

Empirical studies on the effect of ECM matrix derived decellularized bovine mesentery on the behavior of Blastema cells in vitro

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Abstract

Objective (s): Extracellular matrix, in addition to its physical role can also control cell behaviors such as cell proliferation differentiation and migration using its inductive effects. In this study, extracellular matrix derived from bovine mesentery as a three-dimensional scaffold was used to study the behavior of blastema cells. **Materials and Methods:** bovine mesentery, cut into pieces of 5 mm by 5 mm was decellularized by combination of physical and chemical methods including snap freeze-thaw and treatment with sodium dodecyl sulfate (SDS). These parts were assembled as scaffolds inside the blastema rings from rabbits pinna. **Results:** Histological studies and scanning electron microscopy showed the removal of cells while preserving matrix of epithelium and collagen fibers in mesenteric tissue. Furthermore adhesion, polarity, migration and maintenance of blastema cells was observed on mesenteric decellularized scaffolds. Also, in the third week of culture their differentiation to mesothelium-like structures and the establishment of tight junction between these cells as well as differentiation of fibroblasts and adipocytes were observed within the mesenteric scaffold. **Conclusion:** The results of the morphological studies of this study indicate that mesenteric decellularized ECM can have inductive effects on the migration and placement of inflammatory tissue cells and possibly to differentiate them into plain squamous mesothelioma cells, fibroblasts and adipocytes. Although more studies are needed to prove the identity of the cells and other features of the scaffold.

Keywords: Cell matrix interactions, Blastema, Mesentery three dimensional scaffolds, Decellularization, Migration

INTRODUCTION

Mammalian cells are associated not only with each other but with the extracellular matrix ECM, the interaction between the extracellular matrix and cells in cellular orientation and behavior. In order to study the interaction of the matrix cell in vitro, two essential pillars are involved; scaffolds play an important role in providing a three-dimensional structure for cell growth in vitro, as one of the most important pillars of tissue engineering ^[1].

In addition to the scaffold, selecting the appropriate cell source also plays an important role in studying the behavior of cells when interacting with the scaffold and most of the cellular resources used are fibroblastic, stem and cancerous cells. However, in order to further simulate the conditions governing cells in vitro, the use of dynamic tissue culture such as tissues that contribute to the phenomenon of repair in porcely animals can be used ^[2].

Blastema tissue contains a group of undifferentiated cells formed in parts of the body being restored by some organisms and has cells that are capable of replication and differentiation

similar to those of the embryo that are able to be influenced by various factors in a variety of specialized cells ^[3].

Such tissue can also be created two days after punching the rabbit's pinna creating a blastema tissue around the hole. These cells are capable of rapidly replicating and differentiating and from around the inside they create new cells in the hole and within a few days, cartilage, connective

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tissue, abdominal and back skin and other parts are completely similar to the original tissue [4]. So blastema can be a model for migration. In the study of cells obtained from the blastema tissue obtained from the rabbit's pinna the expression of specific genes of ESCs, including Oct4 and Sox2 was identified. In this way, the dynamic tissue of the blastema derived from the rabbit pinna can be considered as a cellular source for the study of cellular behaviors [5].

So far, various synthesized scaffolds have been developed to examine the behavior of cells in three-dimensional conditions but each of these scaffolds has defects such as the lack of control over the adhesion of cells and so on. An extracellular matrix of living organisms can be used to study the migration behavior of cells. To do this, decellularization techniques can be used to remove the effect of tissue cells from the extracellular matrix, so that ultimately only the extracellular matrix can remain from the tissue and can be used as a scaffold. The purpose of the decellularization process is to remove the cellular and nuclear material while maintaining integrity and minimizing any damage to the composition and bioavailability of the extracellular matrix [6].

In the previous research with the aim of studying the interaction of cells and matrix. in this study, for the first time mesenteric bovine was used to natural scaffold. The main objective of this study was to investigate the behavior of blastema cells of New Zealand rabbit in interactions with the mesenteric bovine decellularized matrix.

MATERIALS AND METHODS

Preparation of Blastema tissue

For the preparation of Blastema tissue, New Zealand male rabbits of 6 to 8 months old weighing about 3 kg were used from the Razi Serum Research Institute of Mashhad. At all times, animals were used in animals kept at the university house and placed under a basal diet individually in a cage with a controlled temperature of $20 \pm 2^{\circ}\text{C}$ and 12 hours of light at night. In all stages of this study, the approvals of the principles of working with laboratory animals were observed. After removing the hair of the back and abdomen of the rabbit's pinna by the worm and numbing it, punches with a diameter of 2 mm and after 48 hours, larger punches with a diameter of 4 mm were made at the place of the previous punch in such a way that the impacted tissue was separated from the punched area by a ring. After separating the Blastema ring to prevent contamination, the specimens were washed seven times with the physiological serum and transferred to the laminar hood [7].

Preparation of Mesenteric ECM Scaffold

The mesenteric bovine was prepared according to the relevant ethics, the specimens belonged to young male bovines were free from infectious diseases. The mesenteric parts were kept in a refrigerator at 0°C in the physiologic serum after removing additional pieces the size of the tissues was cut to 5 mm in 5 mm.

Cell decellularization of bovine mesenteric tissue was used simultaneously by both physical and chemical decomposition methods [8]: In the physical method, the components were first placed at -4°C for a week, and then after thawing and washing with normal saline, snap freeze-thaw in liquid nitrogen were performed for 5 cycles [9].

The next step was a chemical decomposition method. In this method, specimens were treated with 2.5% sodium dodecyl sulfate (SDS Merck, Darmstadt, Germany) for 3 hours. The components were then removed from the SDS solution and rinsed with a phosphate-buffered saline (PBS) for half an hour [10].

Evaluation of mesenteric scaffolds using histologic methods

4% paraformaldehyde (Merck, Darmstadt, Germany) was used to prepare the samples for histological and fluorescent studies. After fixing, dehydration of the samples was carried out by upward degrees of ethanol embedded in paraffin (Lab-O-Wax, Milan, Italy) micrometer transverse cross-section with microtome (Leits, Vienna, Austria) Paraffin drainage, watering and staining of Hematoxylin-Eosin (H&E) (Merck, Darmstadt, Germany). DNA contents were also stained with Diamidino 2 phenylindole (DAPI; Sigma-Aldrich, Taufkirchen, Germany) [11].

The placement of Blastema tissue in the vicinity of decellularized scaffolds and transfer to the culture medium

Under the laminar hood and under completely sterile conditions, the scaffolds were placed in the blastema rings and for culture, DMEM (Dulbecco's modified eagle medium) and temperature of 37°C with CO_2 25% were used. In order to prevent microbial contamination and nutrition of the cells, the culture medium was changed every two days. In order to carry out tissue studies. Sampling was done every week from the culture medium.

Optical and fluorescent microscope studies

Histological studies including staining of hematoxylin and eosin and DAPI on the cropped scaffolds were performed to investigate the interactions of the blastema tissue cells with these scaffolds.

SEM imaging microscope studies

In order to obtain images for studies scanning electron microscope (SEM) (Leo VP1450, Germany), samples were fixed with glutaraldehyde (TAAB Laboratories Equipment Ltd. Reading, UK) and Osmium tetroxide (TAAB Laboratories, UK) and were dehydrated ascending grades of ethanol. After drying, the samples were fixed on metal stubs and coated with gold-palladium by sputtering (Sputter coater, SC7620, Sussex, UK) and examined under a scanning electron microscope.

RESULTS

Investigation and study of decellularized bovine mesentery

In this study, mesenteric tissue of the bovine was decellularized in various physical and chemical stages and used as scaffold and inducing agent for differentiation of blastema cells. The hematoxylin and eosin staining technique showed that the cellular components were completely removed from the mesenteric tissue during the decellularization process (Fig. 1)

According to Fig. 2: The normal mesenteric (A-C) and decellularized mesenteric scaffold (D-F) were confirmed using DAPI fluorescence, the complete removal of nuclear material from decellularized scaffolds was confirmed.

In order to further investigate the structural changes of mesenteric tissue during preparation, scaffolds were prepared by using an electron microscope of the image. In confirmation of the results of optical and fluorescent microscopes as shown in Fig. 3, the cells were completely removed, the overall structure of the mesenteric tissue remains after the preserved decellularization process (A) and mesenteric epithelium, such as an empty net of cell (B) and bound to the connective tissue. In addition connective tissue collagen fibers remain healthy and intact (C,D).

Investigating the interactions between blastema cells and decellularized bovine mesentery scaffolds

During the staining of hematoxylin-eosin in Fig. 4, no cell migration from the blastema tissue to the mesenteric scaffold was observed on the first days of the first week after culture (A). However, at the end of the first week, primary penetration of the cells was observed on the mesenteric scaffold (B). The second week witnessed the migration and invasion of cells from the blastema tissue into the cellular migration (cell migration & invasion) (C) and more cell density in parts closer to the blastema ring at the surface and lower cell density in the distal parts of the blastema ring (D) As well as the maintenance of cells in the mesenteric and possible differentiation of fibroblastic cells (C,D). In the third week, widespread penetration of the cells into the mesentery and their possible differentiation into fibroblast cells (E), the adhesion of polarized cells to the mesenteric margin elongation and ductility of the cells at the surface which may indicate the formation of simple squamous cellular epithelium cells or mesothelium-like layers, as well as the placement of a number of cells around the mesenteric cavities and a possible differentiation of adipocyte cells (fat cells) (F).

Fluorescent microscopy studies also showed migration and penetration of cells from the blastema to the mesenteric scaffold in the second week after culturing. Also as shown in Fig. 5, cell density is more closely related to blastema (Fig. 5).

SEM electron microscopy studies, as shown in Fig. 6, show the penetration and initial adherence of the cells from the blastema tissue to the mesenteric scaffold (A), in the first week after the culture and the migrating cells with the pseudopodia ("false feet") to the mesenteric scaffold (B). In the second week after the culture, the invasion of the blastema cells and a significant increase in the deposition of the cells into the mesenteric scaffold (C,D), The formation of a simple squamous epithelium or mesothelium with cellular connections between them (E,F) and a tight junction between the cells was observed (G,H).

DISCUSSION

Natural scaffolds are derived from the Decellularized tissues , by maintaining the natural structure of the extracellular matrix, leading to an increase in adhesion, migration and cell proliferation processes.

[12].

Thus far, several decellularization approaches have been presented to develop extracellular matrix derived scaffolds [13, 14].

Based on various researches reports on snap freeze-thaw cycles are followed by using different detergents which causes the omission of cellular components in tissues [15].

In this research, a scaffold derived from a mesenteric extracellular matrix of bovine was synthesized using a combination of physical and chemical methods.

As a physical approach, snap freeze-thaws cycling in liquid nitrogen were used in five cycles. Stemming from the studies, it is believed that some cellular components such as cell nuclei would not be omitted by this process, nevertheless, the freeze-thaw cycling disrupts the cells and intracellular proteins, and treatment with other reagents causes the necessity to remove cell components completely [16].

Many methods based on detergents are advanced to decellularize various tissues and organs [17]. According to the previous researches, the best detergent for expelling cells and cell debris from tissue is SDS [18].

What is clear from the detergent treatments combination with freeze-thaw is that there is a synergistic impact on omission of cellular components which leads to much bigger success in decellularization [15, 19-21].

In this research, a three-hour treatment with 2.5% SDS made it possible to have total removal of cellular components with preservation of extracellular matrix contents with H. E staining. It is expressed by the previous researches that DNA is quite difficult to remove in comparison with other intracellular components due to its sticky features and strong adhesion towards the ECM proteins [10]. The remains of the DNA after its decellularization are likely to cause

inflammatory responses following implantations [22, 23]. Therefore, the use of more specified methods are of high importance for detection of the DNA. DAPI staining demonstrated total DNA eradication from the decellularized mesentery tissue that was achieved. Moreover, microstructures were analyzed by electron microscopy and approved eradication of cells from the decellularized tissues of the obtained scaffolds. In conclusion, a freeze-thaw cycles combination with a three-hour treatment with 2.5% SDS would lead to the complete removal of cells along with relative preservation of collagen contents of the mesentery ECM.

In order to investigate the effects of mesenteric extracellular matrix on the behavior of blastema cells, the prepared scaffolds were assembled within the rings of blastema tissues from rabbits pinna.

The success in decellularization and the creation of suitable scaffolds for tissue repair and engineering relates to molecular signals generated by the compounds of the remaining extracellular matrix together with protein structures [24].

The present study using optical microscopy, fluorescence and electron microscopy (SEM) showed that the blastema cells migrated to the decellularized mesenteric region showing distinct behaviors.

Blastema-derived stem cells are capable of differentiating into bone marrow, cartilage and fat cells, a characteristic that is a specific trait for mesenchymal stem cells [25]. Also, the undifferentiated cells produced by Blastema in the repair of rabbit ears have a high reproductive capacity and express the ability to express the markers of specific stem cells expressing these immortal OCT4 and SOX2 cells [5].

According to the results, the differentiation of the cells of the blastema tissue was observed in the number of immature cells that appeared to be fibroblast, mesothelial cells (squamous cell) and adipocyte cells in the differentiation pathway.

In one recent study, rat epithelial cells cultured on a three-dimensional collagen scaffold were able to form mature epithelium morphology cells [26]. With respect to Fig.4 (E,F), it seems that pseudo-embryonic blastema cells in a decellularized scaffold have the potential to differentiate into squamous epithelial cells or mesothelioma mesenteric tissue. However, in this study a natural scaffold along with blastema tissue as a cell source was used. The successful use of extracellular matrix-based scaffolds is not unique to a particular tissue. In the study, the extracellular matrix of the small intestinal mucus after decellularization was used as a scaffold to repair the attachment of gastronomy muscle and Achilles tendon in dogs [27]. These scaffolds were recellularized by the culture of the ancestral cells and finally replaced completely with the contraction and tendon muscle [28].

In the third week of culture, the transformation of the cells of the blastema tissue and their placement in the mesenteric margin is also important (Fig. 4E,F). Research has shown that the movement of cells is guided by a gradient from chemical messages and on the other hand physical interactions between cells and substrates can also play a role in this kind of movement [10].

Investigating the induced effects of esophageal decellularized scaffold on epithelial cells, maintaining collagen and elastin of decellularized matrix and showing the behavior of polarity, adhesion, migration and propagation of epithelial cells cultured on these scaffolds [29]. In this way, collagen in the scaffold can be considered as one of the possible causes of migration induction and adhesion of the cells embedded from the blastema tissue to the mesenteric scaffold. Mesothelial cells form a number of junctional complexes including tight junctions [30].

In this regard, SEM electron microscopy studies in the third week of culture, in addition to the formation of squamous cells at the mesenteric surface showed the establishment of tight junction connections between these cells confirming the differentiation of the cells towards the formation of mesothelium cover tissue. During a research that examined the growth pattern of human epithelial cells and fibroblasts cultured on a small intestinal submucosa (SIS) extracellular matrix, fibroblasts and epithelial cells grafted onto the SIS were attached by scaffolds [31]. Also the ability of the scaffold to support the bonding of fibroblasts and epidermal cells to migration, amplification and differentiation led to the presence of our base membrane compositions which might be suitable for studying the interaction of the matrix cell and for the research of substitution of the dermis. According to the results obtained during the third week of culture, Blastema cells were differentiated by mesenteric matrix into fibroblastic cells. In the recent research, human epithelial cells and fibroblasts were used for culture on the SIS scaffold But in the present study we tried to study the cell differentiation as an important component of the blastema tissue as a dynamic tissue adjacent to the mesenteric scaffold which confirms the formation of fibroblasts during the third week (Fig.4F).

Previously, fibroblasts were cultured in a three-dimensional collagen matrix and it was stated that the cell specific skeleton protein used by the cell was consistent with the surrounding mechanical conditions [32]. Another result that was observed after three weeks of culture in stained samples with hematoxylin-eosin, the differentiation of the adipocyte cells into the scaffold. During the evaluation, the degree of proliferation and differentiation of primary human adipose tissue (ASC) cultured on decellularized matrix (PDM) and hyaluronan scaffolds (XLHA) revealed that the Scaffold has a profound effect on the propagation and differentiation of adipocytes from cultured human Asc cells [33]. Therefore, it can be seen from the present study that scaffolds obtained from mesenteric bovine tissue may provide ideal materials for

inducing proliferation and differentiation of squamous cell, fibroblast, and adipocyte. Although the differentiation of these cells in this study was only considered phenotypically and more studies are needed to prove the identity of the cells and other features of the scaffold.

CONCLUSION

The results of this study indicate that Blastema tissue and mesenteric scaffold of cattle are a suitable model for investigating interactions of tissue in a three-dimensional structure. The findings of this study included the formation of Mesothelioma mesenteric (Fig.4E, F) and also the formation of fibroblasts and adipocytes (Fig.4F) the role of the effect of mesenteric extracellular matrix on the differentiation of blastema tissue cells can be considered. Because the extracellular matrix through the specific receptors present at the cell surface can have a profound effect on cellular behavior including adhesion, cell polarity and migration and sequences that regulate cell survival differentiation and proliferation^[34].

Also the use of blastema tissue that is formed during some wounds and has embryonic cell properties can be a suitable model for evaluating the behavior of cells in different tissue conditions. In this study, behaviors such as migration, adhesion and polarity were visible.

Tissue and regenerative tissue engineering efforts are being carried out in today's research for a variety of tissues and organs of the human body. Recent advances suggest that engineering textures may have widespread clinical applications in the future that are designed to provide better conditions for the presence of tissue substitutes in transplantation and repair medicine^[35].

It is also worth mentioning that in the future with the continuation of similar and targeted studies in this field, it is possible to provide conditions for the further attention to the industrialization of these scaffolds and in particular its intelligent type.

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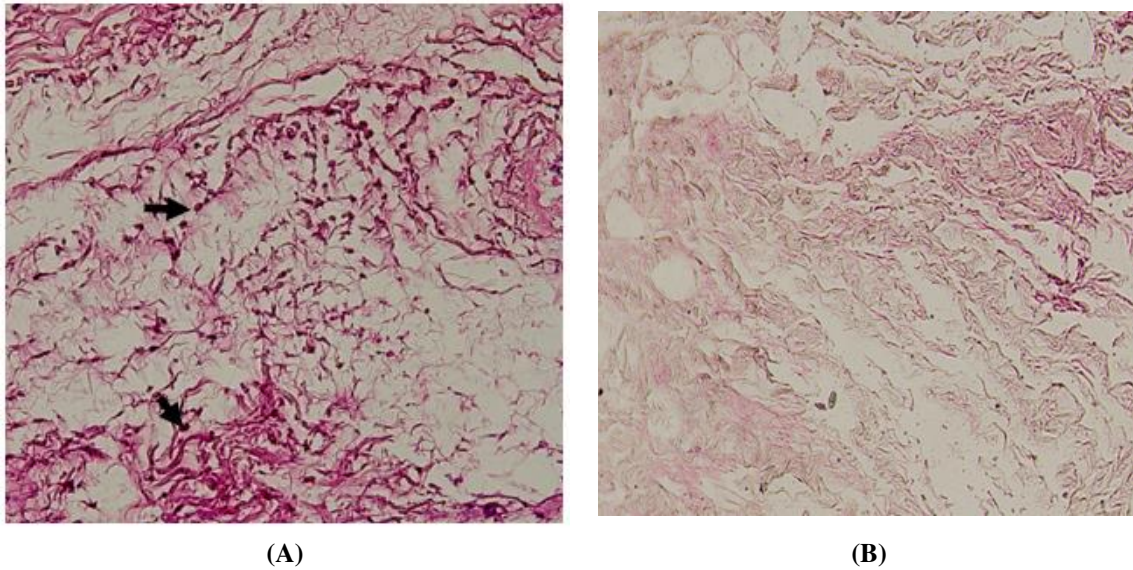


Figure 1: Mesenteric cattle before A and after decellularization (staining with hematoxylin-eosin) arrows indicate the nucleus of the cells.

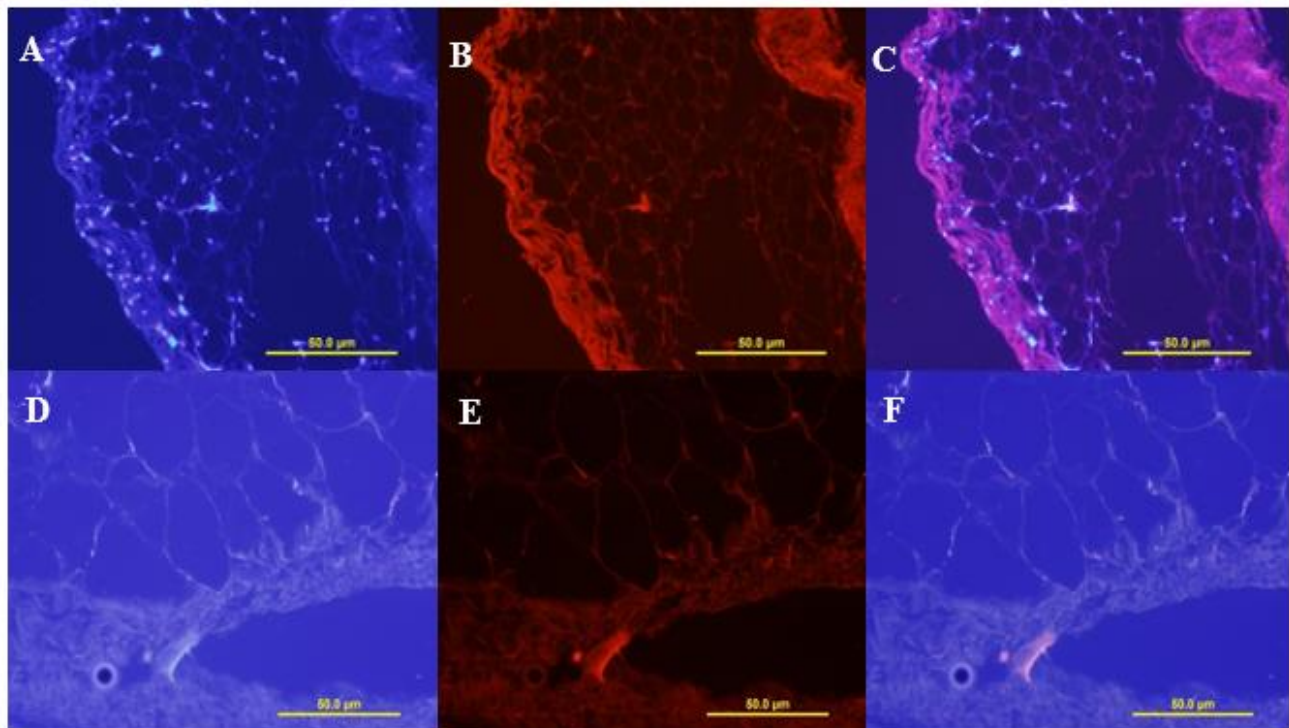


Figure 2: Natural mesenteric display and decellularized mesenteric scaffolds using DAPI fluorescent staining (A,B,C) cores in the natural mesenteric tissue of the DAPI fluorescent dye are bright spots indicating the presence of cells in the tissue. (D,E,F) The decellularized mesenteric scaffold that is darker due to the absence of nuclei.

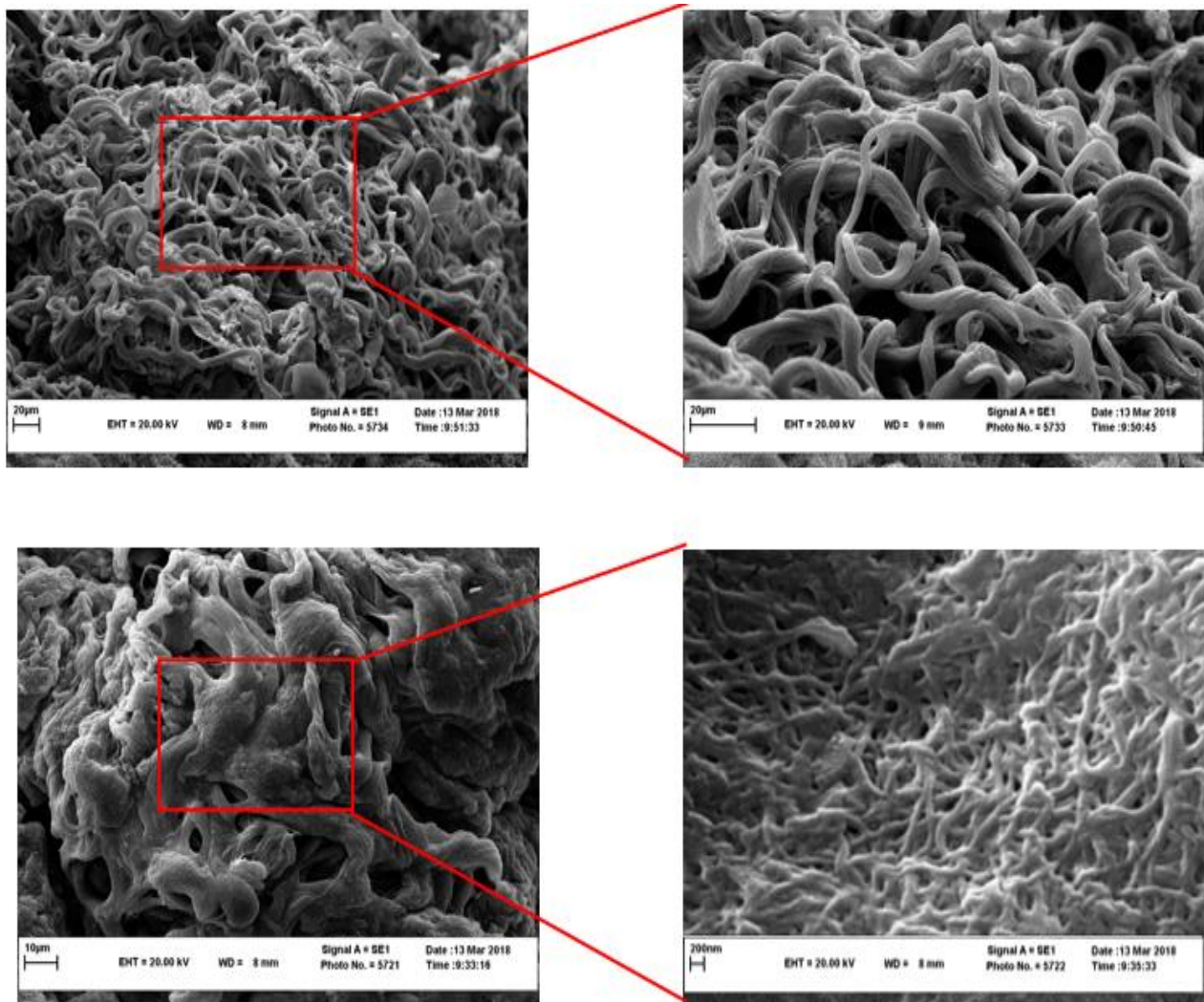


Figure 3: Mesenteresis of bovine after decellularization by scanning electron microscopy

A) Maintaining the overall mesenteric structure of cattle after decellularization. B) shows the mesenteric as an empty net of the cell and Figures C and D show collagen strands after decellularization with a magnitude of 1000, 2500, 2500, 50000 respectively.

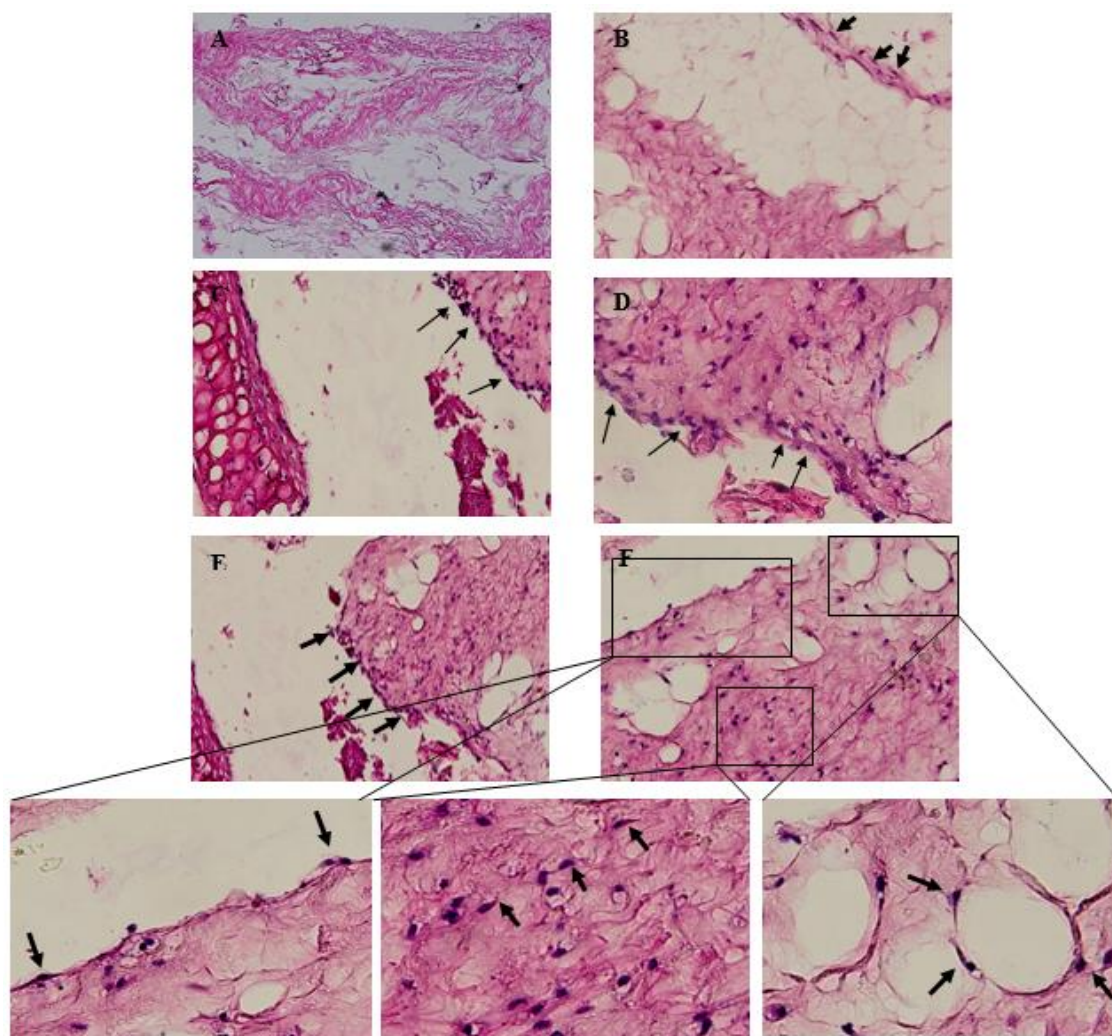


Figure 4: Interaction of blastema cells with mesenteric extracellular matrix by hematoxylin-eosin staining. (A) No migration of cells to the scaffold on the third day after culture, (B) Initial penetration of the blastema cells into the scaffold at the end of the first week after the culture, (C,D) Two weeks after culture the migration and invasion of cells from the blastema tissue into the mesenteric scaffold (cell migration & invasion) the arrows indicate the invasion of the cells from the blastema tissue to the bone scaffold. As well as the greater density of cells in the parts closer to the blastema ring and less density in the regions further away from the blastema ring and maintenance of the cells in the mesenteric, (E) Three weeks after culture, the cell density in the parts closer to the blastema and the wider penetration of the cells into the mesenteric, (F) The polarity of the cells and their adhesion to the mesenteric margin as well as the pulsation of the cells which may, As well as the placement of cells around the mesenteric cavities and possibly differentiation into adipocyte cells.

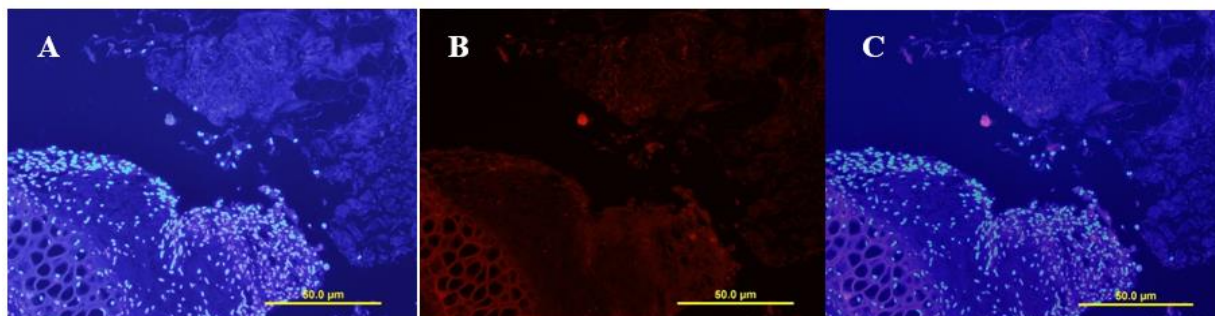


Figure 5: Interaction of blastema cells with mesenteric extracellular matrix using DAPI fluorescence staining in the second week after culture. The bright points representing the nucleus of the cells show the penetration of cells from the blastema tissue to the mesenteric scaffold.

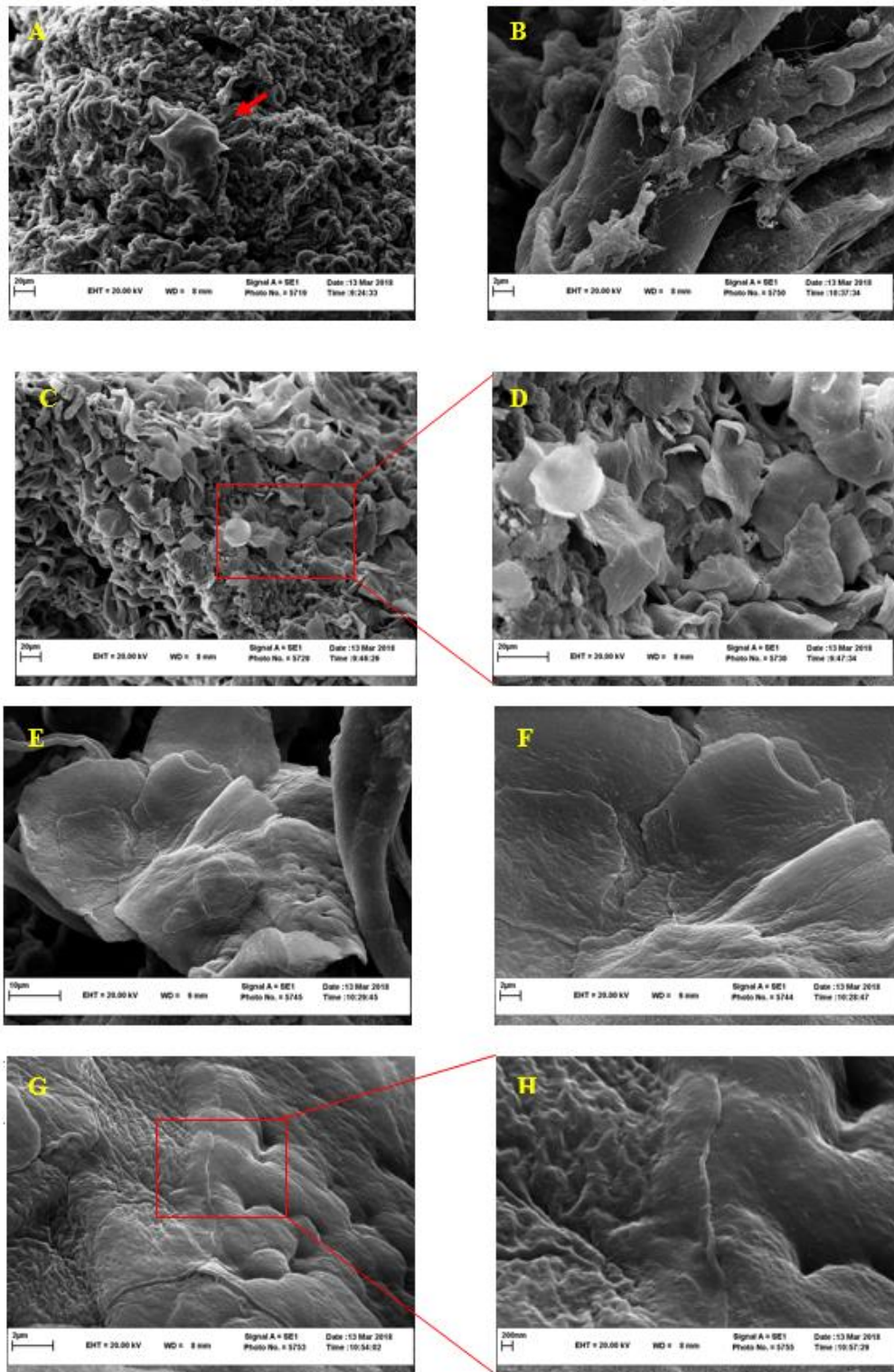


Figure 6: SEM Electron Microscope Investigation of the Blastomy Cells with Mesenchymal Extracellular Matrix: (A) Penetration and Adherence of Blastema Cells to Mesenteric Scaffold in the first week after culture, (B) Formation of idle and false legs in migratory cells to mesenteric scaffolds, (C,D) increase of maintained deployed cells in mesenteric scaffolds, (E,F) The formation of a simple squamous epithelium or mesothelium by cells at a more favorable level and creating intercellular connections between them, (G,H) The formation of tight junction tight junction between cells in the second week after culturing. With a magnitude 1000,10000,1000,2500,5000,10000,20000,50000 respectively.