DOI: 10.15825/1995-1191-2023-1-113-122

BIOLOGICAL AND FUNCTIONAL PROPERTIES OF HUMAN UMBILICAL CORD-DERIVED LYOPHILIZED TISSUE-ENGINEERED MATRICES

A.A. Kondratenko, L.I. Kalyuzhnaya, D.V. Tovpeko, V.S. Sheveleva, R.I. Glushakov
Kirov Military Medical Academy, St. Petersburg, Russian Federation

The use of tissue-engineered products (TEP) from decellularized extracellular matrix (dECM) to treat deep skin lesions is a tissue engineering method that promotes regenerative healing. Cell-free preparations reproduce the hierarchical complexity of tissues, mimic structural, biochemical and mechanical signals that are necessary to attract cells, and are a source of bioactive molecules. The human umbilical cord biomaterial has a fetal phenotype with extra-embryonic origin, and therefore is available and has no ethical limitations in its use. The tissue engineering laboratory at Kirov Military Medical Academy developed and patented a TEP from the highly regenerative human umbilical cord in the form of matrix and hydrogel matrix. To study its regenerative potential, lyophilisates of tissue-engineered solid-state and hydrogel matrices were implanted around mini pig full-thickness wounds in vivo. The external signs of inflammatory response and the histological images of biopsy specimens from the lyophilizate implantation areas were analyzed. The effect of nutrient media, "conditioned" with lyophilizates of both matrices, on the viability and migration activity of fibroblast-like cells, isolated from mini pig skin, was investigated. The matrix lyophilisates showed good biocompatibility and bioactivity in in vitro and in vivo experiments. Implantation of the samples promoted faster formation of mature epidermis compared to the control.

Keywords: tissue engineering, solid-state matrix, hydrogel matrix, human umbilical cord, full-thickness wound.

INTRODUCTION

Skin wound healing is a process of morphogenetic response to injury aimed at restoring the anatomical integrity and physiological function of the injured area [1]. Early and effective epithelialization of the wound defect with mature neoepithelium is required to restore the protective function of the skin [2]. The strategy of applying tissue-engineered products (TEP) from decellularized extracellular matrix (ECM) allows not only to accelerate granulation tissue formation by mechanical filling of the defect, but also promotes healing. The component composition, porosity and biodegradability of TEP serve as factors of biointegration and modulation of the local immune response. At the same time, the characteristics of the initial biomaterial for TEP fabrication, the procedure of cellular material removal and the final consumer form of the product affect its biological effectiveness [3]. The limitations of autografts, allogeneic and xenogeneic materials encourage researchers to look for alternative biomaterials [4].

The unique composition, homologous extra-embryonic origin, availability without invasive procedures and ethical restrictions make the human umbilical cord an attractive biomaterial for creation of highly regenerative TEPs. TEP (which can be manufactured in various forms) from a highly regenerative human umbilical cord was developed and patented in the tissue engineering laboratory of Kirov Military Medical Academy.

Objective: to evaluate the effect of the developed lyophilized TEPs (tissue-engineered solid-state and hydrogel matrices) from highly regenerative human umbilical cord biomaterial on skin cells in vitro and healing of full skin wounds of mini pig.

MATERIALS AND METHODS

Fabrication of cell-free tissue-engineered products from human umbilical cord

Human umbilical cords were obtained from healthy full-term newborns after spontaneous delivery with informed consent of the mothers and using guidelines approved by the Ethical Committee at Kirov Military Medical Academy, Protocol #203 (St. Petersburg, Russia).

To obtain a tissue-engineered ***solid-state matrix***, umbilical cord vessels were carefully removed under sterile conditions. Wharton's jelly was crushed with a blender (Bosch, Germany) and homogenized (gentle MACSTM Dissociator Milteniy Biotech, Germany), h-cord-01-01 program. Cells were removed using 0.05% sodium dodecyl sulfate solution (Biolot, Russia) for 24 hours at room temperature in a shaker at 140 rpm (Biosan, Latvia). Sodium dodecyl sulfate was removed by washing with phosphate-buffered saline (Biolot, Russia).

Corresponding author: Albina Kondratenko. Address: 6, Akademika Lebedeva str., St. Petersburg, 194044, Russian Federation. Phone: (812) 292-32-63. E-mail: kondraa24@gmail.com

To make the ***tissue-engineered hydrogel matrix***, 10 mg of dry tissue-engineered matrix was solubilized with pepsin solution (P/1120/46 Thermo Fisher Scientific, Germany) at the rate of 1 mg of enzyme in 1 ml 0.01 N HCl, pH 2.0, for 72 hours at room temperature and 180 rpm shaking. Pepsin was neutralized with 0.1 n NaOH solution to pH 7.4.

The resulting tissue-engineered solid-state and hydrogel matrices were lyophilized (ZirbusVaCo5II, Germany), sterilized by ultraviolet light in a LAMSYSTEMS microbiological safety box (Russia) with a UV-C radiation flux of 12 W for 15 minutes, and stored hermetically sealed at a temperature of –20 °C for a year.

Study of the dynamics of healing of full-thickness wounds in a mini pig

All manipulations with the animal were carried out in accordance with the ethical principles approved by the Ethics Committee at Kirov Military Medical Academy, Protocol No. 263 (St. Petersburg, Russia) and established by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [6]. A male mini pig No. 43-40 weighing 22.5 kg at the beginning of the experiment was obtained from the nursery of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences.

Zoletil 100 (Virbac, France, 10 mg/kg) and Xylazine (Xyla, Estonia, 0.1 ml/kg) were used intramuscularly for anesthesia. After careful removal of the hair on the back and treatment of the skin with 70% ethyl alcohol solution, DMP08 8-mm dermopunch (Sterylab, Italy) was applied five times at 7-day intervals in two full-thickness skin defects, 1 cm deep. 0.01 g of tissue-engineered solid matrix lyophilisate was placed into the experimental wounds. Observation of animal behavior, local skin reactions were recorded daily throughout the experiment, photographing the wounds with a scale bar in the calibration frame.

After 35 days, biopsies were taken from all experimental and control wounds. Then, two weeks later, the experiment was repeated, but 0.01 g of tissue-engineered hydrogel matrix lyophilisate was introduced into the wounds. Biopsy material was extracted, and the wounds were photographed on similar dates. Biopsy material was fixed in neutral formalin for 24 hours, embedded in paraffin, and histological sections were prepared and stained using Van Gieson's and Heidenhain's stains (Biovitrum, Russia) according to the manufacturer's instructions. The preparations were analyzed using an Axio Imager 2M microscope (Carl Zeiss, Germany).

Histological assessment of acute inflammation (HAAI) was performed at 1 and 2 weeks on a scale of 0 to 15. The following inflammation criteria were assessed: amount of neutrophilic cell infiltration in the dermis (0–3 points); amount of neutrophilic cell infiltration in the hypodermis (0–3 points); edema (0–3 points); bleeding (0–3 points); necrosis (0–3 points). The criteria used to determine the number of cellular infiltrates were as follows: 0, within normal histological limits; 1, scattered; 2, grouped or knotty; 3, diffuse. Histological assessment of edema: 0, no; 1, focal; 2, local; 3, diffuse. Degree of hemorrhage: 0, no; 1, soft; 2, moderate; 3, severe. Volume of necrosis: 0, no; 1, focal; 2, nodal/regional; 3, diffuse [2].

Quantitative data were calculated using ImageJ software. The relative area of the wound defect, the relative area of the vascular bed, the average vessel diameter, and epithelial thickness were determined. To calculate each morphometric index, 5 visual fields in each slice were examined, the total number of measurements n = 10.

Extraction and scaling of fibroblast-like mini pig skin cells

Skin fragments isolated for the production of full-thickness wounds, after removal of the fat layer, were washed three times with saline solution with antibiotics (penicillin, streptomycin for cell cultures, Biolot, Russia). Next, skin fragments were chopped with sterile scissors, placed in 25 cm^2 culture vials (Thermo scientific, USA), and 5 ml of nutrient medium was added. Culture medium DMEM (Biolot, Russia), 10% fetal calf serum (HyClone, USA), gentamicin for cell cultures 100 IU/ml (Biolot, Russia). After obtaining the primary mixed population of fibroblast-like cells, they were subcultured every 7 days at a 1 : 5 dilution in 25 cm^2 culture vials. Cellular material was detached from plastic by 0.02% trypsin-Versene solution (Biolot, Russia) in a 1 : 3 ratio at a temperature 37 °C for 5–7 minutes. After trypsin inactivation with nutrient medium and fetal bovine serum, cell material was precipitated by centrifugation at 3500 rpm for 5 minutes. The cell material was washed twice with nutrient medium under the same conditions. After the supernatant was removed, the precipitate was resuspended, and the cells were counted in a Goryaev chamber according to the conventional procedure.

Evaluation of the effect of lyophilized TEPs on the metabolic activity of mini pig skin cells

Fibroblast-like mini pig skin cells of the 4th passage were cultured in 96-well plates (Sigma-Aldrich, USA), 150 µl at a concentration of 13,000 cells per well. To study the effect of lyophilisates of both matrices on cell viability, conditioned nutrient media obtained by preincubation in the nutrient medium of the above composition of the studied TEP samples (1 mg/ml 24 hours at 4 °C to maintain sterility) were used. The study of cytotoxicity is mainly aimed at studying the effect of soluble substances contained in TEP, including sodium

dodecyl sulfate used for decellularization. The choice of 1 mg/ml concentration was guided by the intended amount and method of clinical application of TEP.

A day after cell adhesion to the surface of the culture plate, the initial medium was replaced with a conditioned medium and the control wells with a standard culture medium (n = 32). The plate was incubated for another 24 hours at 37 °C in 5% CO_2. Then, resazurin dye solution (Biocompass-S, Russia) was added to each well with 11.2 μM/ml as final concentration in the well in a volume equal to 10% of the total volume of culture liquid. Resazurin dye was dissolved in phosphate-buffered saline (PBS), pH 7.4 (Biolot, Russia) before adding it to the well. After 4 hours of incubation and shaking (2 min, 37 °C) on an orbital shaker (Biosan, Latvia), fluorescence levels were measured on a Victor X5 microplate reader (Perkin Elmer, USA) at 590 nm wavelength using an excitation wavelength of 560 nm. The results were expressed as a percentage relative to the control.

Evaluation of the migration activity of mini pig skin cells

Fibroblast-like mini pig skin cells of the 4th passage were cultured in 12-well plates (Sigma-Aldrich, USA) by 500 μl in concentrations of 45,000 cells per well. To study the effect of matrix lyophilisate samples on cell proliferative activity, after reaching 80–90% confluence, a "scratch" was applied with a sterile pipette tip to damage the cell monolayer. Then, 0.5 mg of lyophilized TEPs were placed into the experimental wells and cultivation was continued (n = 4). A day later, calcein AM dye solution (Lenreaktiv, Russia) at 1 : 3000 concentration in PBS (Biolot, Russia) was added to each well and incubated for 30 minutes in a CO_2 incubator. After washing twice with PBS, images were registered using a Zeiss LSM-880 microscope (Carl Zeiss, Germany) at a 494 nm wavelength and 517 nm registration. The relative residual defect area of the cell monolayer was determined.

Statistical processing of quantitative data was performed using nonparametric Mann–Whitney U test. Differences were considered significant at $p < 0.05$ (Statistics 7.0). Quantitative data were presented as median, 25% and 75% quartiles (Me, Q1; Q3).

RESULTS

Human umbilical cord-derived lyophilized tissue-engineered solid-state matrix (Fig. 1, a) is a ready-to-use porous drug formulation. The matrix does not contain cell nuclei of the original biomaterial, consists of collagen, retains glycosaminoglycans (GAGs) and essential basement membrane components. After decellularization of Wharton's jelly, collagen fibers retain their structural three-dimensional organization [7, 8]. Tissue-engineered hydrogel matrix is a product of matrix enzymatic treatment (by pepsin) and contains peptides, GAGs and free growth factors, previously fixed in tissue-engineered matrix structures. The hydrogel matrix has the ability to polymerize and gel under physiological conditions in vivo and in vitro. Tissue-engineered hydrogel matrix is an injectable dosage form that has shown efficacy in healing deep simulated synovial intra-articular cartilage defects [9]. However, lyophilized hydrogel matrix for the treatment of deep skin lesions seems to be the most convenient. Lyophilized hydrogel matrix can be used without the need for pre-processing. The dry form is very hygroscopic and actively absorbs wound exudate (unpublished data). If necessary, the product can be rehydrated into an injectable hydrogel matrix. Lyophilized tissue-engineered hydrogel matrix, similar to lyophilized tissue-engineered solid-state matrix, has a heterosporous structure (Fig. 1, b).

Implantation of both lyophilizate samples did not result in external signs of inflammatory response, such as edema, hyperemia, and temperature rise at the implantation site. As healing progressed, the wound areas gradually decreased. At week 3, the wound area in the control was 60.74% of the original defect area; the wound containing solid-state matrix lyophilizate was

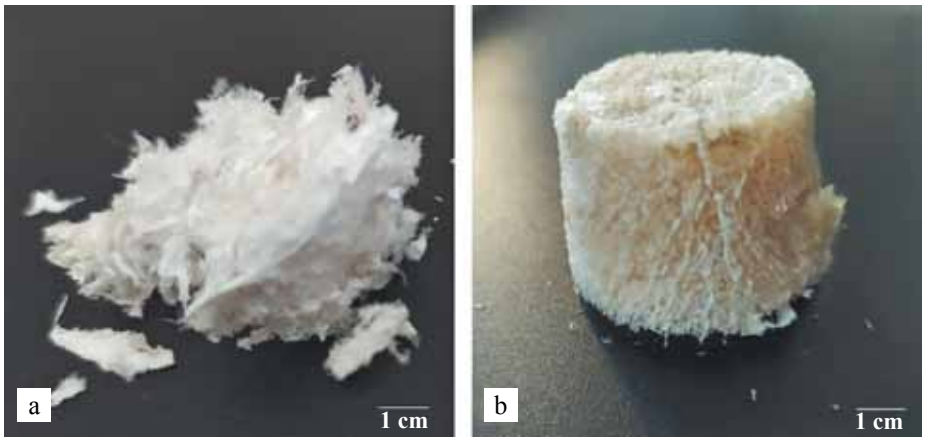

Fig. 1. Lyophilized tissue-engineered solid-state (a) and hydrogel (b) matrices

already 58.89% and the wound treated with hydrogel matrix lyophilizate was 55.02%. At week 4 and 5, planimetry results were as follows, respectively: 53.98% and 48.05% in the control; 51.08% and 30.34% in the presence of solid-state matrix lyophilizate; 50.27% and 42.69% when treated with hydrogel matrix lyophilizate.

Analysis of histological preparations at week 1 and 2 showed comparable values of the HAAI index in wounds with lyophilized TEPs and in the control. At day 7 after wound infliction, the HAAI index was 7.5 (7.0; 9.0) in the control, 7.0 (6.0; 8.0) in specimens containing tissue-engineered matrix and 7.0 (6.0; 8.0) in the tissue-engineered hydrogel matrix. The difference was not statistically significant. At week 2, the values were 6.0 (6.0; 6.0) with the control, 6.0 (6.0; 7.0) with the tissue-engineered matrix, and 6.0 (5.0; 7.0) with the hydrogel matrix. The difference was also not statistically significant. The results indicate that placing TEP (both solid and hydrogel forms) into the wounds did not cause an excessive inflammatory response, but at the same time did not inhibit the physiological process of inflammation.

Intense filling of the defect space with granulation tissue was observed in the experimental and control wounds already at week 1 (Fig. 2).

The density of the vascular bed in the tissues surrounding the wounds with lyophilized TEPs was statistically significantly higher than that in the control. The specific vascular area in the tissues surrounding the wounds at week 1 was 3.65% (3.65%; 3.77%), 5.63% (5.12%; 5.84%), and 4.76% (4.50%; 5.02%) in the control and experimental (lyophilized samples), respectively ($p = 0.009$ for solid-state matrix and $p = 0.028$ for hydrogel matrix compared to control). The mean diameter of vessels in the tissue surrounding the wound was 14.88 (13.92; 15.88), 10.40 (10.03; 10.64), and 10.88 (9.30; 11.57) μm in control and experimental samples, respectively ($p = 0.008$; $p = 0.007$). Specific area of granulation tissue vessels was not statistically significantly different in the control and experimental samples. As the follow-up period increased, the changes in these indicators were not statistically significant.

The most notable differences were observed in the formation of the epithelium when lyophilized TEPs were used. At week 1, epithelialization of the control wound (in contrast to the experiments) did not occur completely, with the thickness of the forming epithelium being 47.45 (46.65; 48.65) in the control wound, 73.76 (71.73; 75.77) in the wound containing solid-state matrix lyophilisate, and 75.35 (70.55; 79.83) μm in the wound with hydrogel matrix lyophilisate ($p = 0.0001$ for both lyophilisates as compared to the control). At week 2: 59.83 (58.22; 61.54), 91.90 (88.27; 92.02), 88.15 (85.73; 90.01) μm, respectively. At week 3: 68.98 (68.89; 71.74), 116.85 (110.74; 119.03), and 109.47 (106.12; 115.47) μm, respectively. At week 4: 88.83 (79.06; 90.84), 140.38 (133.47; 143.43), 132.56 (131.84; 138.45) μm, respectively. At week 5: 124.40 (121.64; 125.38), 159.16 (157.48; 161.99), 155.48 (152.12; 159.36) μm, respectively. The difference in these values in the experimental wounds, compared with the control, at all stages of the study, was statistically significant ($p = 0.0001$).

The forming epithelium of control wounds at week 1 consisted of irregularly shaped cells arranged in a thin layer. With the increase of the study period (3–4 weeks), the basal and spiny layers of the epithelium were clearly distinguished (Fig. 3). Epithelium formation was faster in the experimental wounds (Fig. 4). Clearly visible cell layers with a large number of Rete pegs were observed in the wounds containing hydrogel matrix already in the first week. Keratinocytes of the spiny layer in the wounds containing solid matrix were visualized larger and more mature already at week 2, they had large, rounded nuclei and were easily distinguishable from the cells of the basal and granular layers.

By the end of the study, the morphology of the cell layers of the epithelium of all wounds was indistinguishable from normal porcine skin (Fig. 4). When stained

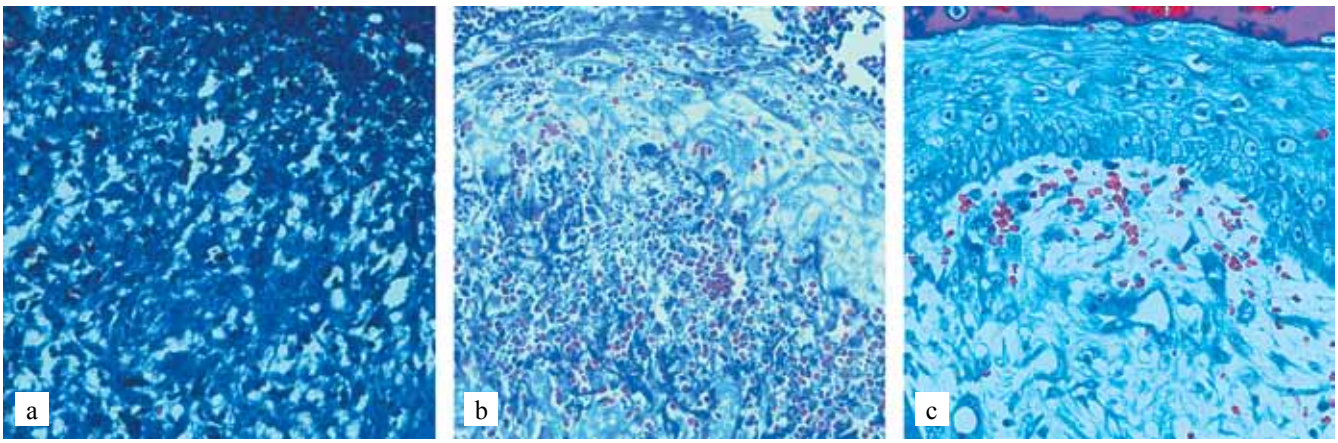

Fig. 2. Full-thickness skin wounds at week 1 in the control (a), wounds containing lyophilisates of tissue-engineered solid matrix (b) and tissue-engineered hydrogel matrix (c). Heidenhain's stain; 400×

with trichrome in samples from wounds containing lyophilized TEPs, formation of collagen structures in the granulation tissue area was observed as early as week 3 of the study. In the control, a similar pattern was observed only at week 5.

The solid-state matrix hydrolysate implanted into the wound bed was visualized in the granulation tissue at the initial stages of the study (Fig. 5) and gradually underwent biodegradation, moving through the papillary and reticular zones into the hypodermis.

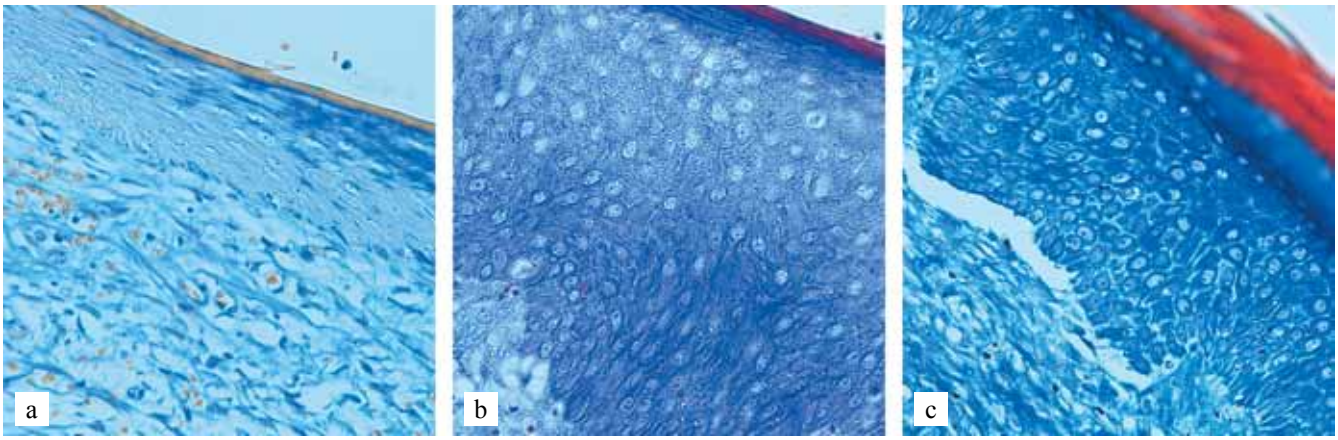

Fig. 3. Neoepithelium in the area of full-thickness wounds at week 3 in controls (a), wounds containing lyophilisates of tissue-engineered solid-state (b) and hydrogel matrices (c). Heidenhain's stain; 100×

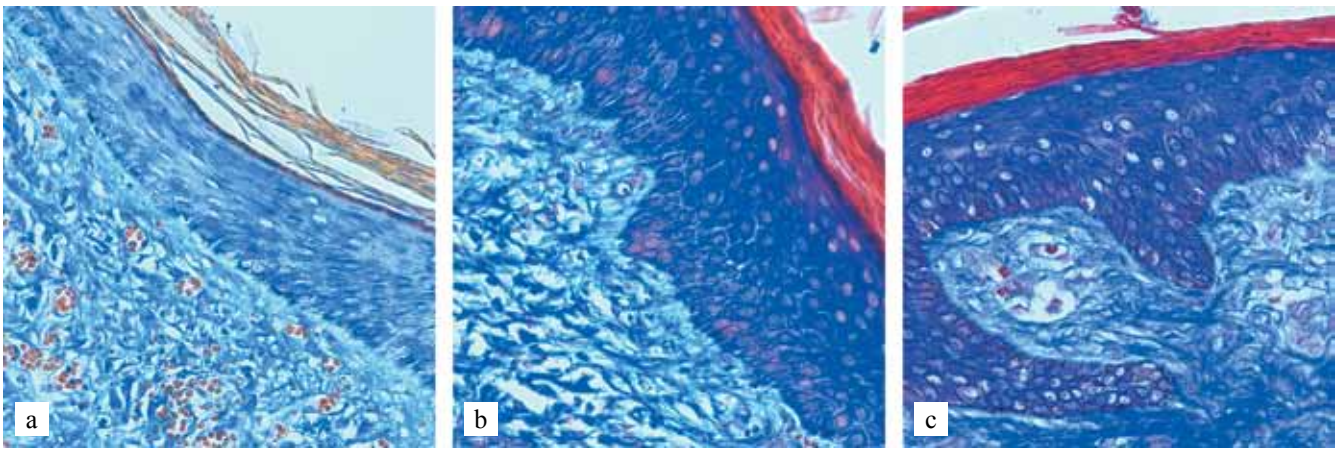

Fig. 4. Differentiated epithelium in the area of full-thickness wounds at week 5 in the control (a), wounds containing tissue-engineered matrix (b) and tissue-engineered hydrogel matrix lyophilisate (c). Heidenhain's stain; 400×

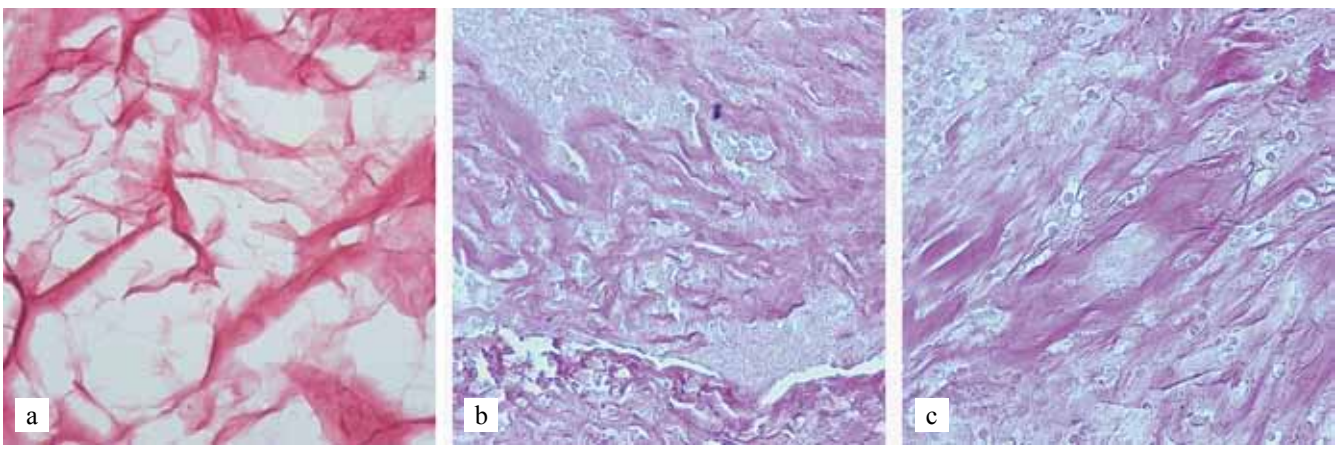

Fig. 5. Tissue-engineered solid-state matrix lyophilisate (a), lyophilisate with blood components at week 1 (b) and integrated lyophilisate of matrix in the hypodermis at week 5. Van Gieson's stain; 400×

The lyophilized hydrogel matrix implanted into the wound bed also underwent degradation and its traces gradually receded into the hypodermis by week 5 of the study (Fig. 6).

Importantly, no dense connective tissue capsule was formed around the degraded lyophilized TEPs and no massive infiltration with leukocytic cells was detected throughout the study period. This may imply that the products were not perceived as foreign by the recipient cells.

An in vitro study performed on cells isolated from mini pig full-thickness skin explants showed the absence of cytotoxic properties of soluble components of lyophilized TEPs. The metabolic activity of the cells in the Alamar Blue assay in the control was taken as 100%, while it was 101.66% (94.54%; 104.72%) and 100.76% (88.82%; 110.69%) for the solid-state and hydrogel matrices, respectively ($p = 0.916$).

The migratory activity of fibroblast-like cells isolated from full-thickness porcine skin explants and cultured in the presence of lyophilized TEPs was statistically significantly greater than that of the control. In the control, at day 1, the applied "scratch" was covered by 68.74% (63.74%; 71.26%) cells, while in the presence of lyophilized solid-state and hydrogel matrices, 75.43% (74.22%; 78.26%), and 76.74% (68.31%; 76.03%), respectively, with a statistically insignificant difference, compared to the control.

DISCUSSION

Skin wound healing is a sequential change in the phases of alteration, hemostasis, vascular responses and proliferation. Disruptions in this well-coordinated process can cause pathological healing. For example, the protective function of neutrophils (phagocytosis), necessary for wound cleansing against microorganisms and necrotic tissues, when in excess, leads to secondary alteration of surrounding tissues by active oxygen radicals, while differentiation of macrophages migrating to the wound defect area determines the outcome of proliferation and healing [10]. High-quality epithelialization is a guarantor of restoration of damaged skin function. These processes are based on numerous biochemical mechanisms and signaling pathways in which ECM plays a leading function [1]. Lyophilized products derived from human umbilical cord ECM that were placed into wounds, partially replenished the lost volume and served as a substrate for cell attachment. This mechanical function of lyophilized TEPs contributed to the rapid stopping of bleeding and filling of the wound bed with granulation tissue as soon as possible.

The results of the use of TEPs from ECM of different origin described in the literature showed that degradation rate, immunogenicity and ability to induce leukocyte infiltration are important predictors of biomaterial compatibility and efficacy [11]. The absence of local acute inflammatory response to the foreign body (lyophilisates of matrix and hydrogel matrix from the human umbilical cord for the pig recipient) and the absence of cytotoxicity of the obtained products for the mini-pig cells in vitro also contributed to the regenerative course of the healing process. Our previously published results indicating the non-cytotoxicity of TEPs for human dermal fibroblasts and for organ cells of different species of laboratory animals, suggest that cell-free products derived from human umbilical cord are biocompatible [8].

The healing outcome largely depends on restoration of the local vascular network delivering nutrients and removing metabolites. A significant advantage of human umbilical cord-derived lyophilized TEPs is the release of a large number of bioactive molecules (growth factors) during its biodegradation, which promote endotheliocyte migration and capillarogenesis. Structural reorganization of Wharton's jelly ECM during decellularization allows growth factors (such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF-β), etc.) that are fixed to scaffold proteins to be present in lyophilized TEPs in higher concentrations compared to the original umbilical cord biomaterial [12, 13]. This study

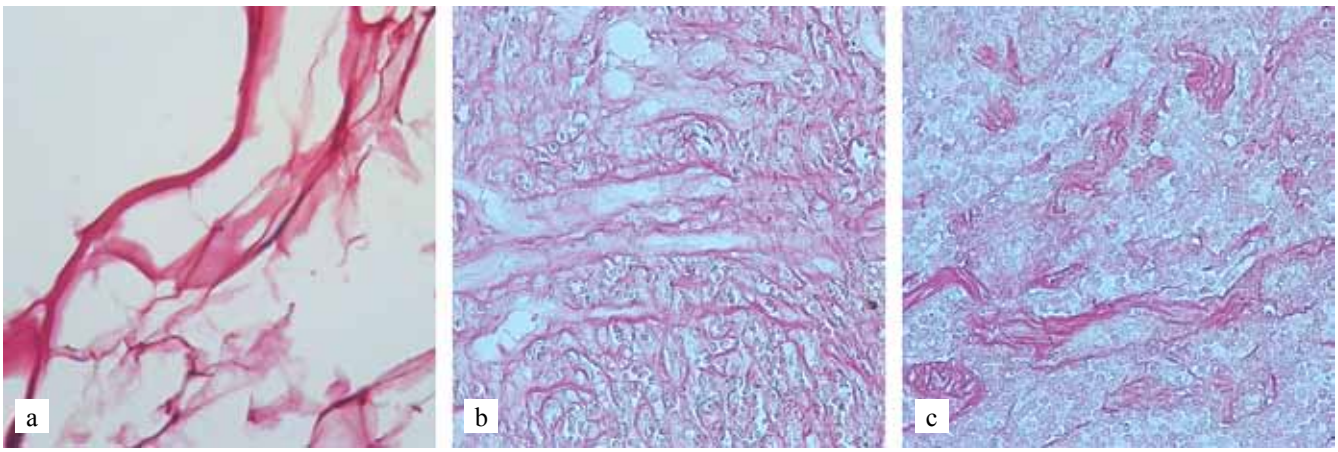

Fig. 6. Lyopholysate of hydrogel matrix (a), hydrogel matrix with blood components at week 1 (b) and integrated hydrogel matrix in the hypodermis at week 5. Van Gieson's stain; 400×

showed an increase in the specific area of the vascular bed in the tissues surrounding the wound with lyophilized TEPs and a simultaneous decrease in their diameter during the phase of fading of vascular responses, which may be a consequence of active formation of new capillaries. A similar phenomenon has been described in the scientific literature [14]. The greater in vitro migratory activity of pig skin cells in the presence of lyophilized TEPs, which we have established, confirms the assumption that lyophilized TEPs have chemotactic properties.

The main granulation tissue substance is deposited in the wound bed by fibroblasts. The origin of fibroblasts that form granulation tissue in deep full-thickness skin injuries has not yet been definitively elucidated. Studies in rabbits have identified vascular adventitia and pericytes below the panniculus carnosus and adipose layer as a source of granulation fibroblasts. In mice, it has been shown that circulating hematopoietic cells with mesenchymal characteristics can be transformed in situ in the wound. It is likely that multiple fibroblast subpopulations together form granulation tissue. In turn, the term "fibroblasts" refers to a very heterogeneous population of cells with different abilities to deposit ECM, differentiate into myofibroblasts and/or contract the wound. Fibroblasts in different skin areas can have different origins, and no single surface marker is intrinsic to all fibroblast lineages. This makes identification of the origin of fibroblasts in granulation tissue an interesting and challenging task [15].

Re-epithelialization of full-thickness wounds occurs exclusively from the wound edges. The suprabasal keratinocyte of the edge of the injured epidermis is elongated along the basal layer cell located under it and reaches the area of the wound bed, acquiring the ability to divide. Closure of the wound surface occurs as the next keratinocyte of the suprabasal layer of the wound edge undergoes the same process. Basal keratinocytes of the advancing epidermal layer have increased mitotic activity, have fewer desmosomes and more gap junctions. When the basement membrane is disrupted, human keratinocytes migrate along the temporal matrix, and it has been shown that their emerging cytoplasmic outgrowths are surrounded by laminin. But it remains unclear whether laminin serves as an adhesion substrate or is produced by cells in response to contact with collagen. Some studies have shown that migrating human keratinocytes do not produce basement membrane components [15].

According to the generally accepted notion, fibroblasts and endotheliocytes of the dermis grow into the primary clot that fills the wound space, while the epidermis grows on top, covering the granulation tissue [10]. There is an assumption that fibroblasts and pericytes are able to migrate from subcutaneous layers, hair follicles, and sweat glands, rather than from the relatively nonvascular and cell-populated reticular dermis [16]. In our study, mechanical filling of the defect area and gradual integration and revascularization of the graft, with formation of the recipient's native ECM, contributed to faster formation of mature epidermis.

Not only the mechanical filling of the wound, but also the qualitative composition of TEP, including structural elements of the basement membrane and functional molecules, play a role in the formation of quality mature epithelium. Statistically significant epithelial thickness was greater in wounds with lyophilized TEP compared to controls. Presumably, this may be a consequence of both the presence of growth factors in them and the basement membrane molecules such as type IV collagen and laminin that we identified in lyophilized TEPs [7]. In their in vitro studies, Deshpande et al. observed that inclusion of fibroblasts into the cell-free dermal matrix lacking basement membrane components had no significant effect on epithelium formation [14].

In wounds with tissue-engineered hydrogel matrix lyophilizate injected, differentiation and maturation of epitheliocytes were most active, not only as compared to the control, but also as compared to tissue-engineered solid-state matrix lyophilizate. At week 1, they showed signs of formation of Rete epidermal thickenings, while in the control wound, even the formation of a thin layer of epitheliocytes was incomplete at this time of the study. The distinct differentiation of epithelial cells, characterizing their maturity, was more noticeable at week 2 of the experiment when hydrogel matrix was used.

CONCLUSION

Our experimental studies on the effect of cell-free lyophilisates of tissue-engineered matrices on isolated cellular material in vitro and healing in an in vivo model showed that these lyophilisates were biocompatible and bioactive. They promote a regenerative type of deep skin wound healing when implanted in vivo more rapidly than in the control, forming a mature epithelial layer. The lyophilized hydrogel form of the tissue-engineered matrix promotes epidermal maturation with the formation of epidermal thickening faster than the lyophilized solid-state matrix. Presumably, this effect can be due to the action of growth factors present in the hydrogel form of the matrix in the unbound state. The results obtained make it possible to develop a scientifically substantiated program of preclinical studies on the safety and efficacy of human umbilical cord-derived lyophilized TEPs in accordance with GOST R 56699-2015 requirements.

The authors declare no conflict of interest.

REFERENCES

1. *Seaton M, Hocking A, Gibran NS.* Porcine models of cutaneous wound healing. *ILAR Journal.* 2015; 56 (1): 127–138. doi: 10.1093/ilar/ilv016. PMID: 25991704.
2. *Liang Y, Tian H, Liu J, Lv Y, Wang Y, Zhang J, Huang Y.* Application of stable continuous external electric field

promotes wound healing in pig wound model. *Bioelectrochemistry.* 2020; 135: 107578. doi: 10.1016/j.bioelechem.2020.107578. PMID: 32534380.

3. *Eweida AM, Marei MK.* Naturally occurring extracellular matrix scaffolds for dermal regeneration: do they really need cells? *BioMed Research International.* 2015; 839694: 9. https://doi.org/10.1155/2015/839694.
4. *Fominykh YM, Mitrofanov VN, Zhivtsov OP, Struchkov AA, Zubritskiy VF, Lebedeva YuN i dr.* Transplantatsiya tkanevykh ekvivalentov v lechenii nekotorykh povrezhdeniy kozhi. *Vestnik transplantologii i iskusstvennykh organov.* 2020; 22 (1): 165–173. https://doi.org/10.15825/1995-1191-2020-1-165-173.
5. *Marques MR.* Enzymes in the dissolution testing of gelatin capsules. *AAPS PharmSciTech.* 2014; 15 (6): 1410–1416. doi: 10.1208/s12249-014-0162-3.
6. Direktiva Evropeyskogo parlamenta i Soveta Evropeyskogo Soyuza 2010/63/ES ot 22 sent. 2010 g. o zashchite zhivotnykh, ispol'zuyushchikhsya dlya nauchnykh tseley [Elektronnyy resurs]. *Garant: informatsionno-pravovoe obespechenie*. Rezhim dostupa: http://base.garant.ru/70350564/ce210ed70e5daea1ed719396b4dabe87/ (data obrashcheniya: 22.10.2022 g.).
7. *Kondratenko AA, Kalyuzhnaya LI, Sokolova MO, Chernov VE.* Sokhrannost' vazhneyshikh strukturnykh komponentov pupoviny cheloveka posle detsellyulyarizatsii kak etapa izgotovleniya vysokoregenerativnogo ranevogo pokrytiya. *Biotekhnologiya.* 2021; 37 (5): 61–65. doi: 10.21519/0234-2758-2021-37-5-61-65.
8. *Kalyuzhnaya LI, Sokolova MO, Chernov VE, Zemlyanoy DA, Chebotarev SV, Chalisova NI i dr.* Vliyaniye beskletochnogo matriksa pupoviny cheloveka na dinamiku rosta i zhiznesposobnost' kul'tiviruyemykh kletok cheloveka i zhivotnykh *ex vivo. Geny i kletki.* 2021; 3: 72–79. doi: 10.23868/202110010.
9. *Kalyuzhnaya LI, Khominets VV, Chebotarov SV, Kharkevich ON, Kudyashev AL, Chernov VE i dr.* Primeneniye biomateriala iz pupoviny cheloveka dlya vosstanovleniya sustavnogo khryashcha. *Profilakticheskaya i klinicheskaya meditsina.* 2019; 4 (73): 45–52.
10. *Tracy LE, Minasian RA, Caterson EJ.* Extracellular matrix and dermal fibroblast function in the healing wound. *Advances in Wound Care (New Rochelle).* 2016; 5 (3): 119–136. doi: 10.1089/wound.2014.0561.
11. *Brown SJ, Surti F, Sibbons P, Hook L.* Wound healing properties of a fibrin-based dermal replacement scaffold. *Biomed Phys Eng Express*. 2021; 8 (1). doi: 10.1088/2057-1976/ac4176. PMID: 34883468.
12. *Dubus M, Scomazzon L, Chevrier J, Montanede A, Baldit A, Terryn C et al.* Decellularization of Wharton's Jelly Increases Its Bioactivity and Antibacterial Properties. *Frontiers in Bioengineering and Biotechnology.* 2022; 10: 828424. https://doi.org/10.3389/fbioe.2022.828424.
13. *Ramzan F, Ekram S, Frazier T, Salim A, Mohiuddin OA, Khan I.* Decellularized Human Umbilical Tissue Derived Hydrogels Promote Proliferation and Chondrogenic Differentiation of Mesenchymal Stem Cells. *Bioengineering.* 2022; 9: 239. https://doi.org/10.3390/bioengineering 9060239.
14. *Konyayeva AD, Varakuta YY, Leyman AY*. Morfofunktsional'nyye izmeneniya sosudov mikrotsirkulyatornogo rusla v slizistoy obolochke polosti rta v khode zazhivleniya ranevogo defekta pri ispol'zovanii polimernoy membrany. *Biomeditsina.* 2021; 17 (4): 57–67. https://doi.org/10/33647/2074-5982-17-4-57-67.
15. *Rittié L.* Cellular mechanisms of skin repair in humans and other mammals. *J Cell Commun Signal.* 2016; 10 (2): 103–120. doi: 10.1007/s12079-016-0330-1.
16. *Konstantinova MV, Khaytsev NV, Kravtsova AA, Balashov LD.* Osnovnyye problemy zazhivleniya ran i ispol'zovaniye zameniteley kozhi. *Pediatr.* 2015; 6 (2): 85–95.
17. *Deshpande P, Ralston DR, Macneil S.* The use of allodermis prepared from Euro skin bank to prepare autologous tissue engineered skin for clinical use. Burns. 2013; 39 (6): 1170–1177. doi: 10.1016/j.burns.2013.02.011.

The article was submitted to the journal on 27.10.2022