COMPARISON OF THE BIOLOGICAL SCAFFOLDS DESIGNED FOR CELL GROWING

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Abstract. Fabrication of the scaffolds for tissue engineering has come a long way since they were came into use. However, even today there are very few products relevant for construction of artificial tissue. Going back, manufacturing of the scaffolds from biological tissue is the way which can resolve a lot of technical, biocompatibility and immunological problems.

The aim of this study was to compare different biological xenogenic extracellular matrix (ECM) relevance for cell growing as well as for xenograft construction, implantation and biocompatibility in the organism.

In our experiments, samples of rabbit's blood vessel, diaphragm, esophagus and intestine were used. All biological tissues were decellularized, the efficiency of host-cell exclusion was proved by scanning electron microscope. In this way, ECM for foreign cell growing was prepared. For artificial tissue fabrication, primary endothelial stem cell line was derived from Wistar rat's pulmonary trunk. Rat cell growing on the rabbits'-origin ECM was verified using MTT test and microscopic analysis. Therefore, artificial tissue was constructed from rabbit's ECM and rat's endothelial cells. Such tissue as implant was inserted under the rat skin to evaluate their biocompatibility. After 2 and 4 weeks, histological examination of the implant and surrounding tissues confirmed biocompatibility of fabricated tissue as well as implanted cell spreading in the host tissue.

According to our studies, the investigated xenogenic ECM were different in structure, but they all were relevant for the cell growing. However, in our experiments, rabbit's blood vessel-nature ECM was the most suitable for rat's endothelial cell growing. After implantation, xenografts with the autologous stem cells were adopted by the organism well.

Keywords: extracellular matrix (ECM), biological matrix, stem cells, tissue engineering

Introduction. Nowadays, implants prepared from biological tissue as ECM are much investigated, some commercially available products already are used in medicine and odontology (Badylak, 2004; Shea, 2010). It is known that ECM is a noncellular part of the tissue composed from the structural proteins (Chaplin et al., 1999). In the organism, ECM provide initial support to the cells, localize them in the appropriate spaces, provide physical and biological cues for adhesion, migration, proliferation and differentiation, and assemble the propagated cells and secreted matrices into functional tissues and organs (Hoshiba et al., 2010). Moreover, the ECM serves not only as a scaffold or supporting material; it is functions also as the regulator of cellular fate: survival, proliferation, differentiation and morphogenesis (Adachi et al., 1997, Giancotti and Ruoslahti, 1999).

Commonly, ECM implants are introduced into the organism without the cells (Teebken et al., 2000). It is probable that cells going to migrate from the recipient's tissue to the implant (Cornwell et al., 2009). Such strategy of tissue engineering relies on the remodeling of the tissue *in vivo* to approach functionality with time; however the tissue engineered from scaffold with the cells must function faster after the implantation (Ratcliffer, 2000). There are quite a lot different investigations, which are trying to create artificial tissue sheet using natural allogeneic or xenogenic matrix and autologous stem cells (L'Heureux et al., 1998; Niklason et al., 1999; Teebken et al., 2000). Scientists are trying to define optimal conditions to attach cells to the matrix and for the matrix to integrate into the organism (Ptaszek et al., 2012). All such investigations are not terminative, because the problem is not solved; there are a lot of questions which are waiting for solution. One of them is to find the most suitable matrix for specific kind of cells to grow. Talking of biomaterials intended for implantation, it is essential to choose such scaffold which acts as a support for cells and promote cell growth, proliferation or differentiation (Han and Liu, 1999; Hoode, 2002).

Although there are a lot of different experiments related with the search for proper ECM and implant construction *in vitro* or *in vivo,* we didn't find any studies where various biological ECM as scaffolds for cell growing are compared with each other. There are studies related with blood vessels (Teebken et al., 2000) or guts, specifically small intestine submucosa layer (Hoshiba et al., 2010). However esophageal or diaphragm matrices are not so popular (Ozeki et al., 2006). Also experiments are different in decellularization methods, upgrowing cells type, experimental animal species. Such a variety of research is because it is necessary to find the most reliable conditions for specific artificial tissue fabrication and

implant construction.

The aim of this study was to compare different biological ECM, their suitability for cell growing as well as for xenograft construction, implantation and biocompatibility in the organism. To achieve the aim, the decellutarized tissue samples were prepared, their micromorphology and relevance for cell growth was evaluated, the tissue was constructed *de novo* and implanted *in vivo*. According to our studies, all the investigated implants were different in structure, they all were relevant for the cell growing as well. However, rabbit's blood vessel – natural ECM – was the most suitable for rat's endothelial cell growing.

Materials and methods. *Animals:* ECM samples were prepared from organs of the euthanized laboratory rabbit; primary endothelial stem cell line was derived from Wistar rat; fabricated artificial tissues were implantated into the Wistar rats. The animals were obtained from the vivarium of the Institute of Biochemistry, Vilnius University. The license for the stem cell research and artificial tissue fabrication was received from Lithuanian State Food and Veterinary Service, 2011-02-12, No 0212. All procedures were performed in accordance with the Order of Director of the State Food and Veterinary service of the Republic of Lithuania, No. B1-866, 31.10.2012.

Tissues for ECM. Diaphragm, esophagus, blood vessel, intestine samples were obtained from the euthanized rabbit. The tissues were transported in the physiological saline with antibiotics (penicillin – 200 U/mL, streptomycin - 200 µg/mL; Invitrogen/Life Technologies, USA).

Decellularization. The samples of diaphragm, esophagus, blood vessel and guts were frozen at -80 °C for 15 min. in the separate tubes without any liquid medium. Later, the samples were thawed at +37 °C for 15 min. These operations were repeated for three times. Following temperature shock, we used enzymatic processing: the tissue samples were moved into the laboratory flasks with trypsin (0. 25 %) and antibiotics. The vessels were put under permanent shaking for 8 h at 30 °C. Trypsin was replaced every hour. For finishing of all the process, decellularized scaffolds were washed with a saline and left in a fridge at +4 °C for 24 h. Before cell seeding, for the sterility assurance the scaffolds were put under UV lamps (distance 20 cm), each side for 5 min.

ECM preparation for scanning electron microscopy (SEM). Hitachi TM-1000 microscope (Hitachi High-Technologies Europe GmbH) was used to analyze the ECM morphology and to confirm the success of decellularization. For the dehydration, the samples were incubated in aqueous glutaric aldehyde (2.5%; Sigma-Aldrich Co. LLC) for 60 min., then washed three times for 10 min. in distilled water. After that, the samples were immersed (for 5 min.) into the increasing concentrations of ethanol (25, 50, 75 and 96 %).

Stem cell culture. Primary endothelial stem cell line was derived from the just euthanized rat's pulmonary trunk endothelium. Briefly: under aseptic conditions, pieces of the vessel were sliced, then minced. The minced tissue was exposed to digestive solution containing 0.125 % trypsin–EDTA (Life Technologies, USA), 1 mg/ml collagenase type V (Sigma-Aldrich Inc., USA) and 0.3 mg/ml hyaluronidase (Sigma-Aldrich Inc., USA) in phosphate buffered saline (PBS). The cell mass was incubated for 15 min at 37 °C in a shaker bath, then washed with Iscove's modified Dulbecco medium (IDMEM; Sigma-Aldrich Inc., USA) supplemented with 10 % fetal calf serum (FCS; Invitrogen/Life Technologies, USA). After the cells were counted, their viability was evaluated by a trypan blue (0.4 % trypan blue in PBS) exclusion test and the cells were dispersed in IDME medium containing FCS (10 %) and antibiotics (penicillin – 100 U/mL, streptomycin - 100 µg/mL). After 1-2 weeks, primary stem cell culture was obtained and following 3–5 passages primary cell line was derived. The cells were passaged twice a week applying trypsin and EDTA mixture. In the experiments, the cells were used from passages 10 to 15.

Study of cell proliferation on the decellularized xenogeneic scaffolds. For the evaluation of cell growth intensity MTT method was used (Mosmann, 1983). To this end, it is necessary to possess an equal area for cell growing. When we are talking about biological scaffolds it is quite difficult to make it equal in size. For the standardization of the area we were preparing special frames from the odontological pre – impression dental mass "Vitasil Putty A" (UAB "Medavita"). Briefly: the biological scaffolds were put down on the polystyrene plate well bottoms, then the frames with equal size holes were lied down, and only then the wells were filled with stem cell suspension (10^5 cells/mL) (Fig. 1).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method is based on the viable cell's mitochondria's enzymes abilities to change MTT reagent's yellow color to violet which was measured with spectrophotometer (Thermo Fisher Scientific Inc., USA) at 570 nm. For this test, solution of MTT (0.2 mg/ml; Life technologies, USA) was prepared in the phosphate buffered saline (Sigma-Aldrich Co. LLC) and added (2 ml) into the wells with cells grown on the tested scaffolds. After 1 h of incubation, MTT solution was removed, and the precipitate solubilized in 96 % ethanol, violet color intensity was determined by measuring optical density. Optical density of the solution is proportional to the amount of viable cells. In our study, number of viable cells which corresponds to the cell proliferation activity was assessed at 96 h of the cell growing on the tested scaffolds.

Imaging of the cells. For the construction of xenoimplant, before seeding, stem cells were stained with DNA specific blue fluorescent dye 4,6-diamidin-2-fenilindol (DAPI; 2 mg/ml in PBS; Thermo Fisher Scientific Inc.) for 12 h. Then the cells were washed in growth medium and seeded on the tested biological ECM. After 96 h maintenance, the samples were visualized with laser scanning confocal microscope Nikon C1 (Nikon Corporation, USA).

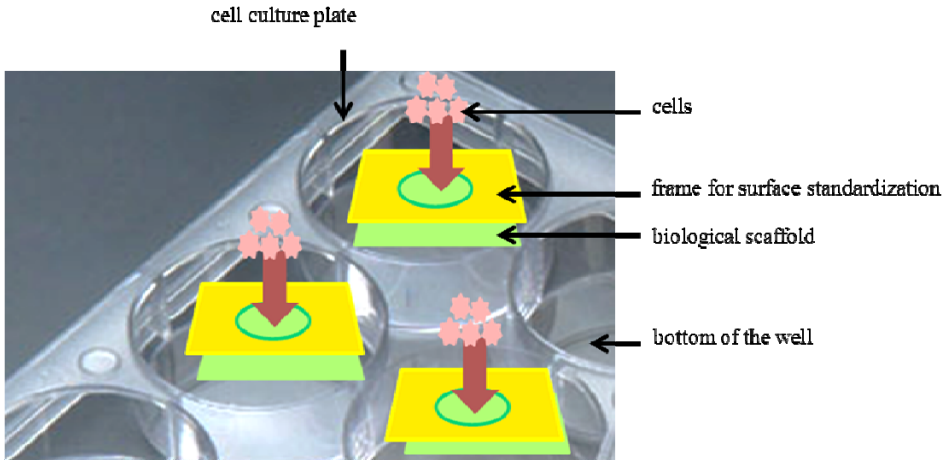

Fig.1. **Equalization of the biological scaffold area for the MTT studies**

Implantation of the xenograft. Stained primary rat endothelial stem cells grown on the rabbit's decellularized scaffolds were implantated under the rats' skin in the dorsal area. After insertion of the xenograft, the skin was closed with 2–3 sutures by using 5–0 polydioxanone by simple interupted suture. All surgical procedures were performed under aseptic conditions. During the operation, animals were anesthetized with a combination of ketamine hydrochloride (70 mg/kg; Vétoquinol, France) and xylazine (7 mg/kg; Alfasan International BV, Netherlands) via intraperitoneal injection (Struck et al., 2011). Postoperative pain was released using Buprenorphine (0.05 mg/kg; Temgesic Essex Chemie AG Lucerne, Switzerland) which was injected subcutaneously for a week (Curtin et al., 2009).

Evaluation of the xenograft biocompatibility. At the end of experiment, rat tissue examination *ex vivo* was performed to detect implanted cell migration and tissue integrity *in vivo.* For this purpose, rats were divided in 2 groups: in group 1 – the implants were left for 2 weeks, in group 2 - for 4 weeks. After the provided time, the animals were euthanized and xenografts with adjacent tissue were removed. Without delay, the examples of tissue were sliced using cryotome (Cryotome™ FSE Cryostats; Thermo Scientific, USA). The prepared 8 µm thickness frozen sections immediately were analyzed by inverted fluorescence microscope Olympus IX51 (Olympus corporation, Japan).

Statistical analysis. Data are expressed as a mean of three independent experiment ± SD. Statistical analysis was performed using Student t-test. Differences are considered statistically significant at $P < 0.05$.

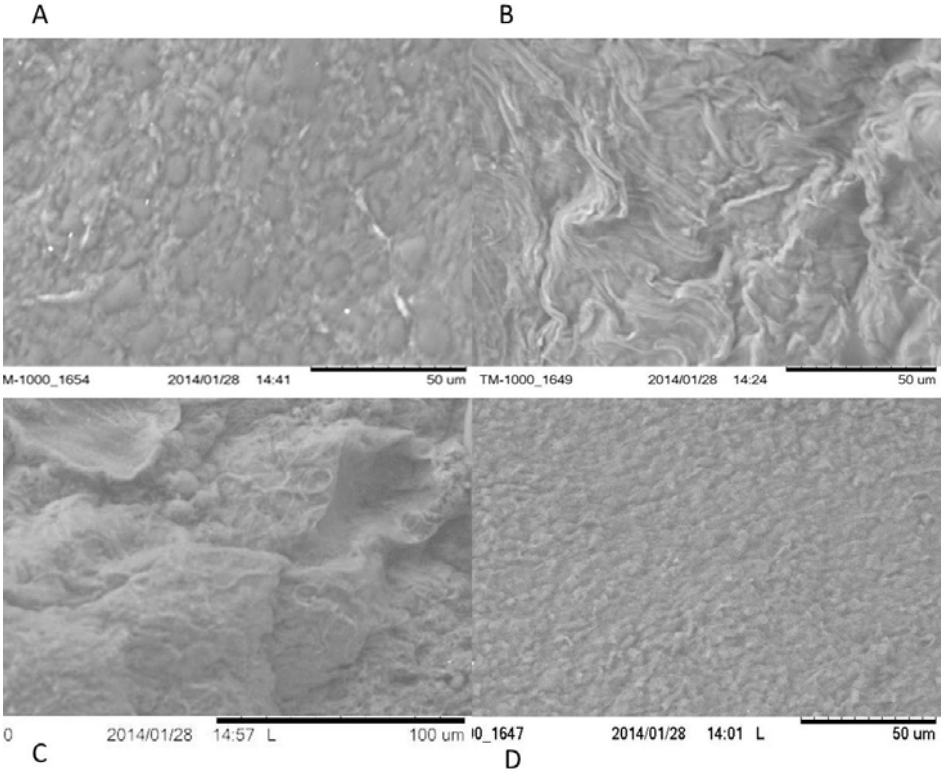

Fig. 2. **ECM of various rabbit's tissues taken on an SEM: A – blood vessel; B – diaphragm; C – esophagus; D – gut**

Results. As the aim of this study was to compare different biological ECM designed for artificial tissue construction, the first step was to prepare natural – origin ECM which would be used as scaffold for endothelial cell growth. There are described a lot of different decellularization methods in the scientific literature, some of them are mechanical, other chemical or enzymatic (Herbikova et al, 2013; Gilbert et all, 2006; Jackson et al., 1991; McFetridge et al., 2004). Our purpose was to choose a relatively simple, fast and effective way to prepare nature-origin scaffold. Therefore, we have combined repeated temperature shock and enzymatic processing. The efficiency of decellularization was examined using scanning electron microscope (SEM). The results showed that after the chosen method there were no more cells on the scaffold. As well, all the structures were highlighted; it was possible to detect the differences in the extracellular matrix of various tissues (Fig. 2). Therefore, the effect confirmed that the combined method of decellularization was efficient enough.

Subsequently, on the prepared rabbit-origin scaffolds rat-origin cells were grown. The primary cell line was derived from the rat pulmonary trunk endothelium. It is a monolayer cell culture; it means that the cells are growing side by side as single, closely packed layer (Fig. 3).

Rat's endothelial cell growth intensity was evaluated by MTT method. The results showed that the cells attached and proliferated on the all tested scaffolds. Their relevancy comparison with each other was not efficient. As the best growth was identified on the blood vessel-origin scaffold, the worst – on the diaphragm, thought the differences were not reliable (Fig. 4, A). Not strict certainty could result because of imperfection of our engineered frame which did not completely limit cell contact with the flexible nature-derived scaffold. It is likely that some of the cells were able to go under the frame. In our tests, the more effective cell proliferation on the decellularizated blood vessel could lead to better mechanical scaffold stretching on the bottom of plate under the frame.

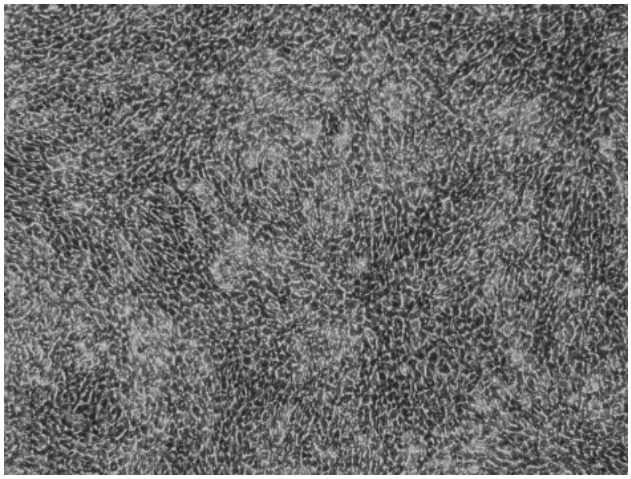

Fig. 3. **A monolayer of rats' endothelial cells on the control polystyrene surface *in vitro*** (10x10)

The results obtained using MTT test were approved using confocal microscope (Fig. 4, B). In the microscopic image of DAPI stained cells on the decellularizated blood vessel, we can see not only the alive blue shining cells, but their localization according to the predictable filaments also.

According to our data, rat cells easily grew on all the studied rabbit-derived ECM. It means that our tested nature-derived decellularizated tissues could be used as scaffolds for artificial tissue construction.

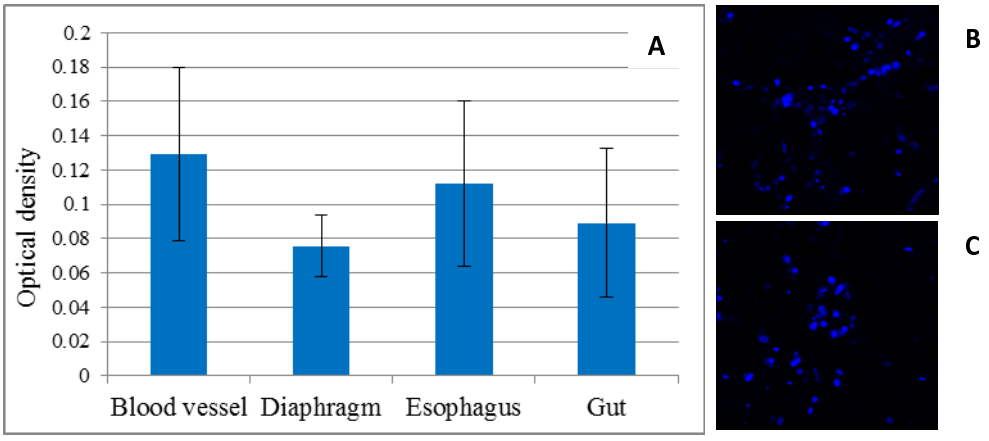

Fig. 4. **Rat's endothelial cell growth on rabbit's decellularizated tissue: A – results of cell proliferation using MTT test, where optical density (OD) is directly proportional to the amount of alive cells;** data are expressed as a mean of three independent experiment ± SD; **B, C – images taken by confocal microscope: DAPI stained (glowing in blue) cells on the blood vessel (B) and diaphragm (C) -derived decellularizated scaffolds.**

For further testing, artificial tissues from decellularizated rabbit diaphragm or blood vessel and DAPI stained rat cells were constructed. Such constructs were implanted under the rat's skin (Fig. 5). Cell viability, their spreading and tissue integrity was studied after 2 and 4 weeks.

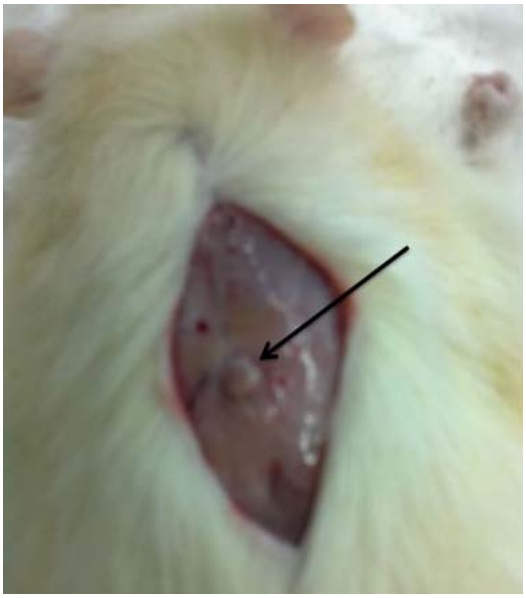

Fig. 5 **Artificial tissue constructed from rabbit's extracellular matrix and rat cells implanted under the rat's skin: an arrow marks the place of the implant (*post mortem*)**

Blood vessel-based engineered implants were removed after two weeks. At necropsy, the construct under the skin looked like without any inflammation, well adapted, with new small blood vessels, surrounded by the roundabout subcutaneous tissues. Diaphragm-based implants were removed after four weeks. Also there was no inflammation under the skin New blood vessels formation was more advanced than after two weeks in a previous trial. This time, it was more difficult to extract the implant *ex vivo*, because it was really overgrowth with a surrounded subcutaneous tissue and was much more integrated into the tissue than the retained for two weeks.

At the end of the experiment, rat tissue microscopic examination *ex vivo* was performed to detect implanted cell migration and tissue integrity *in vivo*. In both cases, micro images taken with fluorescence microscope demonstrate widely spread implanted (blue, DAPI stained) cells in surrounding tissues (Fig. 6, A and C). According to light microscope images, there were not observed any irregularities of tissue integrity (Fig. 6, B and D). Therefore, our experiments show that both scaffolds are relevant for cell growing and implantation *in vivo* or for the construction and usage of artificial tissues.

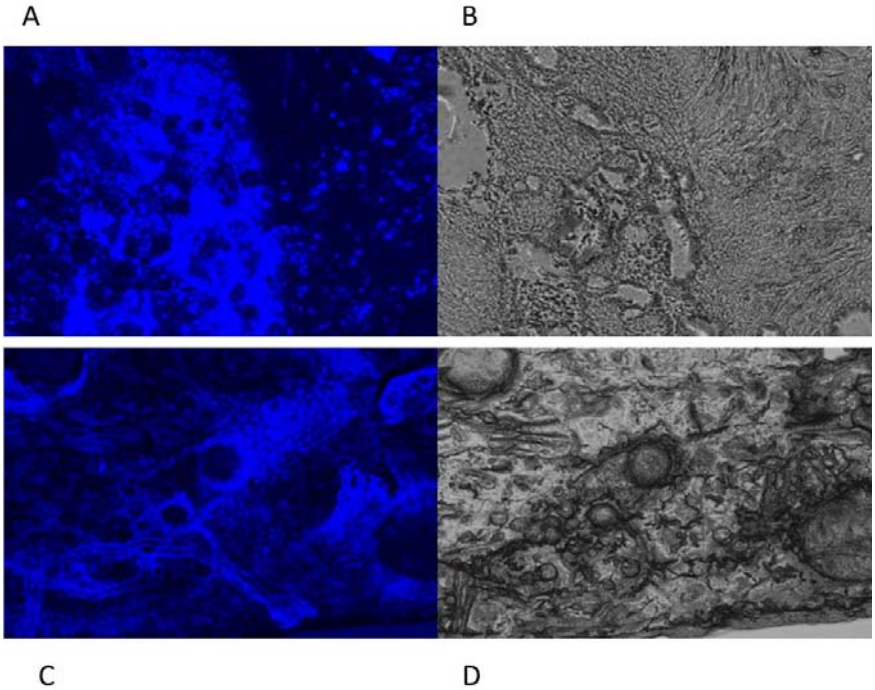

Fig. 6. **Micro images of the implant and surrounding tissues taken by light and fluorescence microscopes: A – slice of the tissue with blood vessel-based construct and a blue shining DAPI stained cells; B – the same image taken with light microscope; C – slice of the tissue with diaphragm-based construct and a blue DAPI stained cells; D – the same image taken with light microscope.**

Discussion. Native ECM used for artificial tissue construction and xenograft implantation represent a very desirable object and are a widely discussed question (Estrada et al, 2012; Jones et al., 2014). The main problem in the preparing of nature-derived scaffolds is a decellularization process which must eliminate cells from the scaffold without destroying its natural architecture. It is considered that for complete reduction of the antigens, totally cell-free samples should be prepared (O'Brian et al., 1999; Goldstein et al., 2000; Da Costa et al., 2004). There are a lot of different decellularization protocols which are not equal in their efficiency and influence for the overgrow process. One of the most investigated is a treatment with trypsin. As Rieder et al. (2004) has noted, this method is one of the most suitable for further cell attachment, however after the processing xenogenic cells

were still visible within the matrix. These remaining cells can serve as unwanted antigens during the implantation of constructed tissue into the organism. In our study, we applied a combination of a physical/enzymatic decellularization protocol which was used in other studies (Crapo et al., 2011). According to our SEM micrographs we can conclude that there were no host cells on the extracellular matrix, as well, this technique did not damage native structure of the scaffold. It is likely that such features of structure result in differences of cell proliferation on the xenogenic scaffolds. MTT test used for the assessments of endothelial cell proliferation showed that the studied different-origin scaffolds were friendly for the foreign-origin stem cells. The results have demonstrated the biggest endothelial cell growth efficiency on the blood-vessel's origin matrix. Confocal microscope analysis indicated that the cells were positioned according to the filaments of the scaffold.

One of the most important properties of the artificial tissue implant is cell proliferation and capability to regenerate the recipient's tissue. Nowadays, none of currently used unnatural scaffolds applied for implant construction has all that. As a result, researchers are developing new innovative materials or trying to adapt the natural resources. Using the methods of tissue engineering, it is possible to create autologous live structure with necessary properties and longer durability from biological material. There are two different ways to create the implants. The first of them is trying to accumulate cells on the decellularized scaffold *in vivo* (Jones et al., 2014; Schaner et al., 2004). Other researchers presume that it is more effective to create an implant *in vitro* and then to implant it (Ratcliffe, 2000). The main difference between them is as follows: remodeling of the tissue *in vivo* creates its functionality with time, and the tissue engineered *in vitro* starts to function immediately after implantation. Both of them require knowledge of the properties of natural-derived scaffolds and their relevance for a particular purpose.

Conclusions.

The investigated native ECM implants were different in structure, but they all were relevant for the foreign cell growing. Although the rabbit blood vessel-nature ECM turned to be the most appropriate for rat endothelial cell growing, the differences between the biological scaffolds were not reliable.

Xenograft constructed from native ECM with the autologous cells is adopted by the organism very well.

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