Elastin Barrier Membranes for Guided Tissue Regeneration Technologies

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Abstract. This article discusses the prospects for the use of new elastin barrier membranes manufactured using adapted technologies for the selective isolation of the elastin component from the extracellular xenogenic matrix of the pericardium ligamentous apparatus: (1) by high-temperature extraction under pressure; (2) cyanogen bromide method. A commercial material, Geistlich Bio–Gide® membrane (BG), was used as a control comparison group. It is shown that the materials of group (1) have a high degree of biocompatibility, exceeding the indicators of the control group BG. Based on the results of an study in a model of subcutaneous heterotopic implantation in rats, it was shown that elastin BM has a chemoattractant effect on the mesenchymal recipient cells and, unlike the control, is able to integrate to a high degree into the surrounding recipient tissues. At the same time, the materials of group (1) had a pronounced proangiogenic effect. Thus, it has been shown that elastin BM groups (1) have a medium-term barrier function and are able to induce full-fledged cellular repopulation and local neoangiogenesis, which can be useful in clinical practice, primarily in GTR technologies (with gingival flap augmentation) or when used together with other BM as an angiogenesis inducer to ensure formation of the vascular bed in GBR technologies of bone tissue.

1 Introduction

Techniques of guided bone regeneration (GBR) and guided tissue regeneration (GTR) are widely used methods in the surgical treatment of periodontal diseases and for the jawbone defects reconstruction. Barrier membranes (BM) provide a barrier between connective and epithelial tissues with different proliferative activity in the GTR technique [1]. With the GBR approach, in addition to the role of a barrier between the alveolar bone and the gingival flap, the membrane can provide stabilization of bone grafts and fillers [1]. The materials used for the manufacture of BM must be biocompatible, non-immunogenic and biostable in order to provide a barrier function throughout the entire period of treatment (bone augmentation) [2].

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Depending on the basis material, BM can be of several types: non-resorbable inorganic and synthetic, and resorbable synthetic and natural BM. Currently, resorbable biomaterials made from natural proteins of bovine or porcine pericardium have the greatest prospects for the development of effective BM. Such biomaterials are convenient to use, have the necessary strength and elasticity indicators, significantly reduce the risk of chronic rejection, and are also easily saturated with bioactive molecules and drugs [2, 3, 4]. In addition, the composition of the main biomaterial, the main component of which is collagen, gives the membrane a number of its own bioactive functions, such as chemoattraction and induction of cell migration, as well as participation in the balance of the pro-inflammatory and pro-regenerative phases of reparative processes [3].

Despite the significant advantages of collagen biomaterials over synthetic and inorganic ones, such membranes are not universal and cannot always provide an effective treatment result. The most pressing problems include the risk of premature loss of the barrier function of BM. It is assumed that the main reason for the premature resorption of these BM is their susceptibility to hydrolytic and enzymatic degradation under the action of extracellular collagenases and body tissue fluid [5]. Therefore, in some cases, additional processing of materials with cross-linking agents (such as glutaraldehyde, etc.) is used to extend the service life of collagen BM, which increases the enzymatic stability of the material, but gives it a cytotoxic effect and can lead to the development of pathological fibrosis, aseptic calcification and chronic rejection [6].

Another problem of collagen BM, which is also characteristic of other types of BM, is a significant slowdown or blocking of regeneration of tissues in contact with the membrane. It is assumed that this effect is associated with insufficient blood supply to the tissues under the membrane [7]. Neoangiogenesis is one of the main factors of successful tissue regeneration and provides not only the necessary level of nutrients and oxygen in the wound, but also delivers immune cells and stem cells to the defect area [7]. It has been shown that the formation of full-fledged microcrovascular vessels during angiogenesis is a necessary factor for the successful reconstitution of the dentin-pulp complex [8]. In GBR techniques using additional bone grafts, angiogenesis plays a central role in providing a functional connection between the implanted material and the surrounding recipient tissues. Mature vascular networks successfully formed during regeneration can promote and accelerate regenerative processes [7]. The induction of both pro-angiogenic and anti-aniogenic factors has been shown for collagen membranes, while which of them prevail and at which stages of the inflammatory-regenerative process is still unknown [9, 10]. To enhance the proangiogenic effect, collagen membranes can be additionally saturated with VEGF, however, the kinetics of the release of molecules from the material remains uncontrolled and does not always lead to a successful result.

This work proposes a new type of barrier materials made on the basis of the elastin extracellular matrix of the ligamentous apparatus of the pericardium. It is assumed that materials such as collagen BM will have a high degree of biocompatibility and the necessary chemoattractant properties. It is also known that elastin peptides have not only a proven osteoinducing effect, but also selective chemotaxis for smooth muscle cells, which can induce local neoangiogenesis within the recipient bed [11].

2 Materials and methods

2.1 Sample preparation

Freshly harvested bovine pericardium was placed in a cold 0.9% NaCl solution (pH 7.4) for transportation. Next, the areas of pericardium ligamentous apparatus were separated from

the base tissue, mechanically cleaned of fat and other tissues and cut into fragments of 10 × 10 mm2.

2.2 Production of elastin BM

Experimental BM was made from the ECM of pericardium ligamentous apparatus using two methods of selective elastin extraction. (1) High-temperature extraction under pressure (according to the protocol of M.A. Lillie et al) [12], (further Group 1). To do this, part of the pericardium fragments were placed in distilled water and incubated for 8 hours in an autoclave (VK-75, KPZ, Russia) at 110kPa. (2) Extraction in formic acid with cyanogen bromide (adapted protocol for Q.Lu et al) [13], (further Group 2). For this method, part of the pericardial fragments were treated in 50 mg/ml CNBr in 70% formic acid (8ml/cm2) at room temperature for 20 h with careful stirring on a shaker incubator (Biosan, Latvia), followed by inactivation in boiling water for 5 min.

Prior to further in vitro and in vivo studies, all finished materials were stored in sterile NaCl (pH 7.4) with the addition of gentamicin (400 mcg/ml) and fluconazole (50 mcg/ml) at 4C.

2.3 DNA measurement

The degree of decellularization of the materials was determined by the amount of residual donor DNA in the tissue. To do this, tissue fragments (n=5) weighing 10 mg were taken from each group of samples and homogenized in Eppendorf-type test tubes using a Merck pestle microhomogenizer (Millipore, USA). DNA isolation from tissue homogenate was carried out using a set of DNA-extran-2 (Syntol, Russia). As a control, an empty test tube was used, with which the same manipulations were performed as with experimental test tubes. DNA measurement in the obtained solutions was performed on a NanoVue Plus spectrophotometer (Biochrom, USA) at 260 nm.

2.4 In vitro biocompatibility assessment

To assess the biocompatibility of experimental materials, cells of mouse embryonic fibroblasts of the NIH/3T3 line (Institute of Cytology of the Russian Academy of Sciences, St. Petersburg) were sown on the BM surface in a 96–well plate (2000 cells/well). After 4 days of incubation on the surface of the materials, the cells were stained with Calcein AM dye (1 mcg/ml) and propidium iodide (2 mcg/ml) (Sigma-Aldrich, USA) in a DMEM medium for 5 minutes at 37 °C and observed using a TCS SP5 confocal microscope (Leica, Germany). At least 500 cells were counted for analysis, and cells seeded on culture plastic were used as a control.

2.5 Assessment of biocompatibility, biointegration, barrier function and inducing bioactivity in vivo.

To study the complex tissue and cellular response of the recipient's body to experimental BM samples, a standard model of heterotopic (subcutaneous) implantation to rats (Wistar males, weighing 180-200 g, n=6 for each group of samples) was used in accordance with the requirements of ISO 109933-6-2021. The implantation of the samples was carried out with full interstitial contact for periods of 4, 8 and 13 weeks. All work with animals was carried out in accordance with the Regulations on Conducting Research on Experimental Animals (Order of the Ministry of Health of the Russian Federation No. 755 of August 12,

1997). The Protocol was approved by the Commission on Biological Safety and Ethics of the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences (05.03.2022, Protocol N26/2022).

2.6 Histological analysis

Fragments of materials before and after implantation were fixed in 4% buffered neutral formalin for 24 hours at room temperature [14]. The recorded samples were washed from excess phosphates and cut on a cryotome (MEV SLEE medical GmbH, Germany) with a thickness of 9 μm. To determine the composition and structure of the obtained materials, differential histochemical coloration for collagen by the Lilly method [15] and elastin by the Weyerhoff-Van Gieson method [15] were used. Micrographs of the stained preparations were obtained using the Nikon Eclipse Ti-E microscopic station (Nikon, Tokyo, Japan). The composition of the materials was evaluated using digitized images (n=5 areas from each slice) using non-commercial ImageJ software (https://imagej.nih.gov/ij /). The protein ratio was calculated as the area of the stained area (collagen/elastin) and expressed as a percentage relative to the total area of the cut. The data were presented as an average ± standard deviation. To characterize the cellular and tissue reaction of the body to the materials, in addition to the above-mentioned colors, an overview staining with hematoxylin-eosin and a combined Alcyan blue G8–PAS method for staining mast cells and glycosaminoglycans (GAG) were also used [15]. To obtain a general picture of the interaction of BM with the recipient's body, using the Nis Elements AR4.13.05 (Build933) software, overview histotopograms of histological preparations were obtained, including both the experimental materials themselves and the surrounding tissues of the host bed and healthy tissues of recipient-rats. The number of newly formed vessels was determined by direct counting in the field of view (n=5 areas from each histotopogram) and presented as an average ± standard deviation.

2.7 Statistical analysis

The results were averaged over 6 samples and the standard error was determined. The research results are presented as an average ± SEM, where SEM is the standard error of the average. All experiments were carried out in at least three repetitions ($n \geq 3$). The statistical significance of the difference was determined using ANOVA followed by multiple Holm-Sidak comparison for two comparison groups, $p \geq 0.05$.

3 Results and discussion

3.1 Characteristics of experimental barrier membranes

To determine the effectiveness of the methods of selective elastin extraction used and the characteristics of the composition of the experimental BM obtained, a histological analysis of the materials before and after implantation was carried out. It was shown that both methods used make it possible to obtain a high-purity elastin matrix (Fig. 1A). The heterogeneity of the control group BG materials structure was also revealed, although the membrane according to the passport was declared to consist of highly purified collagen, some fragments of the membrane contained elastin (up to 40% of the total area of the fragment).

Quantitative analysis of the DNA of the materials after processing confirmed that both methods of isolating the elastin matrix also effectively decellularized the materials (Fig.

1B). In group (2), the residual DNA of the donor was almost completely absent, as in the control group, in group (1) the amount of residual DNA did not exceed the minimum allowable value of 50 ng/mg of tissue.

However, an in vitro assessment of the cytotoxicity of the studied materials showed that Group 2 materials have a pronounced toxic effect on cells, expressed in almost 100% cell death after 96 hours of cultivation. This effect is apparently caused by insufficient deactivation of cyanogen bromide,. For materials of group BG and group (1), the cell viability index corresponded to the control values of the viability of cells seeded on culture plastic (Fig. 2C). The results of the cytotoxic test showed that the materials obtained using method (2) did not meet the basic safety requirements for medical materials, as a result of which group (2), despite the high degree of purity and uniformity of composition, was excluded from further research. The use of the cyanogen bromide method of elastin extraction to obtain elastin BM requires modifications of the existing protocol, primarily aimed at more effective inactivation of cytotoxic cyanogen bromide.

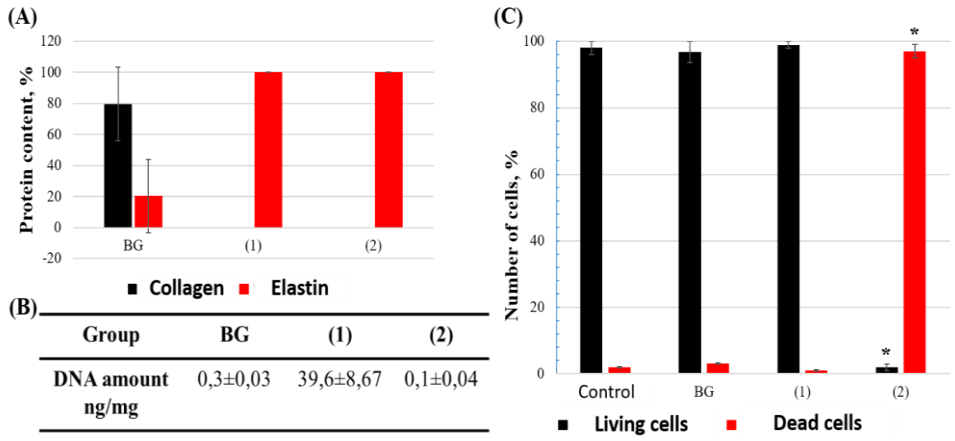

Fig. 1. Characteristics of experimental BM after treatment and before implantation. (A) The relative content of collagen and elastin in the materials after treatment; (B) The degree of decellularization of the materials after treatment, estimated by the degree of their purification from the residual DNA of the donor cells; (C) The cytotoxic effect of BM on NIH/3T3 cells after 96 hours of cultivation.

3.2 Results of evaluation of biointegration, barrier function and inducing bioactivity of experimental materials

In the early stages of implantation (4 weeks), signs of capsule involution, formation and partial structuring of granulation tissue were observed for all the studied materials. The absence of fibrous processes in the border areas was also observed, which corresponds to the normal physiological reaction of the body to the implanted material [16]. A moderate cellular reaction was also observed, which manifested itself in a uniform repopulation of the contact zones of the BM matrix without signs of leukocyte invasion.

The analysis of the biointegration and efficiency of the barrier function of the studied BM in dynamics after 8 and 13 weeks of implantation showed that at the intermediate period of 8 weeks, the control (BG) and experimental (1) groups retained the structural integrity of the matrix and were not subjected to transmembrane vascularization. The cellular reaction to the materials of group (1) in comparison with BG was characterized by a pronounced migration of mesenchymal cells. It is also important to note that in group (1), mast cells without signs of degranulation were found among the cells that migrated into the

thickness of the membrane matrix. It is known that mast cells, as resident connective tissue cells, play a key role in the development of a foreign body chronic reaction , as well as allergic reactions, regeneration processes, angiogenesis and immune protection [17]. According to the literature analysis, mast cells also participate in the processes of biomaterial resorption, however, these processes must be accompanied by degranulation and release of inracellular mediators and proteases [17]. No signs of degranulation were observed for mast cells found in the materials of group (1), while their number did not differ from that in normal healthy tissues of the subcutaneous space of rats that did not receive any materials, which indicates a pronounced degree of biointegration of the materials of this group.

After 13 weeks of implantation, the BG control group underwent intensive resorption, accompanied by pronounced histio-lymphocytic invasion, significantly exceeding those even for early implantation, which indicates the launch of a cell-mediated mechanism for the disposal of foreign material in the recipient's body. In addition to leukocytes, a small number of mast cells, including those with traces of degranulation, were also observed in the membrane matrix. The volume of BG membranes decreased significantly, while the collagen of the membrane matrix was almost completely resorbed.

Table 1. The number of mast cells in the BM matrix and in the surrounding tissues of the recipient

Group	Average number of mast cells per FOV (n=5)	
	Matrix	Recipient tissue
BG	8,8±3,8	47,6±7,7
(1)	40,2±3,6	

On the contrary, there were no signs of utilization resorption in group (1), while signs of structured neocollagenesis and pronounced remodeling processes were found. A small number of migrating leukocytes were observed in the border regions of the membrane, while the membrane thickness itself was densely populated with fibroblasts and a small number of mast cells without any signs of degranulation. Taking into account the correspondence of the number of mast cells in the matrix of materials and in the normal tissues of the recipient (Table 1), it can be concluded that the migration of mast cells in this case is an indicator of the full-fledged biointegration of the material and its settlement with resident connective tissue cells, that is, the implanted material for the cells of the subcutaneous space of the recipient was indistinguishable from its own tissues.

Table 2. The number of newly formed blood vessels in the matrix BM and in the surrounding tissues of the recipient

Group	Average number of vessels per FOV (n=5)	
	Matrix	Recipient tissue
BG	0,8±1,3	9,4±6,1
(1)	17,6±3,6	21,8±2,5

Analysis of the number of newly formed blood vessels showed that elastin BM from group (1) underwent transmembrane vascularization, while not interfering with the barrier function of the membrane. BM (1) also induced intensive contact neovascularization, characterized by the formation of a definitive vascular network in the surrounding tissues of the recipient (Table 2). The manifestation of such a powerful neoangiogenesis only at late implantation dates is apparently associated with the inducing effect of elastin peptides formed during the remodeling of the elastin matrix.

4 Conclusions

The conducted studies have shown that elastin BM, developed using the method of high-temperature extraction, have high biocompatibility and full-fledged biointegration. Like the control BM (BG), elastin membranes retained complete structural integrity up to 8 weeks of implantation and, unlike the control, were not resorbed even after 13 weeks of implantation, which indicates their ability to maintain medium-term or possibly longer barrier function. The ability to rapid biontegration and the pronounced chemoattractant effect of elastin BM for mesenchymal cells indicates the prospect of using these membranes in GTR technologies, primarily in the restoration of gingival flap deficiency. At the same time, the observed intensive induction of neovasculogenesis in the recipient's border tissues indicates that the obtained elastin membranes can also be useful in GBR technologies when used in combination with osteoplastic materials to provide intensive vascular nutrition of the graft implanted in the recipient's bone tissue.

The reported study was funded by RFBR according to the research project № 20-315-90101. Qualitative and quantitative analysis of the samples was carried out by facilities of the Center for Collective Employment of ITEB RAS.

The authors declare no conflict of interest.

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