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DEVELOPMENT AND STUDY OF PERSPECTIVE CLINICAL PROPERTIES OF BIOLOGICALLY ACTIVE MATRIX COMPOSED OF DECELLULARIZED LYOPHILIZED HUMAN AMNIOTIC MEMBRANE IN COMBINATION WITH PLATELET-DERIVED GROWTH FACTORS AND HYALURONIC ACID

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Abstract

Nowadays the development of novel biomaterials which would promote tissue lesions regeneration is one of the crucial tasks of regenerative medicine. In the current research we developed a biological active membrane composed of decellularized, lyophilized human amniotic membrane in combination with growth factors of platelet-rich plasma (PRP) and hyaluronic acid. On the basis of histological examination, scanning electron microscopic examination and DNA content analyses it was drawn a conclusion that Sodium Dodecyl Sulfate (SDS) (Sigma) solution and 1% Triton X-100 solution completely remove all existing cells of placenta and chorionic-amniotic membrane without impairment of their structure. After the processes of decellularization and lyophilization the DNA content was less than 2%.

The PRP growth factors as the components biological matrix provide faster regeneration. The latter are fixed on the surface of cellular structure of decellularized, lyophilized human amniotic membrane by the reservoir of gelatinous hyaluronic acid.

Key words: amniotic membrane; growth factors; hyaluronic acid; morphology; scanning electron microscopy.

Introduction

In the modern period of development of tissue engineering a lot of attention is drawn to the membrane matrix which the cells responsible for tissue regeneration would be integrated on. Scaffolds are the mechanical bases for cell support, cell differentiation and distribution during the entire process of new tissue formation. Amniotic membrane can potentially be used as framework for different bioengineering matrixes for tissue engineering [1, 2]. However the improvement of biostability and biocompatibility of amniotic membrane in order to broaden indications for its use as a framework remains a challenging task. The development of construction technologies of biomaterials with set properties is driven by the need of matrixes for target regeneration [3, 4].

The use of platelet-rich plasma (PRP) is the method of tissue regeneration stimulation due to the local administration of autologous platelet-rich plasma derived from patient's whole blood. The application such modern and potentially highly effective method in the treatment of severe lesions of covering tissues and injuries facilitates and makes the process of wound healing faster [5, 6].

The preparations containing hyaluronic acid (HA) are widely used in medicine and are effective in promoting regeneration. Due to its physical and chemical properties, HA interacts with other molecules (water, protein etc.), which is extremely important, taking into account its potential capacity for modulation of specific processes in the human body [7, 8]. It is known that under pathological conditions the concentration of HA in tissue decreases and cell proliferation and regeneration slow down. The local administration of HA slows down the formation of pathological changes, suppresses the manifestation of inflammatory-proliferative process and triggers the process of physiological tissue regeneration. Such effects of HA are connected with its ability to mediate the migration of fibroblasts into the lesion and stimulate the process of angiogenesis. Both of these have a positive impact on local microcirculation, taking into account the crucial role of microcirculation improvement in sanogenetic mechanisms [9].

So, the development and choice of biomaterials for scaffolds' production is a critical step in tissue engineering [10]. The combination of decellularized lyophilized human amniotic membrane, growth factors and HA in one biological complex can make the process of regeneration significantly faster and facilitate patients' rehabilitation in postoperative period [11-13].

The aim of the research is to develop the method of obtaining a biologically active membrane consisting of decellularized lyophilized human amniotic membrane and PRP in combination with hyaluronic acid fixed on its surface.

Materials and methods

The material for the current study was performed by full-term placentas obtained from donors who gave birth at 37-41 weeks of pregnancy. All women signed the informed consent form for placenta donation. All donors had normal course of pregnancy and gave birth to healthy newborns whose weight varied between 2300 to 3900 g.

The study was approved at the meeting of the Ethics Committee of the Georgian National Institute for Medical Research.

Method of obtaining of decellularized lyophilized human amniotic membrane

Immediately after placental expulsion in the delivery room placenta was washed through the umbilical arteries with 200,0 ml of 0,9% saline diluted with heparin (5000 IU). After that placenta was placed within sterile container system and transported to laboratory of the department of clinical anatomy of Tbilisi State Medical University. Under laboratory conditions placenta was placed on the sterile glass table and the cannulation of umbilical vessels was conducted. To do this, two cannulas performed by polypropylene vascular catheter with diameter of 4 mm and length of 20 cm were inserted in umbilical arteries and fixed by two silk ligatures. The third cannula with diameter of 6 mm and length of 20 cm was inserted in umbilical vein and also fixed by two silk ligatures.

The process of decellularization was initiated through the intraarterial cannulas, using 500,0 ml of 0,9% saline. Flushing was stopped once a clear solution began to flow from the umbilical vein. After flushing the placenta was frozen at -800°C for 24 hours. The frozen placenta was thawed +4°C and washed with PBS in the amount of 500,0 ml through the placental arteries. Then the placenta was perfused with solution containing Sodium Dodecyl Sulfate (SDS) (Sigma) and distilled water for 72 hours, starting with 0,01% SDS for 24 hours, then 0,1% SDS for next 24 hours and 1% SDS for last 24 hours. After that the placenta was washed with distilled water for 15 minutes, and only then 1% Triton X-100 solution was used

for 30 minutes in order to remove residual SDS. Then the placenta was washed with PBS for 1 hour. Decellularized placenta was sterilized in 0,1% peracetic acid solution ('Aldrich-Sigma', Germany) in PBS solution for 3 hours; after that the amniotic membrane was separated from the decellularized placenta.

The separated amniotic membrane was placed on a sterile table, cut into plates measuring 4x4 cm and placed within a lyophilization apparatus (Power Dry PL 6000 Freeze Dryers). After lyophilization the amniotic membranes were packed in disposable plastic packs and sterilized with g-rays at dose 15 kGy.

Decellularized lyophilized amniotic membranes were stored under sterile conditions at room temperature until used [14].

Method of obtaining of PRP. After a rat was euthanized by intraperitoneal administration of lethal dose of 0,5% sodium thiopental solution, the blood was collected in a test tube, which then was placed in a centrifuge Kokusan H-9R (Japan). The blood was centrifuged for 20 minutes at 29°C (1600 rpm). After centrifugation the upper and the middle layers of liquid were removed from the tube and transported to a clean tube, which was again placed in a centrifuge and centrifuged a second time for 15 minutes (400 rpm). Thus, it was obtained a plasma divided into two fractions: the upper layer – platelet-depleted plasma; the lower layer – platelet-rich plasma (PRP).

Method of applying PRP with HA on the surface of decellularized and rehydrated human amniotic membrane. To obtain a biologically active membrane in combination with PRP growth factors and HA 1 ml of PRP was mixed with 0,5 ml of HA in a sterile cup. Lyophilized human amniotic membrane was placed in a Petri dish and rehydrated with 0,9% NaCl solution for 40 minutes. Then rehydrated amniotic membrane was placed on a sterile table and its anterior surface was covered with PRP with HA. After that, the membrane was inverted and its reverse side was also covered with PRP and HA.

HA used for the study was performed by a viscoelastic gel hyaDENT BG containing 2,0 mg of HA and 16,0 mg of cross-linked HA.

Methods of study of human amniotic membrane

1. *Scanning electron microscopy of amniotic membrane before and after decellularization.* For scanning electron microscopy the tissue samples measuring 0,5x0,5 cm x 0,1cm were dehydrated using ethanol solution and then lyophilized. After that dried tissue samples were coated by gold dust. The prepared specimens were studied under the scanning electron microscope Hitachi (Japan).

2. *Method of the quantitative analysis of DNA taken from tissues of normal and decellularized human amniotic membrane.* DNA was isolated from the tissues of normal and decellularized chorionic-amniotic membrane by a standard method using commercial extraction kit (G-spin Kit, iNtRON Biotechnology). Total DNA was determined on a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific) at a wavelength of 260 nm. All samples were normalized to dry weight of human amnion.

3. *Histological methods of research.* The excised tissue pieces of normal and decellularized human amniotic membrane were placed in 10% formalin solution, then embedded into paraffin blocks, which were cut into sections with a thickness of 5 mm. The sections were stained with hematoxylin and eosin. Visualization of specimens was performed on a light microscope ('Olympus', Japan).

Results

The solutions used in the current research that contain Sodium Dodecyl Sulfate (SDS) and 1% Triton-X solutions completely remove all existing cells of placenta and chorionic-amniotic membrane without impairing their structure. It was confirmed visually and by using histological methods of research, scanning electron microscopy and DNA test. The abovementioned is demonstrated on the photographs of normal human placenta (Fig. 1) and human placenta after decellularization (Fig. 2).



Fig. 1. Normal human placenta before decellularization



Fig. 2. Decellularized human placenta

To visualize placental vessels watercolor dyes of red color were administered through the umbilical arteries, and watercolor dye of blue color was administered through the umbilical vein (Fig. 3). The process of separation of decellularized chorionic-amniotic membrane from decellularized placenta is shown in Fig. 4.

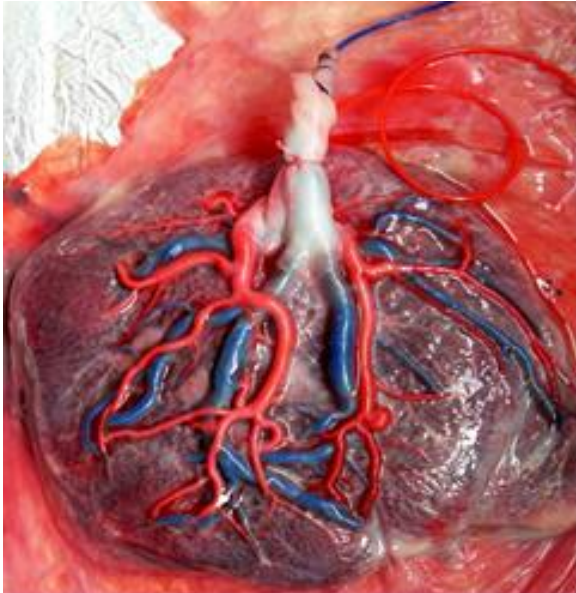


Fig. 3. Visualization of placental vessels (watercolor dyes administered through umbilical arteries [red], and umbilical vein [blue]).



Fig. 4. Separation of decellularized chorionic-amniotic membrane from decellularized placenta

The conducted morphological research (Fig. 5) and electron microscopic examination (Fig. 6) revealed an intact structure of extracellular matrix and the absence of cellular elements. The structure of amniotic membrane extracellular matrix was preserved even after lyophilization and rehydration (Fig. 7).

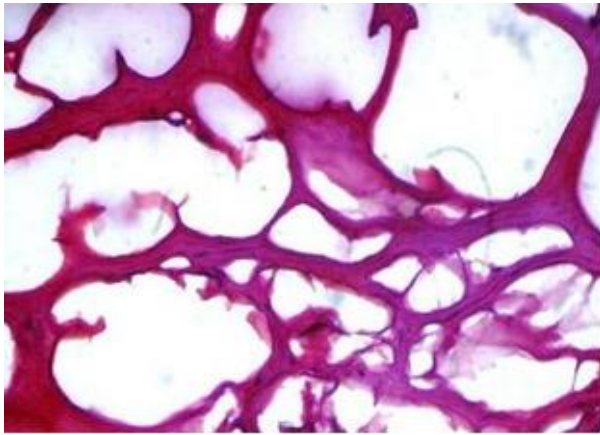


Fig. 5. Decellularized amniotic membrane.
Hematoxylin & Eosin. x 400

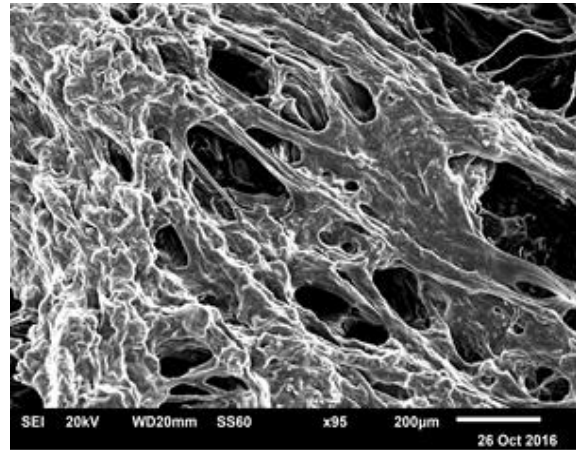


Fig. 6. Amniotic membrane after
decellularization

On the basis of quantitative DNA analysis we calculated the DNA amount in the human chorionic-amniotic membrane before decellularization – the figure was 338 µg/ml. After decellularization and lyophilization the residual DNA content was less than 2%.

According to our data, PRP and HA evenly covered both surfaces of lyophilized and rehydrated amniotic membrane (Fig. 8).

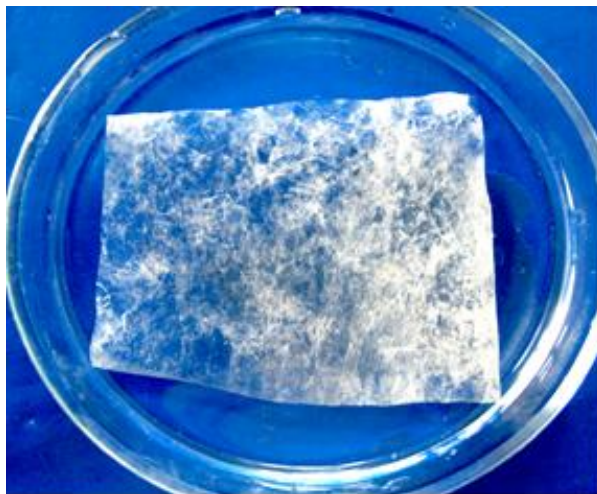


Рис. 7. Decellularized human amniotic
membrane after lyophilization and rehydration

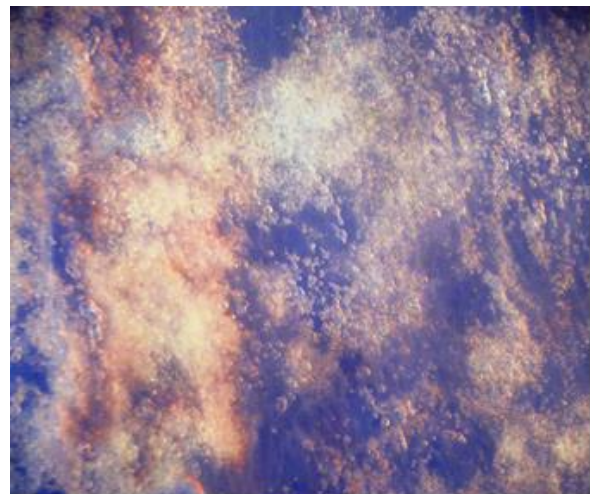


Рис. 8. Adhesion of PRP and HA to the
anterior surface of rehydrated
decellularized human amniotic membrane

Thus, the biologically active membrane with PRP and HA fixed on its surface developed in the current research is characterized by plasticity. Such membrane can evenly cover tissue lesions and firmly attach to epithelial surface; it doesn't require additional expenses, it's easy and convenient in use.

Conclusions

1. Biologically active membrane with a combination of PRP growth factors and HA applied on its surface can be used in tissue engineering.

2. Decellularized lyophilized human amniotic membrane is a collagen basis with cellular structure which makes it suitable for mechanical support and fixation of a combination of PRP growth factors and HA on its surface.

3. Hyaluronic acid in such biologically active membrane acts as reservoir for PRP growth factors.

4. PRP growth factors are biologically active molecules which trigger the process of regeneration; however they are not able to create three-dimensional material. Therefore, they are included in the scaffold, being applied on the 3D-structure of decellularized lyophilized human amniotic membrane and fixed by a HA gel reservoir.

We believe that our further experimental studies will result in deeper understanding of regenerative potential of biologically active membrane for the closure of bone and soft tissue defects and establish indications for their clinical use.

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