or reduce immunogenic reaction, calcification rate and causing less tissue degeneration[14,17]. Some studies have reported that decellulrazation of BP may lead to significant alteration in ECM properties[18,19]. One of the major components of

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the BP tissue is type I collagen networks embedded in extracellular matrix(ECM) which in turn determine antigenicity, structure-function properties and even re-cellularization potential[7,20,21]. Studies have revealed that cell migration and proliferation are influenced by collagen fibers density, spatial arrangement and porosity[22,24]. It has been recently shown that decellularization processes brings about extensive cellular membrane destruction and collagen fibers disruption in BP[25]. Despite the massive amount of data on the biochemical and mechanical changes after decellularization, there is little information about the effects of decellularization on the geometric or topographical changes of the BP and its effects on cell seeding. This study aimed to examine the effects of the decellulrazation processes on the surface architecture of the BP and subsequently epithelial cell seeding onto it.

MATERIALS AND METHODS

The study was approved by Ethical Committee of the North Khorasan University of Medical Sciences. After opening the sternum , pericardium was exposed and three suitable areas of sternal surface of bovine pericardium (BP) (3 years old) (N=3 cow calf) were collected. The material in plastic containers containing a hypertonic solution of NaCl was transported to the laboratory at 4ºC. The samples were washed by isotonic 0.9% NaCl solution and all the fat and adherences were meticulously removed. The pericardium was cut into 4 long strips each about 1×1 cm and treated with phosphate-buffered saline (PBS), streptomycin, penicillin under ultraviolet (UV) about1hour to eliminate any bacterial contamination. The bovine pericardium (BP) was treated according the decellularizing method of Eirini Pagoulatou et al [26] in three steps as briefly as: incubation in distilled water for 2 hours at 4˚C, Decellularization with 1 % Triton X-100 (AppliChem, Germany), 0.1 % sodium dodecyl sulfate (SDS) (Merck),150 mM NaCl (Merck) and 1 % deoxycholic acid (AppliChem,Germany) in 10 mM Tris (Sigma Aldrich) buffer pH 7.4 with protease inhibitor cocktail (Sigma Aldrich). Sample were treated for 12 h at 4 C in the detergent solution. Decellularized pericardium matrices were subjected to several washes in distilled water until complete removal of the detergent. Fresh BP were used as control and only treated PBS and post fixed with glutaraldehyde (GAD)1% and were subjected to washing.

Tissue harvesting and cell culture[27]: Two adult male rabbits were purchased from Razi institute and were housed 2 weeks to accommodate to new condition. The animals were deeply anesthetized by ketamine and chloroform. After deep anesthesia bilateral checks were exposed and the operation field was carefully disinfected and washed as routine surgery. Buccal Mucosal flaps (1×1cm) were carefully incised and transferred to laboratory. The samples were treated by 70 % ethanol (10 seconds) and then washed by PBS and antibiotic thoroughly, muscular and fibro-fatty tissue were removed gently and cut into several small pieces, then the mucosal samples were treated as follow: DMEM+ dispase(2.5%) for 24 hours at 4˚C, DMEM and trypsin(.04%) for 4 hours in 37C , washing several time with DMEM+PBS10%. Finally isolated cells were incubated in culture medium containing DMEM ،30% DMEM / HAM S F-12 ، 20%fetal calf serum(FCS) ،0.1% antibiotic at37C.

Seeding[27]: The scaffolds were placed in a high-attachment 12-well plate and the cell suspensions were then overlaid on the serous side of the BP scaffolds at concentrations2×105 cells/mL. The seeded bio scaffolds (3 times seeding during 3 days) were incubated at 37˚C. The sample were checked each day and after three days the culture media were changed. 10 days later the samples were gently removed and placed in fixation solution containing glutaraldehyed3% and PBS for 24 hours at 4˚C. The fixed samples were sent for SEM study to Ferdowsi University, Mashhad. Iran. The SEM results were analyzed and quantified by Mat Lab software (R2018a x64).

Image processing for surface topography: In order to measure the surface topography and porosity on the surface of BP, All imaging data were imported into Matlab software as a common method in image analysis(Toolbox 7.1 The math Works,Natick,MA,USA) and processed as[28]: A 3-D matrix was made for each image, RGB images were converted to greyscale images, A 50×50 unit was selected for each matrix, units were scanned and black-white points were considered as raised and dropped points(this processes were extended for total size of images), the points were normalized and their surfaces were calculated and finally the surface were compared between two images by t-test.

RESULTS

SEM results of serous side of the BP showed that decellularization process causes a variety of changes in the diameter [Fig 1], arrangement, porosity (extracellular matrix niche) and disruption of collagen bundles of BP [Fig2], while in cellular group collagen fibers showed more compact and organized orientation. The pore surfaces were measured 182.0511.61±μm^2 and 132.4412.35± μm^2 in acellular and GAD BP respectively ($P<0.05$). The con surfaces were measured 93.5413.41± $\mu m2$and 114.7811.67± μm^2 in acellular and GAD BP respectively ($P<0.05$).The average thickness of collagen bundles in decellularized BP and cellular (10 random fields) were obtained 19.56.3± µm and 16.8±.99 µm respectively ($p<0.05$). Additionally, the surface ratio of pore and cons of the decellularized group were calculated based on the 3D plots. The comparison between the ratio of pore and cons showed meaningful difference($P<0.05$) [Table 1]. The results of seeding of the buccal epithelium on BP in cellular group showed scattered cells adherent

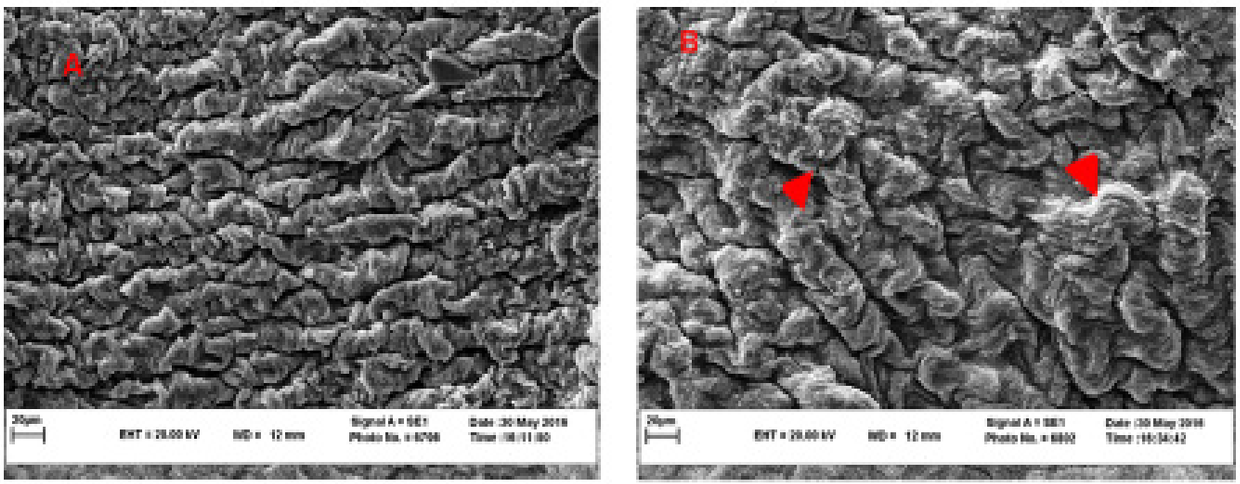

Fig. 1(A,B): Bovine Pericardium in GAD-fixed (A) The collagen fibers are compact and regular. In decellularized pericardium(B), Irregularity (arrowhead) and thicker collagen bundles are seen(SEM x1000).

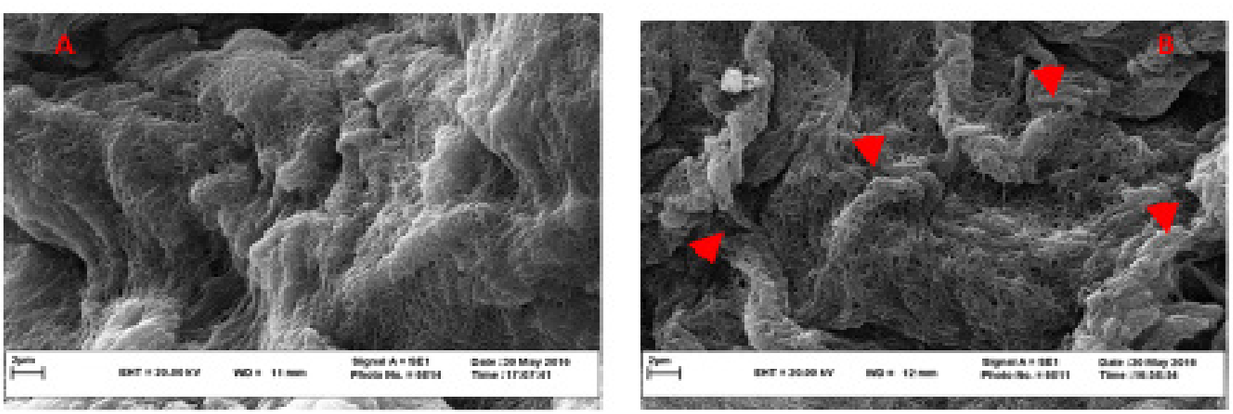

Fig. 2(A,B): In comparison to GAD-fixed (A), there are some collagen fibers disruption and irregularity (arrowheads) seen in decellularized group (B)(SEM x10000).

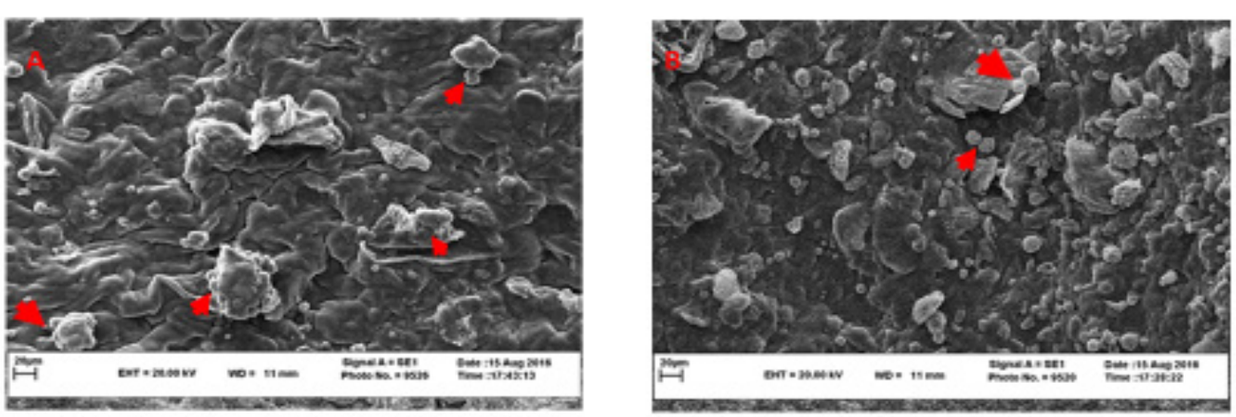

Fig. 3(A,B): Buccal cells seeded onto the GAD –fixed BP(A), show scattered clumping(arrows)(SEM x700) , in acellular group(B)numerous cells and some mitotic cells are seen (SEM x1500).

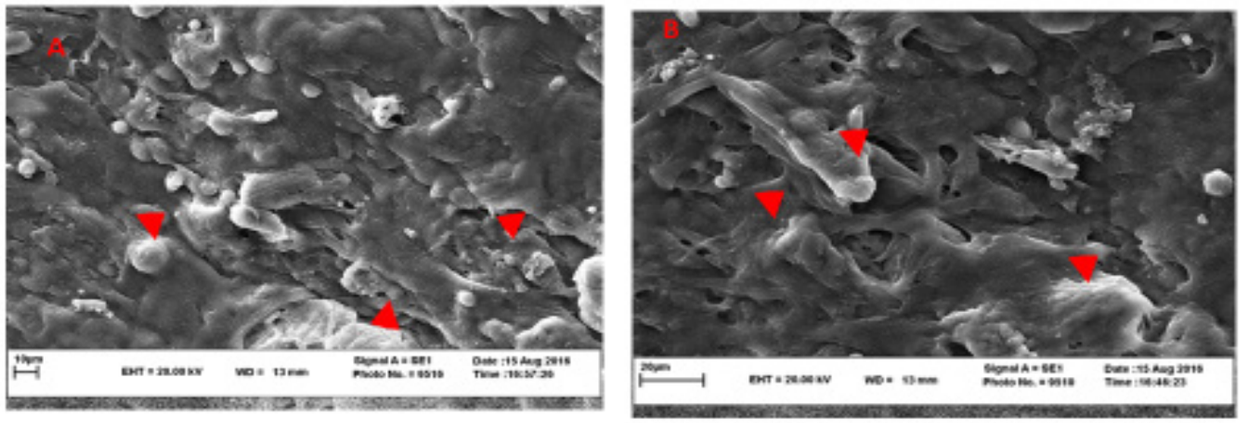

Fig. 4(A, B): cell attachment (arrows) and re-epithelialization (arrow heads) were seen in acellular BP after 10 days

Table 1: Surface geometry of the BP

Group	pore surface	con surface	Ratio of pore to con	Collagen bundles thickness
Acellular	*182.05±11.61µm ^{2}	93.54±13.41 µm^2	1.69±0.08*	19.5±6.3 µm*
GAD BP	132.44±12.35 µm^2	*114.78±11.67 µm^2	1.35±0.09	16.8±.99 µm

The ration of Con to pore in acellular BP showed significant level of difference in comparison to those of GAD BP(*P<0.05). Collagen bundles thickness also showed significant level of difference (*P<0.05).

to the scaffold and also cellular clumps were observed. The results of acellular group showed more attached cells onto scaffold, epithelialization growth, proliferation and spreading on the scaffold, specifically these features were noticeable in ECM niche. Higher magnification revealed embedded cells in the matrix [Figs3 and 4].

DISCUSSION:

The results of this study demonstrated that decellularization brings about extensive topographical and structural changes in the BP tissue. Quantification of collagen architecture after decellularization revealed increased diameter of collagen bundles, larger porosity of BP and extracellular matrix (ECM) niche, disruption in the arrangement of collagen fibers , more cell attachment and remodeling after seeding and widespread epithelialization of buccal epithelium onto the scaffold. The results of GAD –fixed BP showed that it leads to less topographic changes. Additionally, it failed to provide sufficient cell host and only scattered cell clumping was observed. Bovine pericardium consists of a network of type 1collagen and elastic fibers embedded in an amorphous matrix, which in turn is composed mainly of free glycosaminoglycan (GAGs) and proteoglycans[29]. Comparative structural changes have been reported in several studies. For instance Bodnar et al reported that SDS (1%) treatment damaged the pericardium tissue causing extreme fragmentation and swelling of the collagen, together with a significant loss of mechanical properties[30]. In another study Liao et al reported decellularization of BP with SDS (0.1%), triton X-100 and trypsin, results in disruption of collagen network, large pore and lose of fiber organization[25]. Mirsadraee et al stated that lower concentration of SDS (0.1) have no effects on the matrix and mechanical properties of collagen fibers[31]. On the other hand some researchers reported that damage to the matrix was potentially caused by the release of proteases and not by the SDS [32].In this study we added some new parameters to the related previous studies including measuring the surface of pore and con, fibers thickness, and ratio of con/pore which have less been dealt before. The results of the seeding revealed that the decellularized BP is capable of the buccal cell repopulation and proliferation. Furthermore, buccal cells could attach properly to surface of the scaffold and re-epithelialization and conducting/directing of buccal cell proliferation occurred extensively. One possible explanation for these findings in decellularized BP is likely the architecture and surface geometry of the BP. Deep porosities after decellularization could possibly provide a 3D micro pools hosting more cells[33,35] and may act to protect seeded cells from environmental disturbances such as medium changes. The importance of surface geometry and collagen architecture in cellular attachment, integration, migration and differentiation have been documented by several studies[22,24]. Although we could not perform various decellularization methods to examine their effects on surface geometry and subsequently on cell seeding, the obtained results highlight topographical parameters as a pivotal factor in decellularization methods of the BP and in vivo application of the BP. Another explanation

for the obtained results in the decellularized BP may reside in biochemical properties of ECM. It is evident that macromolecules in the scaffold can influence seeded cells behavior. ECM consists of structural and functional molecules including heparin, heparin sulfate, chondroitin sulfate and hyaluronic acid, adhesion molecules such as fibronectin and laminin and transforming growth factor-b, basic fibroblast growth[36]. It has been shown that using SDS based method preserves collagen contents and the glycosaminoglycan (GAGs) such as decorin which is known as modulator in cell attachment to ECM[37]. Conversely, GAD-treated BP failed to provide cellular proliferation and epithelialization. These results could be partly explained by the possible toxicity of residual aldehyde[38] and preservation of topography of the collagenous scaffold of BP. Admittedly behavior of cells in 3D in vitro are affected by a wide range of factors including surface geometry and exposed GACs of ECM[22 - 24,37]. According to our finding topographical niches could provide a suitable growth pool or microenvironment, which in turn can influence cellular attachment and subsequently cell-ECM integration. It is recommended to study the effects of various methods of BP decellularization on the geometry of BP and subsequently cellular behavior.

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