SURVIVAL OF HUMAN CELLS IN TISSUE-ENGINEERED CONSTRUCTS STORED AT ROOM TEMPERATURE

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Tissue-engineered constructs (TECs), the dermal equivalent (DE) and the skin equivalent (SE), are allogenic equivalents of the skin and derm used to treat critical skin loss. Selection of storage conditions that contribute to longer shelf life, thereby expanding the possibilities of logistics and use, is one of the major issues related to the TECs development. The study was aimed to determine the shelf life of the DE and SE TECs stored in normal saline at room temperature by assessing morphology and viability of the cells on their surface, along with the levels of endothelial growth factor (VEGF) secreted by these cells. Using the MTT assay and staining with vital dye, we discovered the following: when TECs of both types were stored in normal saline, the cells viability and metabolic activity decreased by more than 50% by days 3–4 of storage. Furthermore, these decreased faster in DEs than in SEs. Morphology of the cells isolated from DEs and SEs after the 3-day storage remained unchanged. Mesenchymal stem cells on the surface of TECs kept producing VEGF after TECs culture medium was changed for saline solution (confirmed by immunofluorescence assay), which could indicate that the cells retained essential secretory activity.

Keywords: tissue-engineered construct, TEC, skin equivalent, mild hypothermia, keratinocytes, MSCs

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Koltzov Institute of Developmental Biology, RAS (protocol № 51 of 09 September 2021) and conducted in accordance with the principles of the WMA Declaration of Helsinki and its subsequent revisions.

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ВЫЖИВАЕМОСТЬ КЛЕТОК ЧЕЛОВЕКА В БИОМЕДИЦИНСКИХ КЛЕТОЧНЫХ ПРОДУКТАХ ПРИ ХРАНЕНИИ ПРИ КОМНАТНОЙ ТЕМПЕРАТУРЕ

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Биомедицинские клеточные продукты (БМКП) — дермальный эквивалент кожи (ДЭК) и биологический эквивалент кожи (БЭК) — это аллогенные эквиваленты дермы и кожи человека, применяемые для лечения в случаях критической потери кожи. Одним из важных вопросов разработки БМКП является подбор условий хранения, способствующих увеличению срока их годности для расширения возможностей логистики и использования. Целью исследования было определить срок годности ДЭК и БЭК путем оценки морфологии и жизнеспособности клеток в их составе и уровней секреции ими фактора роста эндотелия сосудов (VEGF) в процессе хранения в физиологическом растворе при комнатной температуре. Используя МТТ-тест и окраску витальным красителем, мы установили, что при хранении обоих видов БМКП в физиологическом растворе снижение жизнеспособности и метаболической активности клеток более чем на 50% происходило к 3–4 суткам хранения, причем в ДЭК быстрее, чем в БЭК. Морфология клеток, выделенных из ДЭК и БЭК после 3 суток хранения, оставалась неизменной. После помещения БМКП в физиологический раствор мезенхимные стволовые клетки в их составе продолжали синтезировать VEGF (показано методом ИФА), что может свидетельствовать о сохранении ими необходимой секреторной активности.

Ключевые слова: биомедицинский клеточный продукт, БМКП, эквивалент кожи, умеренная гипотермия, кератиноциты, МСК

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Tissue-engineered constructs (TECs) assessed in this study, the dermal equivalent (DE) and the skin equivalent (SE), are three-dimensional equivalents of the skin and derm that comprise living cells cultured on the surface of biocompatible matrices. One of the TEC disadvantages is the low survival rate of the cellular component when frozen and its short shelf life at positive temperatures, which limit TEC logistics and use. To introduce TECs into a wide clinical practice, it is necessary to find a balance when the cell-based product would not require storage conditions that are difficult to maintain but simultaneously would meet such criteria as functional activity and sufficiently high viability of the cellular component by the moment of transplantation. Developing the reliable method for TEC storage without using xenobiotics would facilitate their widespread integration into clinical practice. Furthermore, an adequate shelf life of the ready-to-use cell-based products

would provide enhanced capabilities for quality control before usage [1].

While successful preservation of the frozen plain TECs has been reported, viability of the cellular component of the cell- and matrix-based 3D systems after thawing usually does not exceed 50% [2–4]. Such viability scores are insufficient to achieve optimal therapeutic outcome. At the same time, there is evidence of successful full-thickness skin graft storage in simple saline solutions at low positive temperatures [5, 6].

These data combined with the need to use complex freezing/thawing modes, specialized equipment, and methods for TEC cleaning of cryopreservation media before usage suggest that the development of the protocol for TEC storage at positive temperatures by the moment of transplantation is promising.

It is known that the range of positive temperatures enabling preservation of cells without significant loss of vitality is +4–+37 °C, while the viability rates at +5 and +25 °C are different. Thus, the cold-induced injury occurs in rat hepatocytes at a temperature below +16 °C, and the injury reaches its maximum at +4–+8 °C [7]. As has been previously demonstrated, human cells can retain 90% viability when stored in isotonic solutions at low positive temperatures for 30 h [8]. There is evidence that adipose tissue-derived mesenchymal stem cells (AT-MSCs) retain all their properties when stored in saline solutions at room temperature [9]. The method for the MSC-based transplant storage under mild hypothermic conditions being an alternative strategy for short-term graft preservation has also been proposed. The authors have earlier shown that the viability of MSCs as a component of TECs is significantly higher at +25 °C than at +4 °C [10].

In view of the above, the sample storage temperature of +22 °C was chosen for this study. The TECs (DE and SE) stability was assessed throughout three-day storage based on the following properties: sample integrity and color; volume, color, and clarity of the liquid in the dish containing the sample; sterility of the sample; the number of viable cells in the sample, metabolic and secretory activity of these cells. The DE TEC was constructed using the biocompatible matrix containing hyaluronic acid and type I collagen and the cellular component (AT-MSCs). The SE TEC was constructed using the biocompatible matrix containing hyaluronic acid and type I collagen and the cellular component (AT-MSCs and skin keratinocytes). The study was aimed to determine the shelf life of the ready-to-use DE and SE TECs stored at room temperature consistent with the criterion of preserving secretory activity and high viability of their cell component.

METHODS

The study was performed in the laboratory conditions. All the procedures involving cell growth and cell culture transfer to biopolymer matrices were performed under aseptic conditions.

TEC engineering and storage

TECs were constructed using human cells obtained from the Cell Culture Collections for Biotechnological and Biomedical Research (Koltzov Institute of Developmental Biology, RAS, Moscow, Russia). AT-MSCs and keratinocytes had been isolated from the skin biopsies of healthy adult donors (40–70 years of age).

The G-DERM, histoequivalent-bioplastic material (G-Group; Russia) made of hyaluronic acid and type I collagen, was used as a matrix for cell growth when culturing TECs.

Thawing and culturing cells

The cryotubes containing cells (1 × 10^6 cells per a tube) were retrieved from the cryogenic storage tanks, thawed at +37 °C, and centrifuged at 200 g. After that the supernatant was removed, and the cellular precipitate was resuspended in the culture medium and transferred to the T25 culture flasks (SPL; Korea).

The α-MEM culture medium (PanEco; Russia) supplemented with 10% fetal bovine serum (FBS)(Hyclon; USA), 1% Glutamax (Gibco; USA), and 1% PenStrep (Gibco; USA) was used for AT-MSCs. Cells were incubated in the CO_2 incubator set to +37 °C, 5% CO_2, and high humidity. The culture medium was changed completely every two days. The cells were passaged after reaching 80% confluence. Passaging was performed in accordance with the following scheme: the culture medium was withdrawn, the cells were washed once with the Versene solution (PanEco; Russia), then 800 μL of the 0.05% trypsin solution (Gibco; USA) were added to the flask and incubated at +37 °C for 5–10 min until the cells became round and detached from the bottom. Trypsin was inhibited by the culture medium. The acquired cell suspension was pipetted and subcultured into new flasks at a 1 : 3 ratio.

The DMEM/F12 culture medium (PanEco; Russia) supplemented with 10% FBS (Hyclon; USA), 10 ng/mL of epidermal growth factor (EGF) (Sigma; USA), 1% Glutamax (Gibco; USA), 1% PenStrep (Gibco; USA), and 1% ITS (Gibco; USA) was used for keratinocytes. The cells were cultured the same way as AT-MSCs.

Construction of the DE and SE TECs

Biocompatible matrix for TECs was prepared as follows: a piece of the G-DERM dry sheet sized 6 × 6 cm was cut and placed into a Petri dish with a diameter of 10 cm, then 10 mL of culture medium was added to the Petri dish, and the sheet was left in the medium for 2 h to swell.

For DEs fabrication AT-MSCs were passaged 6–8 times. The cells were removed from the culture flasks using the Versene solution and trypsin as described above and centrifuged at 300 g. The cellular precipitate was resuspended in the culture medium for AT-MSCs to obtain a concentration of 3 × 10^5 cells/mL. The suspension containing AT-MSCs was applied on the well-prepared biocompatible matrix, 3 mL of cellular suspension per one DE. Two hours after cell seeding the volume of the medium in the dish containing the matrix was adjusted to 10 mL, and the equivalent obtained was incubated in the CO_2 incubator for three days.

Cells of two types were used to prepare SEs: AT-MSCs were passaged 6–8 times, and keratinocytes were passaged 1–2 times before the procedure. At the first stage, AT-MSCs were plated on the matrix as described above. Two hours after seeding AT-MSCs, the second layer of cells (keratinocytes) was applied on the matrix. To do this, keratinocytes were removed from the culture flask and centrifuged at 300 g. The cellular precipitate was resuspended in the culture medium for keratinocytes. The cellular suspension was poured into the Petri dish containing the previously prepared matrix with AT-MSCs based on the ratio of 1 × 10^5 cells/cm^2. After that the TEC was incubated in the CO_2 incubator for three days.

TEC storage

The culture medium was removed from the Petri dishes containing DEs and SEs, and they were washed twice with

DPBS (PanEco; Russia). After that, 10 mL of sterile saline were added to each dish containing TEC, and the dishes were packed in vacuum bags. The ready-to-use TECs obtained by this method were stored in a thermostat at a temperature of +22 °C for five days. Several samples were withdrawn for analysis every day.

Visual assessment of sample quality after storage

Sample integrity and color change, volume, color, and clarity of the liquid in the dish containing the sample, and the sample sterility were assessed after the sample withdrawal from storage prior to further analysis of the cellular component in the following way. After the packages containing samples were transferred to the laminar flow hood, where the secondary packaging (vacuum bag) was removed, the Petri dish was opened, the saline used for sample storage was collected, the liquid volume was measured, and liquid was transferred to the new dish. The saline was inspected using the IX73 inverted microscope equipped with the DP74 camera (Olympus; Japan) at 200× and 400× magnification to reveal the traces of contamination. The sample was examined and tested for integrity using tweezers. Then assessment of the cellular component viability and functional activity was performed.

Assessment of the cells ability to adhere and form cell cultures after being part of a TEC

After storage, cells were isolated from TECs in the following way. The samples were washed with the Versene solution (PanEco; Russia), then treated with 3 mL of the 0.25% trypsin solution (PanEco; Russia) and left for 10 min at +37 °C with intense shaking for fermentation. After that trypsin was inhibited by the equal volume of culture medium supplemented with 10% FBS and pipetted in order to wash as much cells away from the matrix surface as possible. Then the obtained suspension was centrifuged for 5 min at 200 g. Supernatant was removed, while the precipitate was resuspended in the culture medium and plated on the cell culture Petri dishes. A day later, the cells were examined and imaged using the IX73 inverted microscope equipped with the DP74 camera (Olympus; Japan).

Assessment of metabolic activity of the cells on the surface of TECs using MTT assay

To estimate metabolic activity at every time point of storage, TECs were taken out of the packages, cut into fragments sized 1 × 1 cm, and put into separate wells of the 24-well plate. Then MTT reagent (Sigma-Aldrich; USA) was added to the wells to reach the final concentration of 30 μg/mL and incubated for 2.5 h. After incubation, the medium was collected, and 180 μL of DMSO (PanEco; Russia) were added to each well. The plate was placed on the shaker. After 1 min the stained solution was collected and put into the wells of the 96-well plate (60 μL per well) for optical density measurement. Optical density of the solution was measured using the Stat Fax 2100 photometer (Awareness Technology; USA) at the wavelength of 530 nm.

Identification of viable cells on the surface of TECs

To identify viable and dead cells on the surface of TECs withdrawn from storage, the samples were stained using the Calcein AM vital intercalating dye (1 μM) (Sigma; USA) in accordance with the manufacturer's guidelines. TECs were stained in the CO_2 incubator (+37 °C, 5% CO_2) for 30 min. The staining method is based on the activity of intracellular esterases present in living cells only. These esterases cleave the dye, thereby making it emit fluorescence in the green spectral region. No fluorescence is detected in the dead cells. The stained samples were examined and imaged using the IX73 inverted microscope equipped with the DP74 camera (Olympus; Japan). The protocol of the Image J software (LOCI, University of Wisconsin; USA) with the open-source plugin [11], which calculated the share of regions stained with calcein in the field of view, was used to define the percentage of living cells in TECs. The values were obtained via three repetitions of the experiment, and the data calculated by assessing original TEC samples cultured in the CO_2 incubator for three days were taken as 100%.

Assessment of the TEC secretory activity by determination of VEGF levels in the conditioned medium by immunofluorescence assay

The samples of normal saline used for TEC sample storage were collected daily throughout the tree-day storage in the following way. Packages containing samples were transferred to the laminar flow hood, where the secondary packaging (vacuum bag) was opened, and 100 μL of the liquid were collected from each dish containing TEC under sterile conditions. After sample collection, the dishes were sealed again and moved into the thermostat for further storage. Original TEC samples in the culture medium and AT-MSCs (of the same cell line and passage as the cells of the studied TECs), which were seeded in the wells of the 24-well plate and cultured utilizing standard method, were used as a control. To provide the control, the growth medium samples were collected before the beginning of the study along with the samples of the medium used for incubation of the matrix alone during the same period. The VEGF-EIA-BEST (A-8784) kit (Vector-Best; Russia) was used for the assessment.

The analysis was performed in accordance with the manufacturer's instructions. Spectrophotometric analysis of the samples was performed at the wavelength of 450 nm using the xMark Microplate Absorbance Spectrophotometer (Bio-Rad; USA).

We calculated mean optical density (OD) values for the well pairs and plotted a calibration curve of OD as a function of VEGF concentration (IU/mL) using linear coordinates. The curve was used to define the concentrations of the control and test samples in IU/mL in accordance with the guidelines issued by the manufacturer of the kit. The results were taken into account when the value for the control sample calculated based on the calibration curve matched the value specified on the label of the flask in the kit.

Statistical analysis

All the experiments with TECs were performed in three biological replicates; each individual experiment — at least in three technical replicates. After obtaining the values in the experiments involving calculation of the share of viable cells and when assessing the MTT assay results, the values were normalized to the data obtained when assessing intact samples before the beginning of the experiment. The mean and mean standard deviation were calculated in Microsoft Excel (Microsoft Corporation; USA).

Significance of differences in the groups was tested by ANOVA using the Prism 8 (GraphPad Software).

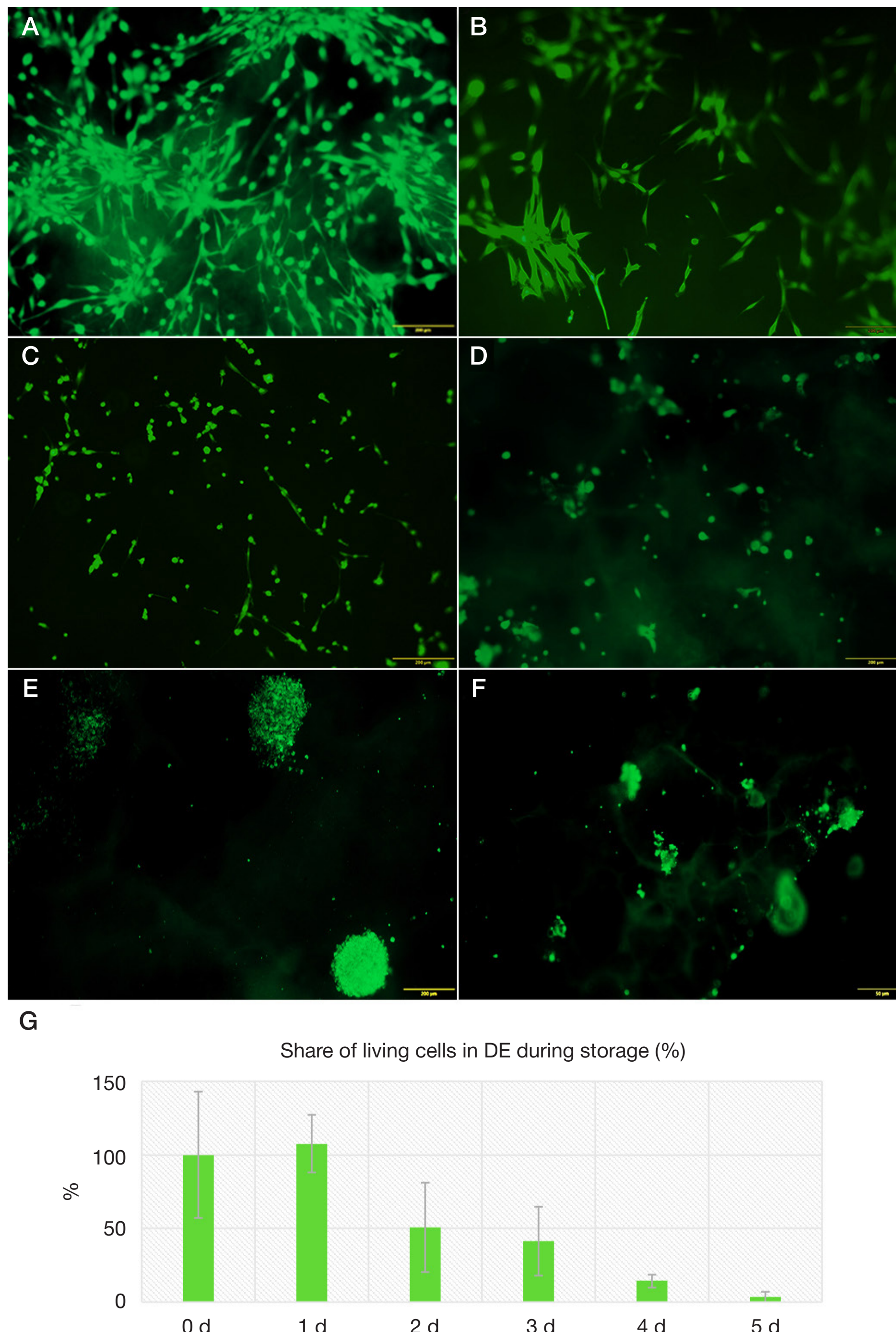

Fig. 1. Identification of living cells stained with the calcein vital dye (green stain) on the matrix surface in the DE TEC at various stages of storage at a temperature of +22 °C (**A–F**): at the beginning of the experiment (no storage, zero day) (**A**), day one (**B**), day two (**C**), day three (**D**), day four (**E**), and day five (**F**) of storage. **G**. The graph showing the percentage of living cells in the DE TEC as a function of the sample condition on the zero day of storage. The data are presented as the mean share of green pixels in three fields of view for three technical repetitions, error bars ±SD (%)

RESULTS

All the samples seemed to be preserved after removing the secondary packaging when withdrawing from storage: the normal saline in which the TECs had been stored remained clear, no volume reduction or contamination by microorganisms was revealed. The samples remained unimpaired and retained their original pinkish-beige color throughout the study.

Viability of the cells in the cultured and packed ready-to-use TECs that were stored at +22 °C was assessed by two methods: by staining with the calcein vital dye and by MTT assay.

The results of the experiment showed that the number of living cells decreased faster in the DE TECs. By the second day, a two-fold decrease in the number of calcein-positive cells was observed in the DE (Fig. 1), while in the SE no decrease was revealed (Fig. 2). Furthermore, the AT-MSC morphology alterations were observed in equivalents of both types on day three: the cells that were normally spindle-shaped became less elongated, several cells started to detach from the surface of the matrix. The critical decrease in the number of living cells in both TECs, to 14% in DE and to 22.3% in SE (the confirmed significance for both TEC types (p-value) ≤ 0.005), was detected on day four of storage (Fig. 1, 2).

The second method we had selected to assess the state of the TEC cellular component involved measuring the intensity of the formazan crystal formation in the cell environment when

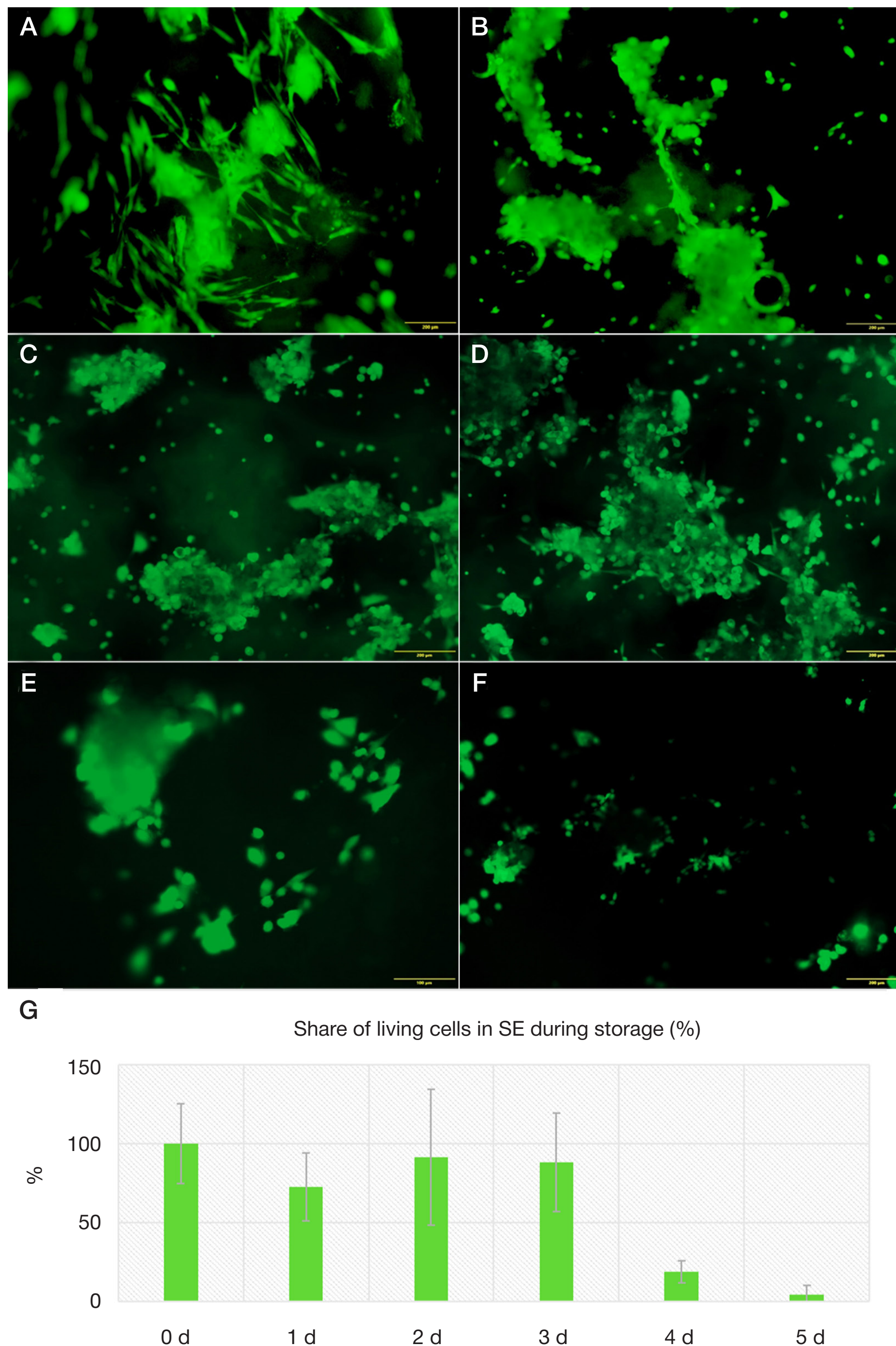

Fig. 2. Identification of living cells stained with the calcein vital dye (green stain) on the matrix surface in the SE TEC at various stages of storage at a temperature of +22 °C (**A–F**): at the beginning of the experiment (no storage, zero day) (**A**), day one (**B**), day two (**C**), day three (**D**), day four (**E**), and day five (**F**) of storage. **G**. The graph showing the percentage of living cells in the SE TEC as a function of the sample condition on the zero day of storage. The data are presented as the mean share of green pixels in three fields of view for three technical repetitions, error bars ±SD (%)

exposed to the cell metabolites that correlated with the metabolic rate of the cells, which was considered reflecting cell viability. This test has shown that cell metabolic activity decreases faster in DE than in SE, since metabolic activity reduction to 44.6% compared to the beginning of the storage has been revealed in DE ($p \leq 0.005$), while in SE it has been still at 79.1% by day three (the difference from the baseline value at this stage was non-significant). Only 18% of the cell baseline metabolic activity was observed in DE by day five, while in SE metabolic activity significantly decreased to 33.7% ($p \leq 0.005$) (Fig. 3A, B).

When assessing concentration of VEGF secreted by cells into the normal saline used for sample storage, VEGF was revealed in all samples. The levels of this factor in the storage solution decreased slightly by day three (Fig. 3C, D). Thus, about 506 ± 136 IU/mL of VEGF were revealed in the samples of the solution used for storage of DEs in the first day, while there were 493 ± 136 IU/mL by day three (the difference was non-significant); 1583.333 ± 189 IU/mL and 1733 ± 208 IU/mL were revealed in the samples of the solution used for storage of SEs at the same stages, respectively (the difference was non-significant).

The cellular component was isolated from TEC on day three. As previously described, cells of only one type, AT-MSCs, were used to create DEs, while cells of two types, AT-MSCs

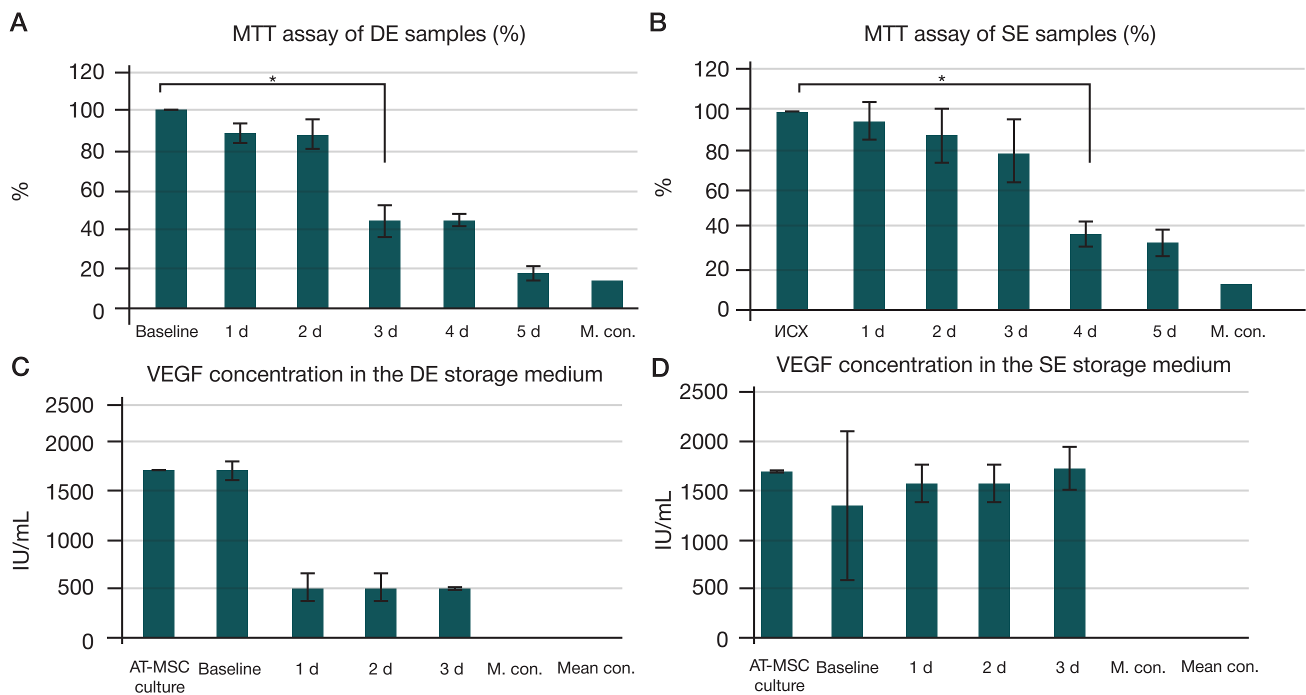

Fig. 3. The changes in the cells' mitochondrial activity assessed by MTT assay in the DE (**A**) and SE (**B**) TECs and presented as mean optical density values obtained at various stages of storage vs. data obtained before storage (zero day), percentage obtained in three technical repetitions, error bars ±SD (%). Significance of the metabolic activity decline observed on day three in DEs and day four in SEs is calculated by ANOVA ($p \leq 0.005$). The data on the VEGF levels in the normal saline used for the DE (**C**) and SE (**D**) TEC sample storage measured by enzyme immunoassay are presented as mean optical density for nine repetitions, error bars ±SD (%). Matrix with no cells, culture medium, and original AT-MSC culture used to construct TECs were used as controls. There were no significant differences between the normal saline samples in the storage groups within three days

and keratinocytes, were used to create SEs, that is why the cells isolated from DEs and SEs were different in morphology and number. All the isolated cells showed normal ability to adhere. These cells quickly attached to the bottom of the cell culture dish, spread, acquired standard morphology (Fig. 4A, C), and retained it throughout the observation period (up to four days) (Fig. 4B, D). Visual inspection showed that the number of attaching AT-MSCs isolated from SEs was significantly higher than that of AT-MSCs isolated from DEs (Fig. 4).

DISCUSSION

The study was focused on the defining the storage duration at room temperature of the DEs and SEs created in our library, when their cellular components retain functioning and viability. Our previous studies (unpublished data) showed that TEC freezing at –70 °C and –20 °C using various cryoprotectants (DMSO, glycerol), specialized freezing media for sensitive cell lines, such as Bambanker (Lymphotec; Japan), and for multicellular objects, such as Cryoderm (PanEco; Russia), resulted in the loss of cell viability after thawing. A significant decrease in the TEC cell viability within 24 h was also reported at a temperature of +4 °C. According to the literature, specialized commercially available culture media (such as Synth-a-Freeze) are often used to freeze TECs, and the freezing/thawing protocols are carefully developed [4, 12, 13]. This complicates and increases the cost of TEC storage and logistics. As noted by our colleagues from the Privolzhsky Research Medical University in their review focused on cryopreservation of MSCs and TECs, there is little research on cryopreservation of the cell-based 3D structures and TECs, and there is no the whole picture of the mechanisms underlying freezing of such products [14]. The conditions are developed specifically for each product, and TEC cryopreservation method was designed initially for longer storage. According to global practice, bioengineered tissue constructs are delivered on ice “just-in-time”. The exceptions include the FDA-approved commercially available tissue-engineered Dermagraft and Apligraf, which are stored and shipped at –75 °C and +20–+23 °C, respectively [15]. Thus, the task of developing the conditions for the short-term storage of tissue-engineered constructs is quite important. The literature analysis has shown that the solution is usually found out for each variant of the product.

Our study was aimed to determine the possibility of the DE and SE TEC storage under the most technically feasible conditions: without the use of any expensive imported reagents, accessory equipment or specific thermal modes. Furthermore, storage in the sterile saline, which represents an approved medicinal product, simplifies TEC preparation for use, since there is no need to wash TECs from saline before transplantation to a patient.

We managed to show that DEs and SEs generally retained their main quality parameters after three days of storage under most simple conditions (room temperature and normal saline). Furthermore, SEs showed better results compared to DEs. The higher cell survival rate in SEs is probably due to the presence of two cell types that have a beneficial effect on the viability of each other. After assessing the rate of viability decline in the studied samples we concluded that storage for more than three days under the current conditions resulted in the reduction of the viable cell number by more than 50% in TECs of both types. Consequently, we did not examine the samples stored for more than 72 h in further research.

As the expected therapeutic effects of the DE and SE TECs include not only mechanical skin wound closure, but also the wound healing regenerative effect, the cellular component secretory activity is an important indicator of the TEC quality. Mesenchymal cells, such as MSCs, are known to play a key role in regulation of the cell-cell interaction and maintaining tissue homeostasis [16]. The AT-MSCs can stimulate angiogenesis

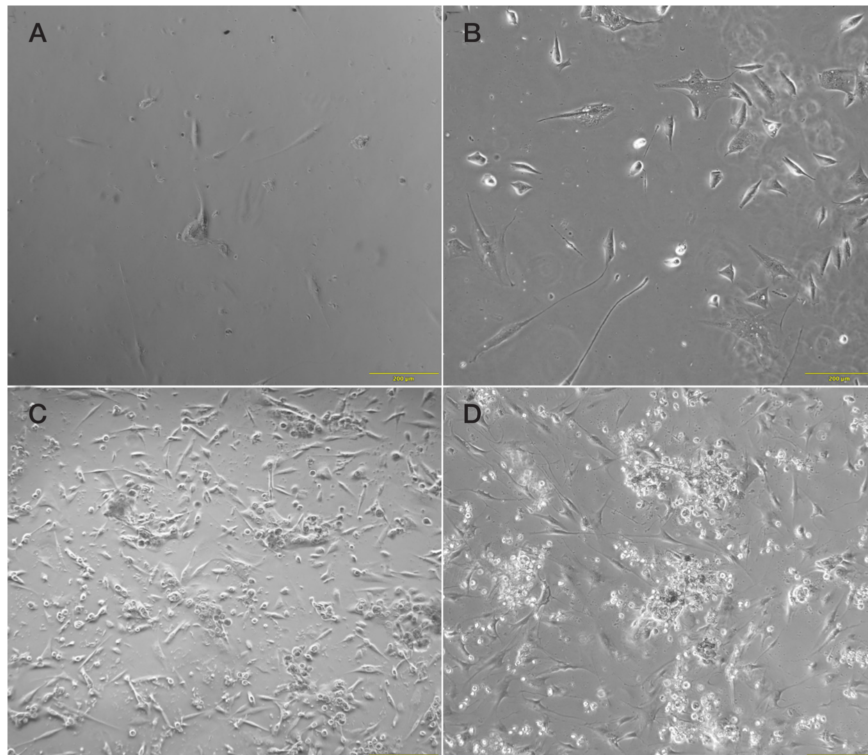

Fig. 4. The culture of cells extracted from DE (**A**, **B**) and SE (**C**, **D**) TECs after 72 h of storage. Cells after one day (**A**, **C**) and three days (**B**, **D**) after extraction (light microscopy, phase contrast)

via activation of blood vessel growth and stabilization of the growing vessels [17]. Growth factors of the VEGF family produced by these cells can induce directed cell migration, proliferation, and differentiation of endothelial cells in the region of the defect via interaction with their VEGF-R receptors, which is reflected in the reduced defect area, the increased number of blood vessels, and higher blood vessel density [9]. Normally, the cultured cells of TECs actively secrete growth factors, such as VEGF. In our experiment, VEGF levels in the culture medium were 1600–2000 IU/mL or higher. Furthermore, we showed that AT-MSCs in TECs we had constructed kept producing VEGF in the normal saline, and the VEGF secretion levels were comparable with its levels in the culture medium used for incubation of TECs before packaging and storage.

The results of the experiment that involved measuring VEGF concentrations for SE and DE suggest that keratinocytes contribute to the AT-MSCs survival and promote VEGF secretion by them.

CONCLUSIONS

When the SE TEC is stored under mild hypothermia conditions at room temperature (+22 °C), its cellular component viability remains at the level over 50% of the value obtained at the beginning of the three-day storage. Viability of the DE TEC cellular component decreases faster: the values that do not exceed 50% of the baseline value are observed by day three. The demonstrated ability of AT-MSCs on the surface of TECs to secrete VEGF when stored under selected conditions can be an indicator of paracrine activity exerted by these TECs that can play a therapeutic role in treatment of skin wounds of different etiology and can be considered an indirect sign of the cell functional activity in the ready-to-use product. The SE cellular component viability and metabolic activity during storage are significantly higher than that of DE. These findings are essential to accelerate implementation of the tissue engineering and regenerative medicine methods into clinical practice.

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