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Histological Study of Interaction between Blastema Tissue and Decellularized Three Ɖimensional Matrix of Bladder

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Abstract

***Background*:** The purpose of this study is to determine the interaction between Blastema tissue and decellularized three-dimensional matrix of ovine bladder in vitro.
***Materials and Methods*:** In order to produce relaxation of decellularized ovine bladder, combination of physiochemical methods were used. Thus, pieces of bladder were put in -4 C and then samples were put in liquid nitrogen; then all samples were decellularized using chemical combination of 1% SDS (Sodium Dodecyl Sulfate). To produce Blastema tissue, a few 2 mm diameter holes were made in auricles of male New Zealand rabbits. After 72 hours, The Blastema ring was removed and it was put under sterile conditions of decellularized scaffolds inside the Blastema ring and moved to the medium. After histological stages, medium samples were stained with Hematoxylin-Eosin and Peak Indigo Carmine. The quantitative data were analyzed by ANOVA and TUKY test at the level of $p<0.05$.
***Results*:** Light microscopic studies at different culture days showed that blastema cells migrated into the decellularized matrix of bladder so that the maximum migration to the scaffold occurred on the 20th day ($p=0.001$). Blastema cells differentiation into the epithelial, fibroblasts and adipocyte cells is obvious on the day 15 and 20.
***Conclusion*:** Bladder decellularized matrix can be a suitable scaffold to induce and differentiate blastema cells.

Introduction

The study of the interaction between cells and tissues is the infrastructure of tissues engineering studies and development of cells and tissues. The three main pillars of tissue engineering include cell, matrix as the scaffold and growth factor [1]. In 1993, Langer defined tissue engineering as a new field of studies in which the principles of engineering and biology are applied to repair the damaged live tissue, renew the repair and preserves the tissue work [2]. From 1990s, tissue engineering has been discussed as the biological successors of body tissues to reconstruct the tissue [3]. Since most cells of mammalians have a tendency, they will be destroyed if no suitable substrate is available for cell adhesion. Therefore, it is necessary to use appropriate scaffold and levels for cell culture [4]. Different biodegradable scaffolds have been successfully used in reconstruction of the urinary organs. The material of biological scaffolds that has been used in repair and tissue engineering includes small intestinal submucosa, decellularized dermis and amniotic membrane tissue [5]. Various factors induce differentiation of cells including the physical contact of cells with adjacent cells i.e. the intracellular interactions and specific molecules present in the surrounding environment [6]. Thus, the adjacent cells, growth factors and extracellular matrix components mediate and regulate the processes of differentiation, division and apoptosis of cells. Extracellular matrix has different effects on cellular behaviors [7]. In fact, the extracellular matrix is a dynamic structure which contains proteins and various compounds such as Fibronectin, Collagen, Laminine and other compounds and provides a complex structure [8]. Urinary tract and bladder are susceptible to a variety of pathological conditions from fetal development period to the adolescence. A number of synthetic materials and natural tissues have been investigated to reconstruct functional defects of the bladder. These natural materials include bladder allografts, gastric-intestinal parts, skin, muscle, pericardium, pair, dura, small intestinal submucosa layer and the bladder decellularized matrix [9]. Auricles of white male New Zealand rabbits are a good model for study of these kinds of studies, so that Blastema tissue cells of rabbit auricles are cells with characteristics of embryonic cells and to some extent, characteristics of stem cells. These cells can be differentiated in different aspects while influenced by environmental factors, including extracellular matrix [10]. Processes of migration, cell proliferation and acceptance are strongly affected by the combination and structure of extracellular matrix. Therefore, the three-dimensional environment

originated from the bladder tissue can be effective in the bladder tissue repair and replacement. The purpose of the present study is to study the interactions of Blastema tissue cells and auricle of male New Zealand rabbits in decellularized matrix of Ovis Aries bladder as the three-dimensional scaffold in the medium.

Materials and Methods

In the present study which is an experimental study, 5 male New Zealand white rabbits weighing approximately 2-3 kg and 4-6 month old were purchased from Pasteur Research Institute of Tehran and then were transferred to the animal house of science faculty of University of Mashhad. Rabbits were put under 12 h light and 12 hours of darkness in the average temperature of 21° C and they were daily fed with rabbit food. At all stages of this research, legislations of the ethics of working with laboratory animals including free access to food and water, painless killing, prevention from the pain from punching such as the use of lidocaine have been observed. Notably, this research has been conducted in 2010 for 12 months.

To produce the decellularized scaffold of Ovis Aries bladder, a few Ovis Aries bladders have been taken from Mashhad slaughterhouse, and were transferred to the laboratory. Then, the desired tissue was divided into smaller equal pieces and transferred into a Falcon tube. Then, bladder was physically decellularized. Given that there are many effective factors for decellularization of every tissue and organ and these factors depend on several factors including the number of cells of tissue, density, lipid content and the tissue thickness, [11] the combination of physical [12, 13] and chemical [14, 15] methods were used for bladder decellularization. So that bladder pieces were put in the freezer of -4°C for 24 hours and were placed in sterile distilled water once every 6 hours, for 10 minutes. After 24 hours, the pieces were put in the freezer of -20°C and -40°C respectively for 2 hours and 1 hour and then, they were prepared to be placed in liquid nitrogen. Bladder pieces were placed in liquid nitrogen by Cryo Tubes through five 2-minute stages and then, they were washed with phosphate-buffered saline (PBS).

Thereby, decellularization ended at physical stage. At the chemical decellularization stage, all parts of the bladder were put in solution of 1% Sodium Dodecyl Sulfate (SDS) with smooth rotation for 24 hours. Then, the samples were washed by sterile distilled water and were sterilized by ethanol 75% and acetic acid 0.2%, and were finally put in PBS for 24 hours. Thus, scaffold of ovine bladder was prepared to use. This scaffold was kept in freezer of -20°C for 4 days to prepare Blastema ring. For this purpose, a rabbit was selected and put in the rabbit maintenance device (inhibitor) to punch auricle and prepare Blastema tissue. After removing hair of the ears with hair cream and using 10% lidocaine spray all ear surfaces were completely anesthetized after about 30 minutes. Further, 5 holes of 2 mm diameter were made in the right ear and 5 holes were made in the left ear using a special puncher. The rabbits were kept under quite sterile conditions for 3 days. At the end of the third day, after anesthetization of ear, a new hole was made on the previous holes with a puncher of 4mm diameter and a Blastema ring was obtained. Then, Blastema rings were completely sterilized in 7 stages, consisting of six stages of placement in sterile saline and a stage of placement in medium. Notably, research conducted using total of 500 ml DMEM (Dulbecco's Modified Eagle's Medium) and 100 ml FBS (Fetal Bovine Serum) were used, so that each time of preparing the medium for consumption, includes 85 ml DMEM and 15 ml FBS and 500 ml penicillin-streptomycin [16].Then, the scaffold of the prepared bladder was transferred to the laminar hood under sterile conditions and each ovine bladder scaffold was placed in the center of a Blastema ring. Then, all samples were placed in the 6-house medium plates and 3 ml of medium was added to them and the plates were transferred to the CO_2 incubator. On the day 5, 10, 15, 20, 25, and 30 after exit from the medium and histological stages, samples were stained with Hematoxylin-Eosin and Peak Indigo and cellular behaviors, including migration, differentiation and proliferation of Blastema quasi-embryonic cells from the ring into the bladder decellularized matrix as the scaffold were examined by light microscopy.

Hematoxylin-eosin staining was used as regular staining. Hematoxylin made the nucleus purple and eosin made the cytoplasm pink. Peak Indigo staining (combination of 100 ml of saturated Picric acid plus 0.1g Indigo Carmine powder) is a specific color with different acidophil intensities. In Hematoxylin-eosin staining, we used Peak Indigo instead of eosin. Through this color, the background which is the matrix of connective tissue and collagen turns into pistachio green along with epithelium and nuclei will turn into light brown with hematoxylin stain. Data analysis was performed by Minitab-16 statistical software. One-way ANOVA and TUKY test were conducted at a significance level of $p<0.05$.

The first index is the mean of the number of cells migrated to the scaffold on different culture days. Thus, microscopic eye lens were used to measure the number of cells migrated to the scaffold. All slides were examined in magnification ×40 and 5 hypothetical points were measured in the field of vision of all iterations. In addition, all cells inside the lens area were measured. A special microscopic eye lens with measurement ruler was used to study the second indicator which is the mean of size changes of the nucleus of the cells migrated into the scaffold.

Each unit of this ruler in a magnification 40× will be the unit of measurement by microns when multiplied by 3.2. Also since there is a variety of nuclei shapes ranging from spherical, oval and drawn in the cells migrated into the scaffold, for an accurate measurement for nuclei in the field of vision of each nuclei, the small and large diameters were measured, added and divided by 2 to obtain a precise number of size of the nucleus of the cells migrated into the scaffold.

Results

The light microscopy study of sections the parts stained with hematoxylin-eosin showed that the use of combination of physical and chemical methods has been appropriate to produce scaffold with preserving extracellular matrix structure of the bladder.

In below, ovine bladder tissue is shown before decellularization (Fig. 1) and bladder tissue is shown after decellularization (Fig. 2).

Control sample in each culture day was a decellularized scaffold without Blastema tissue which was put in medium. No cells were observed in any of control samples in different culture days. In the 5th culture day, no Blastema cell migration was taken place from Blastema ring into the bladder scaffold. The control sample was also decellularized in this day.

Comparison of control samples on the 10th culture day showed that the Blastema cells have migrated to the bladder scaffold. Comparison of control samples on the 15th culture day showed that the Blastema cells have migrated to the bladder scaffold as connected cell colonies. The use of the specific peak Indigo staining showed that Blastema cells migrated to the scaffold in this day have been differentiated into epithelia cells and some events of forming the bladder epithelium polymorphs in the scaffold are happening (Fig 3).

In addition, the statistical analysis of the mean of size of the nucleus of cells migrated into the scaffolds showed that the greatest nuclei size is related to this day (Fig. 4).

Comparison of the control sample on the 20th culture day showed that the Blastema cells, migrated into the scaffold in this day, have been differentiated into fibroblast cells (Fig. 5). In addition, the differentiation of several Blastema cells into adipocyte cells was also observed in the scaffold (Fig. 6). It seems that some events of angiogenesis process are happening in the scaffold in this day.

Statistical analysis of the mean of the number of cells migrated into the scaffolds showed that the greatest migration of Blastema cells into the scaffold has occurred on this day, that these cells have been differentiated into fibroblasts (p=0.001) (Fig. 7).

The 20th day shows the highest number of cells migrated and the 30th day shows the lowest cell (p<0.05). The column that contains at least one common letter is not statistically significantly different at the level of p<0.05. On the 20th culture day, the presence of cell colonies that seems to be the Blastema cells differentiated into the epithelia cells is detectable in the scaffold. Statistical analysis showed that the nuclei size of these cells is smaller than the cells on the 15th culture day (p=0.001) (Fig. 7).On the 30th culture day, migration of Blastema cells to the scaffold is continued, but the cells migrated into the scaffold are scattered and not connected. Statistical analysis of the mean of size of nuclei of cells migrated into the scaffold showed that the smallest nuclei size is related to this day, which can indicate passivity of these cells (Fig. 7).

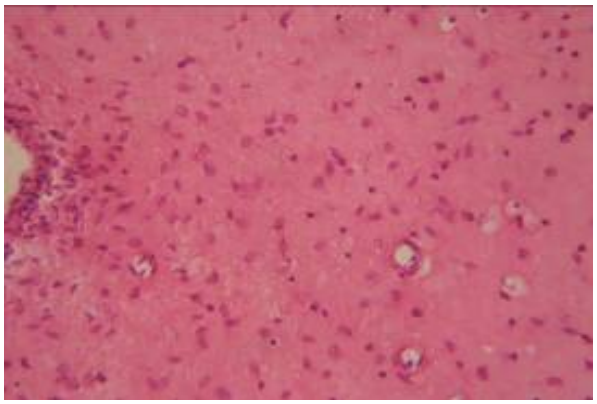

Figure 1. A microscopic view of ovine bladder tissue before decellularization Hematoxylin-eosin staining, magnification: ×40

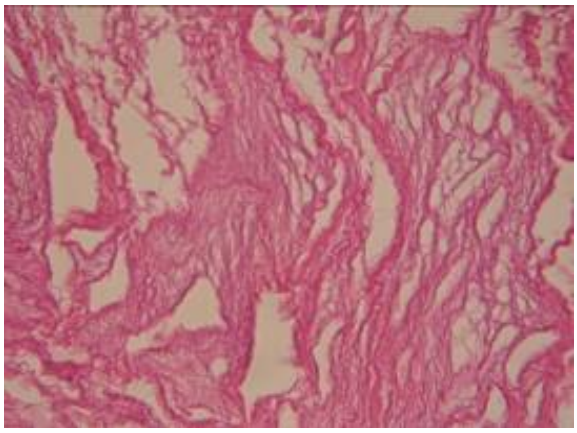

Figure 2. A microscopic view of ovine bladder tissue after decellularization in which no cells are observed in and only extracellular matrix is visible in pink. Hematoxylin-eosin staining, magnification: ×100

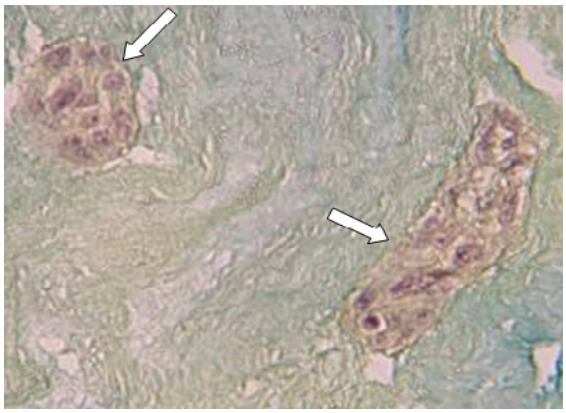

Figure 3. A microscopic view of the 15th culture day in which differentiation of Blastema cells into epithelia cells is evident. Arrowhead: It shows Blastema cells differentiated into epithelial cells. Indigo peak staining, magnification: ×40

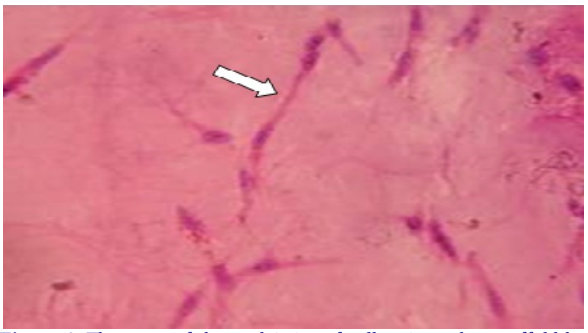

Figure 4. The mean of the nuclei size of cells migrated to scaffold by microns. The largest nucleus size is related to day 15 and the smallest size is related to day 30 (p<0.05). The columns that contain at least one common letter, have no statistically significant difference at the level of p<0.05.

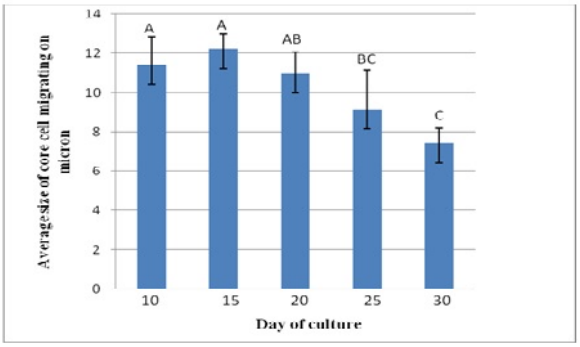

Figure 5. A microscopic view of the 20th culture day in which differentiation of Blastema cells into fibroblast is evident. Arrowhead: It shows Blastema cells differentiated into fibroblast cells. Hematoxylin-eosin staining, magnification: ×40

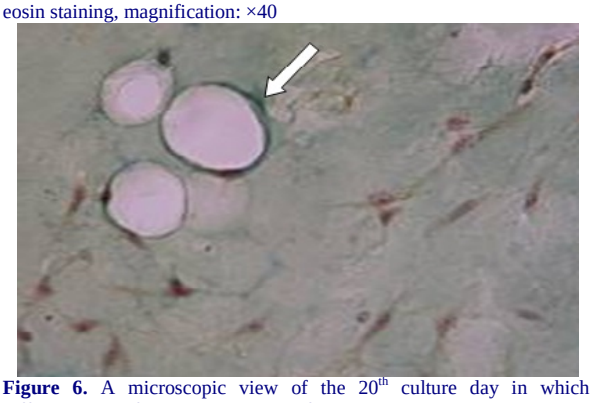

Figure 6. A microscopic view of the 20th culture day in which differentiation of Blastema cells into fibroblast and adipocyte is evident. Arrowhead: It shows Blastema cells differentiated into adipocyte cells. Hematoxylin-eosin staining, magnification: ×40

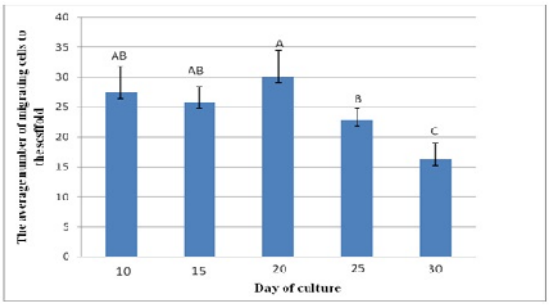

Figure 7. The mean number of cells migrated into the scaffold

Discussion

The results obtained in different days of culture revealed that migration of Blastema cells from Blastema ring into the bladder scaffold began 10 days after the culture and reached its maximum on the 20th day of culture. However, the lowest migration of Blastema cells to the scaffold occurred on the 30th day. Cell differentiation was typically observed as an important component of this study on the 15th and 20th days so that staining the samples of the 15th day with peak Indigo showed that the Blastema cells migrated to the scaffold has been differentiated to the epithelial cells. In addition, on the 20th day, differentiation of Blastema cells into fibroblasts and adipocyte cells in the scaffold was evident.

Bolland et al could decellularize ovine bladder tissue through maintaining the original compounds. They showed that the obtained matrix is biocompatible with cells originated from the bladder and has the appropriate potential for use in urinary tract surgeries and tissue engineering applications [17]. In the present study, such compatibility was observed between decellularize ovine bladder matrix and Blastema quasi-embryonic cells; this matrix has the ability to conduct and differentiate cells. In 2005, through a series of experiments, Kawamura repaired the bone tendon through macrophages cells which were in the early stages of development. He proposed that these cells will be gradually settled in the tendon graft again [18].

In the present study, such behavior was observed from Blastema quasi-embryonic cells; so that these cells migrated to the tissue that contained only extracellular matrix and its cells had been destroyed through physical and chemical methods. Extracellular matrix consists of a different complex of structure and functions of proteins, which plays an important role in morphogenesis and protection of cell and tissue structure and function [19]. The extracellular matrix can be considered as a morphogenetic code which is interpreted according to the cells that are in contact with. Through the specific receptors on the cell surface, information of extracellular matrix can has a profound effect on cell behaviors including adhesion, cell polarity, migration and signals that regulate cell survival, differentiation and proliferation. Cell backgrounds are highly variable in different tissues and each type has been allocated to a specific function.

The major components forming cell background are proteoglycans, glycosaminoglycan and collagen and non-collagen glycoproteins [20]. In the present study, it seems that Blastema quasi-embryonic cells in the decellularized matrix of ovine bladder tissue cells have been differentiated into fibroblast, transitional epithelium and adipocyte. The studies conducted in three-dimensional environments on the behavior of fibroblast cells, showed that three-dimensional matrices not only displace cells, but also cause changes in the matrix modeling [21]. Such results are consistent with the present study; because according to the histological studies of this research, it was observed that the Blastema quasi-embryonic cells

have migrated into the scaffold and have been differentiated into fibroblast after 20 days being influenced with the obtained matrix. Therefore, research and investigation on fibroblasts in three-dimensional collagen matrix environments suggests new opportunities for understanding the interaction and compatibility occurring between cells and matrix surrounding the tissues. Lindberg examined the growth pattern of human epithelial cells and fibroblasts which were cultured from the extracellular matrix originated from Small Intestinal Submucosa (SIS).

This researcher observed that fibroblasts often invade the scaffold when cultured with epidermal cells on SIS. Also, the scaffold ability to provide a setting to connect fibroblasts and epidermal cells and migration, proliferation and differentiation with the presence of basement membrane components, suggested us that this model may be suitable for studies on the cell-matrix interactions and dermal alternative studies [22]. However, in the study of Lindberg, human epithelial cells and fibroblasts are used to culture on the SIS scaffold; whereas, in the present study, cell differentiation has been investigated as an important component which is confirmed by the fibroblast formation on the 20th day, through using Blastema tissue as a dynamic tissue in the vicinity of the bladder scaffold. The studies of Ferrtti have shown that Blastema tissue cells are capable of differentiation in different aspects [23, 24]. Other studies have been conducted on proliferation and differentiation of adipocyte cells in artificial medium. Flynn has proliferated and differentiated the adipose originated from stem cells on the natural scaffold, which was a combination of decellularized pair matrix and hyaluronan [25]. In the results of the present study, Blastema quasi-embryonic cells have the ability to differentiate into fat cells in such a scaffold. In addition, the report of Halbleib has shown that hyaluronic acid scaffold is considered an appropriate three-dimensional environment for differentiation of human adipocyte precursor cells in the medium [26]. Saxena took esophageal epithelial cells from young rats and cultured them in artificial conditions and could make epithelial cells through mature epithelial morphology. They also studied the feature of survival of these cells on the three-dimensional scaffold of collagen [27].

According to the studies conducted in this research, it seems that quasi-embryonic cells have been decellularized in the scaffold and have the ability to be differentiated to the transitional epithelium cells of bladder tissue. The results of this study indicate that the natural scaffolds obtained from ovine bladder tissue, can be a good platform for studying cell behavior and proper models as the first steps towards healing and tissue engineering applications by preserving the main compounds. Such natural scaffolds have the ability of the differentiation, migration and proliferation of stem and quasi-embryonic cells. It seems that preparation of such scaffolds would be an important part of future research of biological knowledge that could have broad applications in regenerative medicine science and tissue engineering.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

No conflict.

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