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BIOMEDICAL CELL PRODUCT MODEL FOR PRECLINICAL STUDIES CARRIED OUT ON A LARGE LABORATORY ANIMAL

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Objective: to develop a model of a biomedical cell product that is consistent with the "homologous drug" strategy based on protocols for preparing the cell component and scaffold carrier for preclinical studies on a large laboratory animal (pig). **Materials and methods.** Biomedical cell products and skin equivalents (SE), were formed using plasma cryoprecipitate prepared from blood plasma of healthy donors and mesenchymal stem cells (MSCs) of human adipose tissue. Cryoprecipitate from pig blood plasma and human adipose tissue-derived MSCs were used to form model skin equivalents (mSE). Bright-field microscopy, phase-contrast microscopy (Leica DMI 3000B) and fluorescence microscopy (Cytation 5 imager; BioTek, USA) were used to monitor the state of cells in the culture and in the composition of the equivalents. Scaffolds for equivalents were tested for cytotoxicity (MTT test, direct contact method). The cell distribution density was characterized by author's method (Patent No. 2675376 of the Russian Federation). **Results.** An mSE was developed for preclinical studies on a large laboratory animal (pig). In the mSE, components that change from halogen to xenogenic conditions during transplantation to the animal were replaced. A comprehensive approach to preparing mSE was presented. It includes sampling of primary pig biomaterial, extraction and characterization of adipose tissue-derived MSCs, preparation of a scaffold carrier for the corresponding "homologous drug" strategy. Cytotoxicity of the mSE scaffold was evaluated. It was shown that mSE provides mechanical support (similar to SE) to cells, as well as comparable development of cellular events during cultivation. **Conclusion.** A model of a biomedical cell product was developed. This model is consistent with the "homologous drug" strategy for preclinical studies on a large laboratory animal (pig). The paper presented a comprehensive approach to developing a model equivalent based on protocols for preparation and testing of the cellular component, the scaffold carrier and the ready-to-use model equivalent.

Keywords: skin equivalent, scaffold, mesenchymal stem cells, preclinical studies, homologous model.

BACKGROUND

In vivo experimental models used for testing new treatment methods remain the golden standard at the preclinical stages of developing drugs and new products of tissue engineering. Currently in vivo preclinical studies are most commonly performed on small laboratory animals, such as mice, rats and rabbits. At the same time there is no doubt regarding the advantages of using large animal models which result from the fact that they possess organs the size and phenotype of which are comparable to human ones. The latter enables to use equipment and methods which are developed and used in people for implantation, follow-up and analysis of the results of using tissue-engineered products in preclinical studies using animals [1]. Longer periods of life for large laboratory animals enable to perform long-term studies. The results of the studies for animals whose physiological parameters and metabolism rates are similar to those in humans may be extrapolated to people with a sufficient degree of certainty.

While selecting an animal model in order to study biomedical cell products (BMCP) it is necessary to pay special attention to the species-specific features of the animals and to the mission of the BMCP. For example, in the course of studying BMCPs intended for restoration of damaged or missing skin tissues it should be taken into account that there exists an enormous diversity of animal types whose skin structure and anatomy varies greatly. Interspecies anatomic and histological skin differences are extremely significant while selecting an animal model whose skin structure would be close or similar to human skin [2]. Relatively recently pigs have been widely recognized as a model for studying human skin tissue repair, as the anatomic and physiological skin structure of a pig is very similar to that in humans. Unlike free-skin animals (these include rabbits, rats, mice) pigs, same as humans, have a strong connection between the derma and the fascia [3]. The thickness and structure of the epidermis and derma are comparable in pigs and in humans [4]. Another important feature that human and pig skin have in common is the absence of a thick body hair layer. Epidermal enzyme patterns, keratin proteins, histological location of dermal collagen IV, fibronectin and vimentin, turnover time of the epidermal tissue for

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porcine and human skin have a high degree of comparability [5]. Moreover, the orientation and distribution of blood vessels in pig derma are similar to the vessel distribution in human skin [6]. Therefore many specialists believe that pigs are the most relevant animal models to study the effectiveness of using skin equivalents and wound healing.

At the same time while developing the design of preclinical studies not only physiological similarity of the chosen animal model should be taken into account but also the existing differences. For example it is known that pigs' and human immune systems are quite similar. However there is also a number of significant differences [7]. Summerfield A. et al. (2015) have shown that immunocompetent porcine skin cells also possess a set of characteristics which distinguish them from human ones [8]. The regenerative process development in the skin tissues is closely associated with the work of the immune system. The immune response is even more significant when tissue-engineered products are transplanted into the wound defect area. It is generally known that tissue-engineered products are not inert but possess high biological activity. The latter may be related both to the cellular component and to the non-cellular component (artificial extracellular matrix/scaffold). The presence of biological activity inevitably leads to the question regarding histocompatibility and immunogenicity of such products. In the development of tissue-engineered products histocompatibility and immunogenicity are taken into account in reference to the human body. For example often one of the key BMCP components are Mesenchymal Stem Cells (MSCs). They possess a number of properties which stimulate the development of a regenerative process in the damaged tissues and are characterized by hypoimmunogenecity related to absence of class II HLA receptors and MSC immunomodulatory activity [9]. The latter expands the potential of using such constructs under allogenous conditions. However in the course of preclinical studies on animal models tissue-engineered products advance from allogenous to xenogenous conditions, which a priori increases the risk of the recipient's organism with the implanted construct. Therefore in the course of preclinical studies phylogenetical differences should be taken into account even if an animal model which is very close to humans is used. During preclinical studies aimed at proving the effectiveness of BMCP use, in order to minimize negative results due to the body's reaction to the xenogenous component the developed products may be modified towards the 'homologous drug' strategy. In this context developing homologous BMCP models for certain types of laboratory animals is currently important.

Objective of the research: to develop a biomedical cell product model which would be consistent with the 'homologous product' strategy on the basis of protocols for preparing the cellular component and the scaffold carrier for preclinical studies on a large laboratory animal (pig).

MATERIALS AND METHODS

1.1. General principles of the work

All the procedures related to cell isolation and cultivation, working with blood and its derivatives, work aimed at the development and cultivation of hydrogel scaffolds took place at class C premises under sterile laminar flow unit conditions (class A) at the laboratory of biotechnology at the Federal State Budget Education Facility of the Volzhsky Region – the Privolzhsky Research Medical University of the Russian MoH. During the course of cell and hydrogel scaffold cultivation the growth media underwent regular control for sterility, mycoplasmatic and viral contamination, presence of fungal flora. The study protocol was approved by the local ethical committee at the Federal State Budget Education Facility of the Volzhsky Region – the Privolzhsky Research Medical University of the Russian MoH and affirmed by the Academic Council of the same University. Each person included in the study submitted voluntary informed consent.

1.2. Obtaining primary donor material

The peripheral blood of 3 healthy volunteers was used as a source of human blood plasma. The blood was taken in the morning (fasting) from the cubitus vein and placed into 'RAVIMED' polymer containers (Poland) with CPDA-1® hemopreservative solution. The blood plasma was separated by centrifugal method at 3000 rpm during 20 minutes. After centrifugation blood plasma was withdrawn. The obtained blood plasma was frozen and stored at –40 °C. The source material for obtaining mesenchymal stem cells was adipose tissue obtained in the course of cosmetic surgeries at the department of reconstructive and plastic surgery, University clinic of the Federal State Budget Education Facility of the Volzhsky Region – the Privolzhsky Research Medical University of the Russian MoH. The material was obtained from three young women whose mean age was 29.3 years (20 to 34 years old).

1.3. Separation and cultivation of human mesenchymal stem cells

Human mesenchymal stem cells were obtained from human adipose tissue Human Adipose-derived Stem Cells – hASCs) by means of thermal enzyme treatment with collagenase (Sigma-Aldrich, Germany) and cultivated in full growth media (media α – MEM, 20% fetal calf serum (FSC), glutamine, antibiotics penicillin/streptomycin) under absolute humidity, +37 °C, 5% CO_2 (CO_2 temperature regulated chamber, "Sanyo", Japan). The media and agents used were produced by "Gibco®"

(UK), the plastic vessels by "Costar" Co. (USA). After obtaining a subconfluent monolayer (60–70%) the culture was subcultured. Cultures after the 3rd–4th passage were used for experiments. The hASCs used for work had a confirmed differentiation potential in adipogenic, osteogenic and chondrogenic directions. Cell viability before their introduction into the experiment was 98–99%. The immunophenotype of the cells was typical for mesenchymal stem cells: the cells expressed CD 90, CD 105, CD 73, CD 44, CD 10 and did not express CD 45, CD 14, CD 34, HLA DR, which corresponded to the criteria determined by the International Society on Cell Therapy for mesenchymal stem cells.

1.4. Assessment of culture concentration and viability of the cells

Concentration and viability of the cells was counted by means of "Countes" cell counter (Invitrogen, USA), using trypan blue intravital stain (Sigma-Aldrich, Germany).

1.5. Determining the immunophenotype of human mesenchymal stem cells

Before starting the experiment the cell phenotype was determined using the monoclonal antibody panel CD 90 FITC, CD 105 PE, CD 73 PE, CD 44 FITC, CD 45 PC5, CD 34 PC7, CD 14 PC5, CD 10 PC7, HLA-DR PC7 (Becton Dickinson, USA) with appropriate isotypic controls on a BD FACS CANTO II flow cytofluorimeter (Becton Dickinson, USA).

1.6. Obtaining material for porcine mesenchymal stem cells and porcine blood plasma

Mesenchymal stem cells obtained from porcine adipose tissue (Porcine Adipose-derived Stem Cells – pASCs) were used as a test culture. Porcine blood plasma was used in order to determine a model equivalent of skin. The study protocol was approved by the local ethical committee of the Federal State Budget Education Facility of the Volzhsky Region – the Privolzhsky Research Medical University of the Russian MoH (protocol ## 8). All the procedures related to working with animals took place at a vivarium at the operating room, observing aseptic and antiseptic regulations and the requirements stated by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (№ 123 of 18 March 1986; ETS No. 170, June 22, 1998, Strasbourg)

Three piglets (Landrace breed, age 8 weeks, weight 13–15 kg, female) were used as a source of pASCs. The animals were introduced into the experiment after a 14 day quarantine. Before the operation the animals were carefully washed using a soap solution. At the operating room 10–20 min before general anesthesia premedication was performed using intramuscular administration of XylaVet (Pharmamagist Ltd, Hungary). The material was obtained under combined anesthesia: after intravenous administration of Propofol ("B-Braun", Germany) the animals were intubated, later anesthesia was maintained using Sevorane ("Abbott", UK). During analgesia the narcotic drug Fabius CS ("Dräger Medical GmbH", Germany) was used, pulse and arterial blood oxygen saturation monitoring was performed using a Nihon Kohden monitor (Nihon Kohden, Япония).

The surgical field was successively processed with 5% alcohol iodine solution, then 70% ethyl alcohol. In order to obtain adipose tissue MSCs 10–30 cm^3 of subcutaneous tissue were removed from the animals' dorsal area. Tissues were obtained using an ACCULAN® 3Ti Dermatome GA 670 electrodermatome (Braun, Germany). A layer of skin and subsequently the upper layer of the subcutaneous adipose tissue (1.2 mm) were removed from the surgical field by the electrodermatome. After that subcutaneous adipose tissue samples were obtained (graft thickness 1.2 mm, graft width up to 10 mm, layer depth up to 2.4 mm). The subcutaneous adipose tissue were rinsed repeatedly and carefully in order to remove blood admixtures in Hanks' solution with antibiotics (100 U/ml penicillin, 100 mcg/ml streptomycin, Sigma, Germany).

Blood sampling in order to obtain blood plasma was performed from the femoral vein. Vein catheterization was performed under ultrasound control (SonoSite SLL Ultrasound System, USA). Blood was collected into 'RAVIMED' polymer containers (Poland) with CPDA-1® hemopreservative solution. The procedure of separating porcine blood plasma was similar to the procedure of separating human blood plasma as described in section 1.2. Euthanasia was performed by means of air embolism under anesthesia.

1.7. Separation and cultivation of porcine mesenchymal stem cells

Cells were obtained from adipose tissue by means of mechanical disaggregation and thermal enzyme processing with 0.2% type I collagenase solution (Sigma, USA) during one hour. After that the cells were cultivated in full growth media in 25 cm^2 culture flasks. Media and agents used were produced by "Gibco®" Co. (UK), plastic materials – by "Costar" Co. (USA). The first change of growth media was performed in 72 hours. Later the media were changed twice a week. After obtaining a subconfluent monolayer (60–70%) the culture was subcultured. Cultures after the 3rd–4th passage were used for experiments. Cell cultivation at all stages took place in a CO_2 temperature regulated chamber ("Sanyo", Japan) at 37°C, 5% CO_2, absolute humidity.

1.8. Differentiation potential assessment

Cell differentiation potential was assessed on 3rd passage cultures. The Hyman Mesenchymal Stem Cell Functional Identification Kit (R and D systems, USA) was used for differentiation.

1.9. Determining the immunophenotype of porcine cells

Determining the immunophenotype of porcine cells was performed by multicolour assay method using the direct immunofluorescence method. The monoclonal antibody panel CD 44 FITC, CD 90 PerCP-Cy5.5, CD 10 PC7, CD 45 PE (Becton Dickinson, USA) was used in this work with corresponding isotypic controls on a FACS CANTO II cytofluorimeter (Becton Dickinson, USA). The measurement parameters have been set once and standardized using particles for BD Cytometer Setup and Tracking Beads (BD™CS&T Beads). Stained cells were incubated for 30 minutes and then rinsed in order to later determine the immunophenotype. The results were expressed as the ratio of cells bearing the corresponding marker (in percentage points).

1.10. Forming of BMCP (skin equivalents / hydrogel scaffolds)

In order to form BMCP – skin equivalents (SE) / hydrogel scaffolds (Pat. № 2653434 RF, 11.04.2017; [10]) blood plasma obtained from healthy donors was used. Frozen blood plasma was defrosted at +2 °C and placed into a centrifuge at +4 °C for 15 min at 1500 rpm in order for the cryoprecipitate to settle. Later 85% of the supernatant from the initial frozen blood plasma volume was collected. The cryoprecipitate was placed into a temperature-regulated chamber at 37 °C till full dissolution. The amount of fibrinogen in blood plasma, blood plasma cryoprecipitate and in the supernatant was determined. The cryoprecipitate was standardized by fibrinogen amount till the final concentration 6 g/l was achieved. For standardization purposes supernatant with low fibrinogen content remaining after cryoprecipitate isolation was used. For the forming of skin equivalents / hydrogel scaffolds a blood plasma cryoprecipitate pool from three donors was used.

For pegylation of the protein part of the cryoprecipitate PEG-NHS (Sigma-Aldrich, Germany) was used. A 2% solution of type I acetous collagen was added to the pegylated cryoprecipitate (collagen obtained from cod skins (Pat. № 2567171 RF… dated 06.10.2014) [11]) which had been pre-neutralized with NaOH. To the obtained composite a gentamycin solution was added – 0.26 mg/ml of the composite (Therma Fisher, USA). Later a cell (hASCs) suspended mixture in a phosphate-buffer saline (PBS) was added to the composite at the ratio of 7:1 correspondingly. The cell concentration was 1.2×10^5 per 1 ml of the composite. The resulting composite was transferred to a form – a 3.5 cm diameter Petri dish pre-treated with silicon. In order to achieve polymerization of the composite a thrombin-calcium mixture was added to it: 80 ME/ml of bovine thrombin (Sigma-Aldrich, Germany) in a 1% $CaCL_2$ solution.

The resulting skin equivalents (SE) were kept in a form for 20 min at +22–25 °C. Then they were transferred to cultural dishes (Corning 60 mm × 15 mm; USA) and covered with 6 ml of full growth media. The cultural dishes with the SE were transferred to a CO_2 temperature regulated chamber (37 °C, with humidified atmosphere and 5% CO_2 content) and cultivated during 6 days, the growth media being changed twice a week [12].

In order to form a model skin equivalent (mSE) as part of SE during its formation the human blood plasma cryoprecipitate and hASCs were replaced with porcine blood plasma cryoprecipitate and pASCs. The method for obtaining porcine blood plasma cryoprecipitate (a pool from three animals) and the method of forming mSE were identical to methods for obtaining human blood plasma cryoprecipitate and forming SE.

Studies ## 1.12.1 and 1.12.2 were carried out on acellular hydrogel scaffolds formed on the basis of human blood plasma cryoprecipitate and or on the basis of porcine blood plasma cryoprecipitate. Scaffolds were formed according to the method described above and under the same conditions. In the course of forming acellular scaffolds cell suspended mixtures in the PBS were substituted by an equal volume of PBS. Acellular hydrogel scaffold samples were used in the studies immediately after production.

1.11. Microscopy

The human and porcine cell structure condition was followed up at all cultivation stages. In order to observe the condition in the hydrogel scaffold structure a bright field and phase contrast method were used. Microscopy and video archiving were performed by means of Leica DMI 3000B inverted microscope equipped with a LAZ.V.4.3 image visualization programme.

In order to visualize the cells and confirm their viability in the scaffold structure fluorescent microscopy performed on a Cytation 5 image tool (BioTek, USA). In order to visualize viable cells Calcein AM was used (catalog № 564061, BD). In order to visualize cell nuclei (cytoblasts) Hoechst 3334 (USA) was used. Staining was performed in accordance with the manufacturer's protocol.

1.12. Studying the impact of hydrogel scaffolds on the hASCs culture

1.12.1. Cytotoxicity assessment – MTT test

The samples studied were cell-less hydrogel scaffolds (6 scaffold samples on the basis of human blood plasma

cryoprecipitate – SE scaffold and 6 scaffold samples on the basis of porcine blood plasma cryoprecipitate – mSE; the sample diameter was 33 mm, thickness – 2 mm). Each sample was placed into cultural dishes (Corning 60 mm × 15 mm; USA) and covered with 6 ml of full growth media. The samples in the media were placed into a CO_2 temperature regulated chamber and incubated under standard conditions for 1 day (3 SE scaffolds, 3 mSE scaffolds) and for 8 days (3 SE scaffolds, 3 mSE scaffolds) in order to obtain an extract. After the control period (1 day, 8 days) the extract over the samples was removed.

The hASCs in the concentration of 1×10^5/ml were inoculated on a flat bottom 96-well plate and cultivated in a growth media with 2% fetal calf serum at 37 °C, 5% CO_2 and absolute humidity during 3 days. After 3 days of cultivation the growth media over the cells was substituted by the extract taken from the samples and a growth media with 2% fetal calf serum in the concentration 0:1; 1:0; 1:1; 1:2; 1:4; 1:8, correspondingly. After 24 hours of cultivation with the extract the MTT test was performed. Optical density was registered at 540 nm on a Sunrise analyzer (Austria).

1.12.2. Cytotoxicity assessment – direct contact method

Hydrogel cell-less scaffold samples were used, their diameter was 15 mm, thickness 2 mm (9 SE scaffold samples and 9 mSE scaffold samples). The samples were transferred to the hASCs culture and covered with 5 ml of full growth media. 24 hours before the beginning of the experiment the hASCs were inoculated on cultural dishes (Corning 60 mm × 15 mm; USA) with density being 20,000/cm^2. The hASCs with scaffold samples were cultivated in a CO_2 temperature regulated chamber under standard conditions. The control was hASCs culture cultivated under the same conditions and in the same concentration but without the samples. After control periods (24, 72, 144 hours) the condition of hASCs was assessed (morphology, density, viability). After the completion of the experiment (144 hours of cultivation with samples) the immunophenotype of the cells was determined (# 1.5).

1.12.3. Comparative study of 3D hASCs cultivation in SE and mSE scaffolds

In order to carry out the study SE (5 samples) and mSE (5 samples) were formed according to the method described in # 1.7, introducing into their content hASCs composite. The samples were placed into cultural dishes with full growth media and cultivated up to 6 days in a CO_2 temperature regulated chamber under standard conditions.

1.13. Cell distribution density characteristic in equivalents

In order to characterize cell distribution density in equivalents fragments the area of which was 0.64 cm^2 were separated from studied samples (SE – 5 samples, mSE – 5 samples) by means of a template. The number of cells was determined by counting the nuclei 24 hours after the beginning of forming SE and mSE. In order to achieve this the selected sample fragments were transferred to a 24 well plate "Black Visiplate^{T-M}TC" (Wallac Oy, Finland). Later analysis of the cell number was performed according to the quantitative analysis method for the cellular component of the scaffold (Pat. № 2675376 RF… dated 17.07.2017 [13]). The method includes intravital staining of the cell nuclei in the scaffold using Hoechst 3334 (USA), fluorescent microscopy with Z-stack function (Cytation 5 image tool with Gen 5 Imedge+ software; BioTek, USA). 5 microphotographs from each sample were analyzed which were taken from different viewpoints (enlargement: 4× lens, 10× eyepiece) at random sites in the thickness of the samples. Objects were registered at 530 μm sites along the Z axis. In order to obtain a characteristic of the cell distribution density in SE and mSE cross-linked Z-stack microphotographs were used with calculation of cell nuclei number and subsequent recalculation of cell number per 1 mm^3.

1.14. Statistical analysis

The results of the studies have been processed by distribution-free statistic methods using Mann–Whitney criteria and Wilcoxon pair comparisons, with the help of STATISTICA 6.0 programme package.

2. RESULTS

2.1. pASCs characteristics

After the cells were recovered from the adipose tissues their adhesion to plastic material was recorded in 24 hours. As a rule, by day 6–10 the cells formed a subconfluent monolayer (60–70%). After the formation of the subconfluent monolayer the cultures were subcultured. During the whole follow-up period cells obtained from porcine adipose tissue were morphologically uniform, had a typical fibroblast-like structure and spread out well on the plastic material. The cells were characterized by clear contours, pronounced projections which formed intercellular contacts. In the centers of the cells oval nuclei were noted with dense nucleoli.

In the course of evaluating the differentiation potential of cultivated cells obtained from porcine adipose tissue on day 6–7 after introducing a differentiating media the appearance of adipose vacuoles was noted in the cells which were stained by a specific Oil Red stain. Further cultivation in a differentiated media led to a further increase in the number of cells with adipose vacuoles

(Fig. 1, b). Osteogenic differentiation was indicated by the appearance of calcium salts in the cells which were stained by a specific stain – alizarin red (Fig. 1, d). On day 13–15 osteogenic differentiation was confirmed by osteocalcin staining of the cells obtained from adipose tissue. Formation of small spheroids (pellets) typical for chondrogenic differentiation was recorded in vitro already one day after adding DMEM/F12 media with differentiating additive. In these spheroids on day 6 type II collagen deposit was recorded by polyclonal antibodies (Fig. 1, f).

The cell culture phenotype was determined in the process of culture growth for passages 2 through 6. In the process of study it has been noted that the pan-leucocyte antigen CD 45 was not noted on the cells derived from adipose tissues at all the stages of the study (less than 1%). CD expression on the 2nd passage amounted to an average of 56%. In the process of culture growth the percentage of cells expressing CD 44 increased and reached 99.7% by passage 6. A typical MSCs marker CD 90 was expressed by about 75% of the cells at passage 2. In the process of growth the percentage of cells expressing CD 90 increased and amounted to about 90% by passage 6 (Fig. 2, a). The number of cells expressing CD10 was increased from passage to passage in all cultures and by passage 6 the number of cells expressing CD10 amounted to about 70%. Thus the phenotype of cells obtained from porcine fatty tissue may be described as CD 90+, CD 44+, CD 10+, CD 45–, which corresponds to the MSCs phenotype.

The phenotype of ASCs obtained from several animals (Fig. 2, b) and dynamic changes in the expression of the main markers were similar. Increased CD 44 and CD 90 expression was noted up to passage 3–4. At later periods the expression of these markers stayed at an invariably high level. Analysis of CD 10 expression showed that expression of this antigen increased continuously till passage 6. CD 45 expression by the cells remained at an invariably low level (below 1%).

2.2. Comparative study of the influence of hydrogel scaffolds on hASCs culture

Comparative study of the cytotoxicity of cell-less hydrogel scaffolds on the basis of human blood plasma

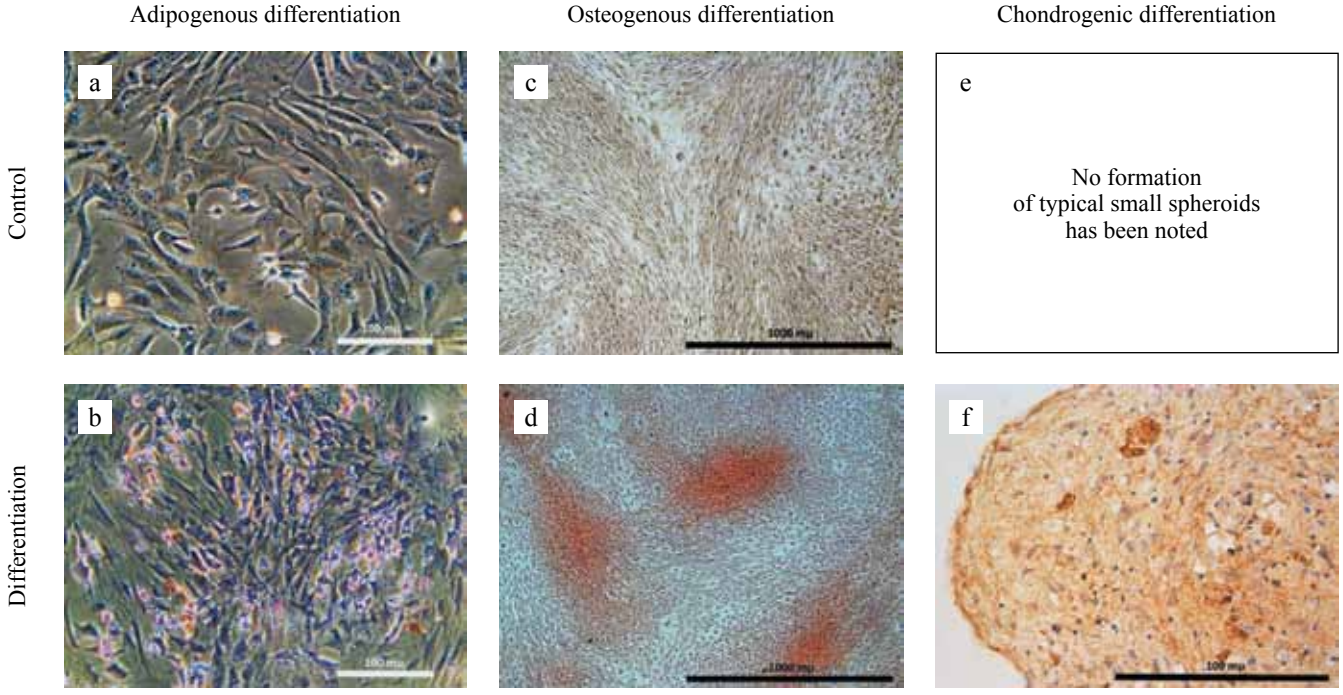

Fig. 1. Differentiation of pASCs. a, b – Adipogenous differentiation of pASCs, Oil Red staining, (cultivation day 15; objective lens 20×, eyepiece 10×; phase contrast): a – control – pASCs culture, third passage without use of differentiating media, the subconfluent monolayer is formed by typical fibroblast-like cells. No adipose vacuole formation has been noted in control culture cells; b – experimental pASCs culture after cultivation in a differentiating media. In the culture cells stained by a specific Oil Red stain adipose vacuoles are clearly noted which demonstrates the pASCs capability of adipogenous differentiation; c, d – Osteogenous pASCs differentiation. Osteogenous pASCs differentiation, staining by alizarin red (cultivation day 15; objective lens 5×, eyepiece 10×, light microscopy): c – pASCs control culture is presented in the form of a subconfluent monolayer formed by morphologically identical spindle-shaped cells, no calcium deposits noted; d – pASCs culture, experimental series after cultivation in a special differentiating media. The culture appears as a subconfluent monolayer. Calcium deposits are clearly visualized which are stained by a differentiating stain (alizarin red); f – pASCs chondrogenic differentiation (light microscopy). a – objective lens 5×, eyepiece 10×; b – Staining in a spheroid formed by pASCs culture after cultivation of type II collagen by polyclonal antibodies in a chondrogenic differentiated media (Abcam, ab34712; objective lens 40×, eyepiece 10×). Formation of small spheroids and type II deposition in them by the cells indicates chondrogenic differentiation of pASCs

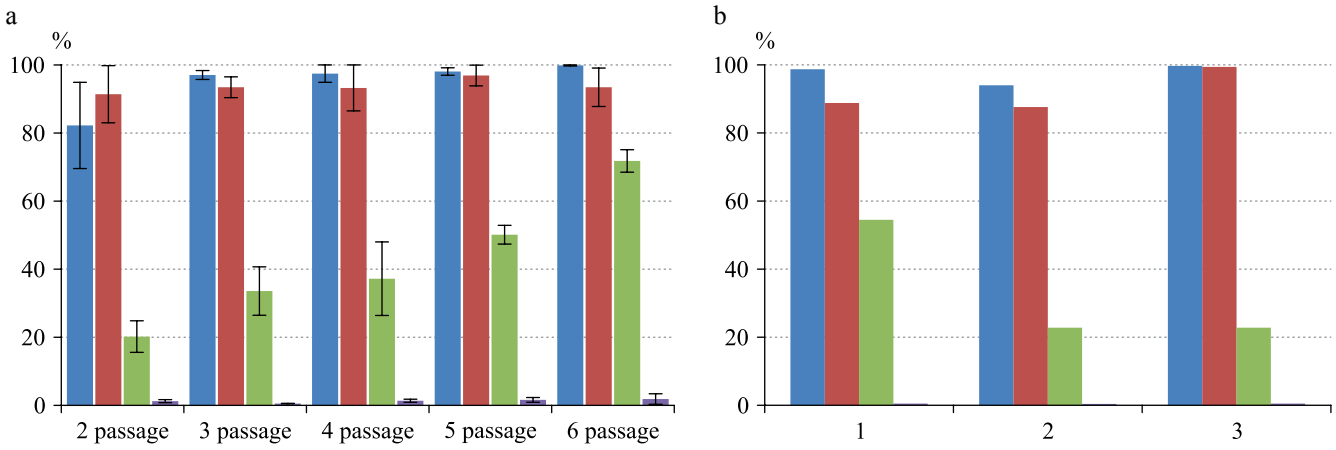

Fig. 2. Expression of differentiation markers for pASCs. (a) example of the changing of cell phenotype from one of the experimental animals in the course of cultivation. From passage to passage an increased number of cells is noted which express CD 44 (blue), CD 90 (red), CD 10 (green). The number of cells in the pASCs culture which express CD 45 (purple) was less than 1% during the whole cultivation period; (b) comparative diagram of MSCs phenotype obtained from the adipose tissue of three different animals (passage 3). In all the experimental cultures the number of cells expressing CD 44 (blue) exceeded 90%, CD 90 (red) – 87% and CD 10 (green) – 22%. Expression of the pan-leucocyte antigen CD 45 on the cells of the cultures under consideration amounted to less than 1%

cryoprecipitate (SE scaffold) and on the basis of porcine blood plasma cryoprecipitate (mSE scaffold) showed an absence of cytotoxic activity on human MSCs (Table 1). Analysis of MTT test results showed a pronounced stimulating action of the extract obtained from cell-less scaffolds under examination on the proliferative activity of hASCs. Thus, after exposure to the extract collected after the first day of incubation from SE scaffolds the number of metabolically active cells in the culture was increased by over 1.5 times as compared to the control group. A similar effect was noted after exposure to the extract from SE scaffolds after 8 days of incubation. After exposure to the extract collected from mSE scaffolds (days 1 and 8 of incubation) the number of metabolically active cells was increased by over 1.8 times as compared to the control group. Rarefaction of the extracts led to a decrease of their stimulating effect. At the same time, even when the extracts were diluted by half there was a statistically significant increase in the number of metabolically active cells as compared to the control group. During comparison of the action of extracts from cell-less SE and mSE scaffolds on the hASCs culture no significant differences according to the results of the MTT test have been obtained (Table 1).

In the course of human hASCs cultivation in the presence of cell-less hydrogel SE and mSE scaffolds no negative effect of the scaffolds on the hASCs culture has been noted. There was no statistically significant difference between cell density in the culture and their absolute number in the cultural dishes under investigation (SE scaffold, mSE scaffold) and the control group at all times during the study (Table 2). The cells in experimental cultural dishes and control dishes were well spread out on the plastic material and formed a subconfluent level. The hASCs were morphologically homogeneous and had a characteristic fibroblast-like form with clear contours, pronounced projections which formed intercellular contacts. All the cells were characterized by high viability (Table 2). The phenotype of the cells from control and experimental cultural dishes did not vary and corresponded to the MSCs phenotype. Cells expressed CD 90, CD 105, CD 73, CD 44, CD 10 and did not express CD 45, CD 34, CD 14, CD HLA DR.

Human adipose tissue hASCs incapsulated in SE and mSE scaffolds were characterized by equal activity in the

Table 1

Cytotoxicity study of cell-less hydrogel scaffolds

Extract withdrawal	Scaffold	Control 0:1	Extract 1:0	Extract 1:1	Extract 1:2	Extract 1:4	Extract 1:8
Day 1	SE	0.341 ± 0.007	0.518 ± 0.011*	0.537 ± 0.026*	0.442 ± 0.020*	0.423 ± 0.020*	0.334 ± 0.007
	mSE	0.322 ± 0.011	0.568 ± 0.026*	0.442 ± 0.029*	0.403 ± 0.014*	0.370 ± 0.011*	0.393 ± 0.016*
Day 8	SE	0.327 ± 0.010	0.519 ± 0.021*	0.512 ± 0.014*	0.437 ± 0.011*	0.371 ± 0.020	0.348 ± 0.022
	mSE	0.282 ± 0.006	0.557 ± 0.032*	0.505 ± 0.025*	0.405 ± 0.022*	0.364 ± 0.016*	0.323 ± 0.017

Note. * – $p < 0.05$, comparison vs control, Wilcoxon criterion; ■ – $p < 0.05$, comparison of SE vs mSE, Mann–Whitney criterion.

course of 3D cultivation. Thus, already after 24 hours the cells demonstrated active matrix-cell adhesion and began sprouting cellular projections (Fig. 3, a, b). In the process of further cultivation dynamic 3D cell growth was noted with formation of multiple projections and intercellular contacts. On day 6 formation and development of cellular

Table 2

Comparative study of the influence of cell-less model skin equivalent on hASCs culture

Cultivation time	Cell density ($\times 10^3/cm^2$)	Absolute number of cells ($\times 10^3$)	Cell viability (% of viable cells)
	Control		
24 hours	26.73 ± 1.17	534.67 ± 23.33	98.70 ± 0.15
72 hours	39.47 ± 1.81	789.48 ± 36.15	99.52 ± 0.18
144 hours	40.64 ± 0.95	812.81 ± 18.94	99.58 ± 0.10
	SE scaffold		
24 hours	27.894 ± 0.90	557.89 ± 17.97	99.25 ± 0.31
72 hours	39.29 ± 1.47	785.810 ± 29.30	99.55 ± 0.18
144 hours	45.17 ± 2.34	902.35 ± 46.95	99.63 ± 0.12
	mSE scaffold		
24 hours	27.96 ± 1.05	559.11 ± 21.03	99.77 ± 0.15
72 hours	38.68 ± 2.36	773.59 ± 47.14	99.13 ± 0.10
144 hours	42.51 ± 1.97	850.12 ± 39.34	99.65 ± 0.16

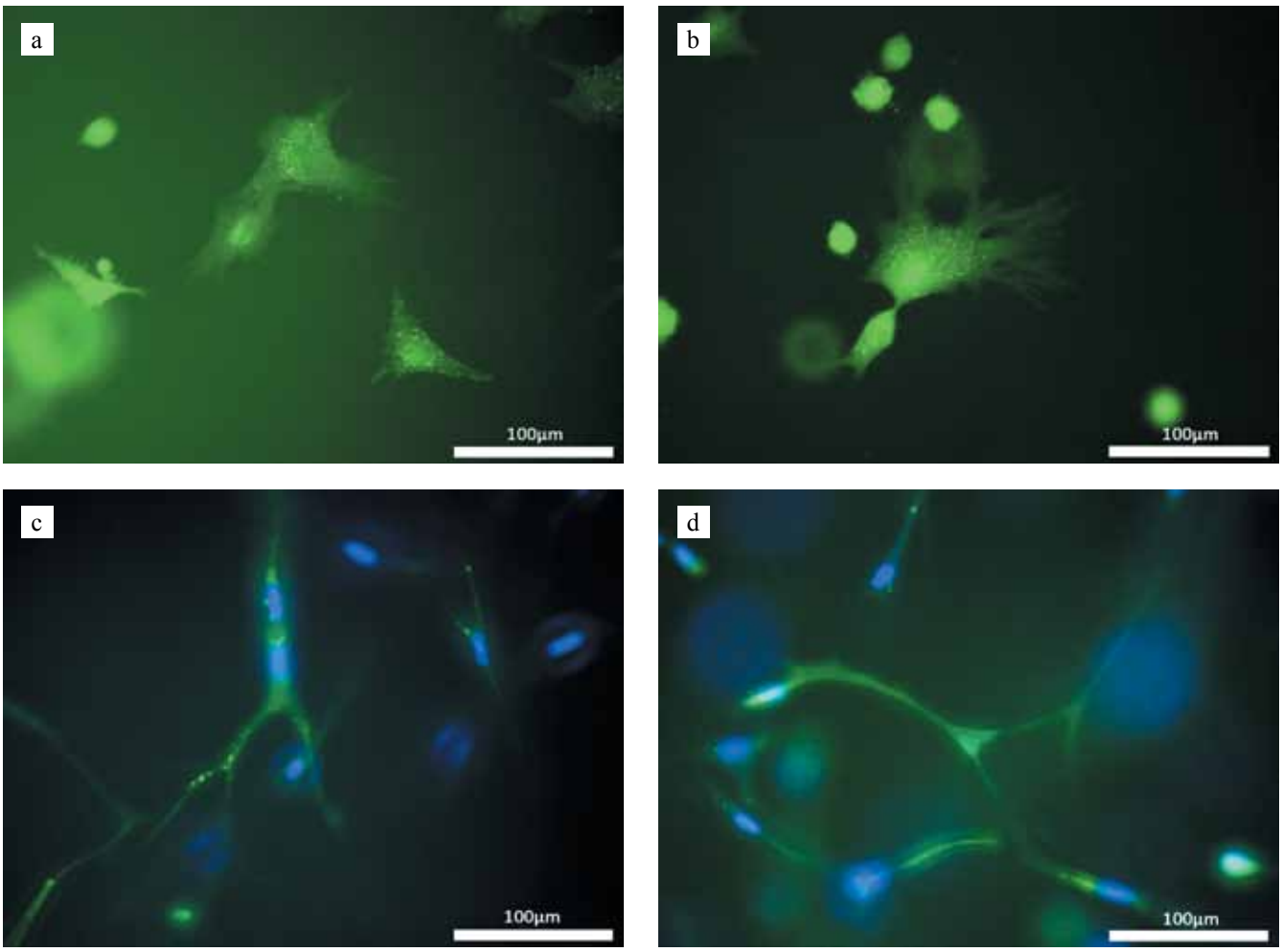

Fig. 3. hASCs cultivation in SE (a, c) and scaffold on the basis of porcine blood plasma cryoprecipitate (b, d). Cellular events in the course of hASCs cultivation in SE and in the scaffold on the basis of porcine blood plasma cryoprecipitate had a similar character: a, b – 24 hours of cultivation: the cells are spread out, sprout cellular projections; c, d – day 6 of cultivation: 3D cell growth with formation of multiple projections and intercellular contacts. Fluorescent microscopy: viable cells are stained with the specific fluorescent Calcein AM stain (green); b, d – cell nuclei (blue) are stained with Hoechst 3334 fluorochrome. *Note:* cells and nuclei lying in other layers of the 3D SE and mSE scaffold structure are out of focus (related to the layer where the cells are in focus)

networks was noted in SE and mSE scaffolds (Fig. 3, c, d). Viability of the cells cultivated in SE and mSE scaffolds was confirmed by staining with Calcein AM.

2.3. Comparative analysis of SE and mSE

Model skin equivalents formed on the basis of porcine blood plasma cryoprecipitate with pACSs possessed characteristics similar to those in skin equivalents formed on the basis of human blood plasma cryoprecipitate and hASCs. SE and mSE had a form-stable, elastic, transparent hydrogel structure (Fig. 4, a, b). The cells were spread uniformly along the whole structure of the equivalents, both in SE and in mSE (Fig. 4, c, d). There was no statistically significant difference between the number of cells detected on 1 mm^3 24 hours after the formation of the equivalents in SE and mSE, which amounted to 135.70 ± 24.78 and 124.93 ± 22.81 correspondingly.

In the process of SE and mSE cultivation no significant differences have been detected in the development of cellular events. 24 hours after the formation of the equivalents matrix-cell adhesion was noted, cellular projection formation and the spreading out of the cells started. In the process of further cultivation human adipose tissue MSCs and porcine adipose tissue MSCs demonstrated active 3D growth (Fig. 4, e, f). The cells formed multiple projections in different directions and cellular contacts which later led to the formation of a cellular network.

3. DISCUSSION

The development of biomedical cell products inevitably involves the stage of preclinical studies using laboratory animals. At the same time studies which involve the use of large laboratory animals is a less common practice than the practice of studies using small laboratory animals, such as rodents. Therefore while preparing study protocols it is not always possible to be guided by similar works and fully utilize standard or generally accepted methods.

In order to develop a comprehensive approach to forming a BMCP model corresponding to the 'homologous drug' strategy for preclinical studies on a large laboratory animal (pig) skin equivalent was chosen as the object of study. The main components of the skin equivalent presented in this work are human blood plasma cryoprecipitate, type I cod collagen, bovine thrombin and human adipose tissue mesenchymal stem cells. Blood plasma cryoprecipitate and hACSs as components of SE are in allogenous conditions related to the recipient (human). When SE are used in studies on a pig model these components will change from allogenous conditions to xenogenous as related to the model recipient (pig). It is known that the same cell products and tissue engineered products may have a significantly higher immunogenic activity under xenogenous conditions as compared to allogenous. In a number of experimental studies it has been shown that the results of using xenogenous MSCs are significantly inferior to the results obtained while using allogenous or autogenous cells and may lead to the development of negative effects. For example, P. Niemeyer et al. (2010) have found that in the groups of animals who received treatment with xenogenous MSCs the results of bone tissue regeneration were worse by all the parameters considered compared to the group of animals who received treatment with autogenous MSCs [14]. In order to avoid misrepresentation of results related to species-specific immune response in the course of future preclinical studies we have developed a model skin equivalent for preclinical studies on pigs. Based on the correspondence to the 'homologous drug' strategy while developing a model skin equivalent as part of SE in the course of its formation it was necessary to substitute the human blood plasma cryoprecipitate and hASCs by porcine blood plasma cryoprecipitate and pACSs. Sea collagen and bovine thrombin which are included in the composition of SE did not require substitution in our opinion due to the fact that they are xenogenous components both for humans and for pigs.

Having analyzed literature sources we did not find any generally accepted protocol for isolation, cultivation or assessment of pASCs. We have prepared and tested relatively simple protocols which are applicable for experimental work. Their fundamental principles are described in ## 1.6–1.9 of the 'Materials and methods' section. The presented approaches enable to obtain biomaterial from a specific area of adipose tissue, which enables to get uniform primary material and standardize the tissue recovery technique. Using a dermatome enables to obtain adipose tissue grafts from a certain depth. The thickness of the grafts (up to 1.2 mm) simplifies the primary material processing as no coarse primary mechanic homogenization or additional procedures aimed at forming thin grafts are required before the mechanical disaggregation stage, as, for example, in the recovery of large volumes of adipose tissue [15].

The selected approaches enabled to perform a characteristic description of the cells obtained from porcine adipose tissue according to the main parameters required for their identification and use in experimental work: viability, morphology, differentiation potential, immunophenotype. The data received by us regarding isolation and description of MSCs from porcine adipose tissue, same as those found in literature sources [16, 17], demonstrate the capacity of pASCs for osteogenic, adipogenic and chondrogenic differentiation, manifestation of typical morphological characteristics and a certain phenotype.

One of the main components of the SE presented in the work is blood plasma cryoprecipitate. In the model SE human blood plasma cryoprecipitate was substituted by porcine blood plasma cryoprecipitate. The procedures

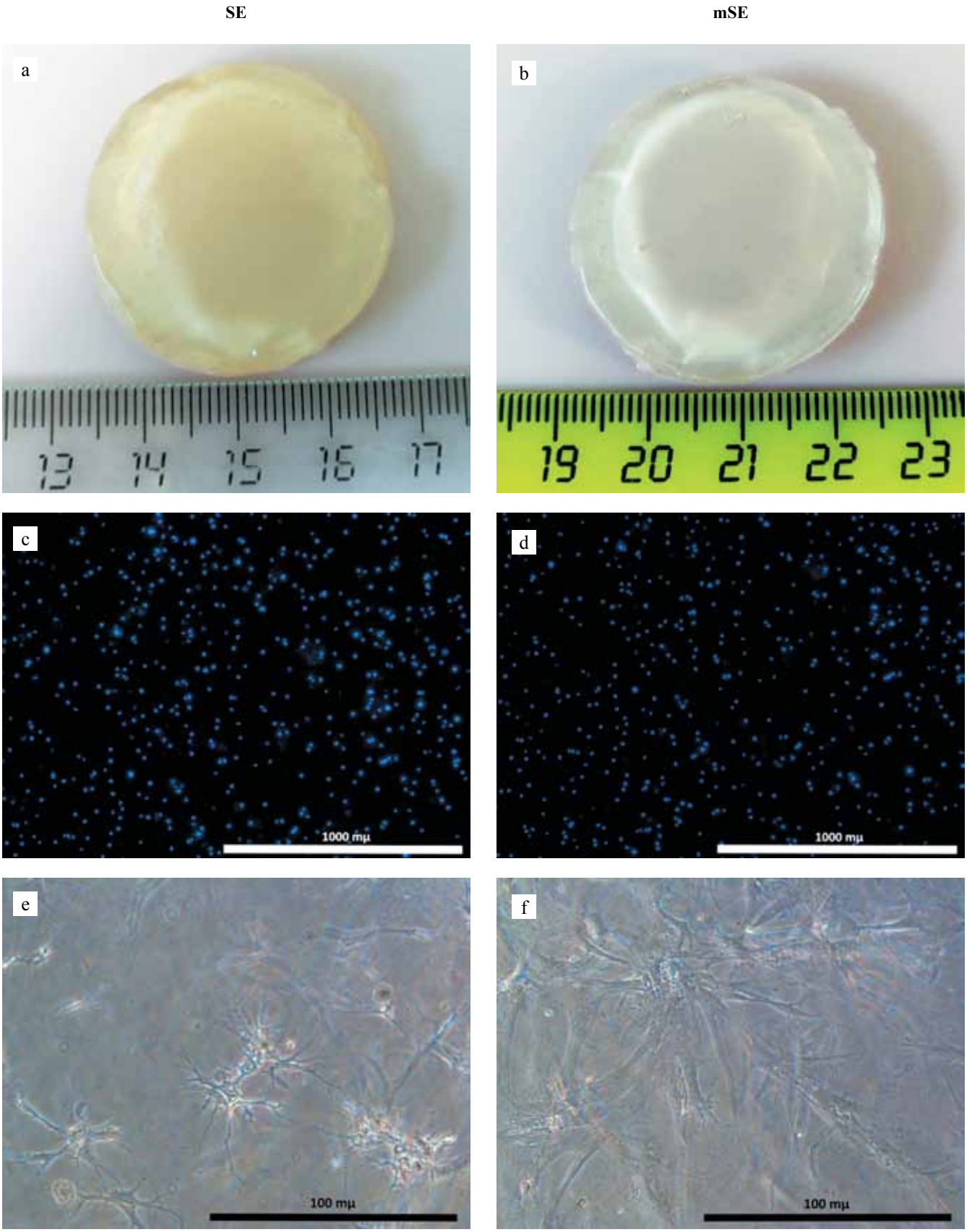

Fig. 4. SE and mSE: a, b – external appearance of the equivalents; c, d – cell nuclei, fluorescent microscopy: crosslinked microphotographs Z-stack (530 mμ), cell nuclei (blue) stained with Hoechst 3334 specific fluorochrome; e, f – MSCs cultivated in equivalents (day 3 of cultivation; phase contrast, objective lens 40×, eyepiece 10×)

related to obtaining porcine blood plasma and preparing cryoprecipitate technologically fully corresponded to those which were used in the process of preparing human blood plasma cryoprecipitate for SE formation. The basis of the SE formation method is the enzyme hydrolysis reaction which takes place in the presence of thrombin. The main structure-forming protein for the forming of the SE scaffold is blood plasma cryoprecipitate fibrinogen, the amount of which in the composite exceeds the amount of collagen by over 22 times. Thus the process of SE forming imitates the process of blood coagulation. The porcine blood coagulation system does not have any essential difference from the human blood coagulation system [18]. Based on this we used the same method for forming mSE that was used for forming SE. The possibility of using mSE in preclinical studies is based on the assumption that mSE should not have any significant differences in their properties as compared to SE. In order to check this assumption a comparative assessment of cytotoxicity and influence on hASCs culture was performed for cell-less SE and mSE scaffolds which were serving as scaffold cell carriers. Data of the MTT test confirmed the absence of cytotoxicity for mSE scaffolds. The results of the MTT test showed that the number of metabolically active cells in the presence of SE and mSE scaffold extracts grew significantly. This data indicates an increase in the cell proliferative activity in the presence of scaffolds. Both scaffolds are formed on the basis of blood plasma cryoprecipitate. Human blood plasma and porcine blood plasma contain quite many biologically active substances (amino acids, proteins, microelements, etc) [19]. It is well known that the concentration of many biologically active substances (BAS) in blood plasma cryoprecipitate is much higher that in blood plasma [20]. Blood plasma components may take active part in cell growth regulation. Thus, for example, calcium cations are present in blood plasma. M.N. Lee et al. have shown that increased Ca^{2+} concentration in the cultural medial leads to increased MSCs proliferative activity [21]. Also exogenous calcium which is included in the thrombin-calcium mixture used in scaffold forming is present in the sample scaffolds of the studied equivalents. Therefore the noted stimulating effect is most probably related to the action of biologically active substances extracted from scaffolds.

The direct contact test results confirmed absence of a negative influence on behalf of SE and mSE scaffolds on the viability, adhesion, morphology, proliferation and immunophenotype of hASCs. At the same time the results of this test did not show any stimulating action on behalf of cell-less scaffolds on the proliferation of hASCs. Most probably this is due to low concentration of BAS which are washed out from the scaffolds in this experiment. It is known that during cell cultivation the presence and concentration of BAS in the cultural media largely determines cultural growth [22]. Thus, in the course of the MTT test the extract was collected from 33 mm scaffolds which were incubated in 6 ml of the media. In the course of the biocompatibility test 15 mm sized scaffold samples and 5 ml of the media were used. At the same time the amount of cells impacted by BAS released by the samples in the MTT test was much lower as compared to the number of cells in the direct contact test. Therefore the results of the direct contact test may to a certain degree be compared to the results of the MTT test with highly diluted extract (1:8). In case of high dilution of the extract the number of metabolically active cells varied little from the control, and the results of the extract's influence on the cells were comparable with those obtained after cell cultivation in a growth media.

It is known that mesenchymal stem cells encapsulated into hydrogel scaffolds demonstrate 3D growth [23]. In order to determine to which extent an mSE scaffold ensures 3D cell growth and how comparable will cell events be in mSE and in SE the same hASCs cultures were encapsulated into both scaffold types. hASCs encapsulated into scaffolds supported their vital activity and demonstrated 3D growth. Both scaffolds are hydrogels. It is known that the hydrophilic nature of hydrogels facilitates the exchange of nutrients and metabolic products between the cells within the 3D structure, thus supporting high viability of the cells [24]. Cellular events noted in SE and mSE scaffolds did not show any significant differences throughout the observation period. It is known that viability, growth, morphology, migration, proliferative activity, etc. are largely determined by the scaffold content and structure [25]. The latter enables to suggest that the content and structure of SE and mSE scaffolds which influence cellular behaviour are comparable.

The latter statement is also confirmed by data obtained in the course of SE and mSE formation. Thus, it is known that the properties of a scaffold as a mechanical supporting structure for the cells are determined by its inner architectonics. In the course of SE and mSE formation the cells were distributed relatively uniformly throughout the whole structure of the equivalents with equal density. The SE and mSE structures are formed primarily by the fibrin network. Self-assembly of the fibrin fibers depends on the concentration of fibrinogen, thrombin and calcium [26]. Literature data bears evidence that protein pegylation also has a significant influence on the forming fibrin fibers [27]. The composite used to form scaffolds also includes collagen. Collagen of various origins is used quite extensively in scaffold technologies and has shown advantages as a structure-forming biopolymer for the formation of mono- and polycomposite scaffolds [28, 29]. Thus, the collagen used to form equivalents may also take part in forming SE and mSE structures. At the same time, concentrations and proportions of the abovelisted components in the course of developing mSE were the same as the ones used for forming SE. 3D cultivation of pASCs as part

of mSE confirmed the comparability of cellular events developing in mSE and in SE.

Summarizing the obtained results we can make a conclusion that the conditions of forming equivalents (content, proportions and concentrations of the main components which take part in structure formation, methodological approach) enable to obtain structures which provide similar mechanical support of the cells and allow to create comparable conditions for placement and interaction of MSCs.

4. CONCLUSION

A comprehensive approach has been presented for the development of a biomedical cellular product model on the example of a skin equivalent to be used in preclinical studies on a large laboratory animal, taking into account the 'homologous drug' strategy. In the course of preparing the model BMCP the components of SE (developed to be used in humans) which change from allogenous conditions to xenogenous in the course of transplantation to an animal were substituted. Compliance of the characteristics of cells obtained from porcine adipose tissues to requirements for MSCs has been confirmed. In the future the set of methods and approaches used in the work for obtaining, cultivating and characterizing pASCs, as well as obtaining porcine plasma, may be useful while carrying out work based on using animal cells, for example in the course of developing protocols for xenotransplantation of cells and cell-based constructions. Lack of cytotoxicity has been confirmed for the mSE cell-less scaffold. It has been proved that the mSE scaffold provides similar conditions for cell placement and 3D growth as the SE scaffold. Cellular events developing in the course of mSE and SE cultivation were also comparable. Therefore, mSE on the basis of porcine blood plasma cryoprecipitate and pASCs can be used for conduction of studies on large laboratory animals (pigs) as a model corresponding to the 'homologous drug' strategy. The authors hope that the set of methods and the protocol of work used in the development of this model equivalent will be useful in the course of preparing and carrying out preclinical studies of similar biomedical cell products.

The authors declare no conflict of interest.

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