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EXPERIMENTAL ORTHOTOPIC IMPLANTATION OF TISSUE-ENGINEERED TRACHEAL GRAFT CREATED BASED ON DEVITALIZED SCAFFOLD SEEDED WITH MESENCHYMAL AND EPITHELIAL CELLS

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Objective: to study the viability of a tissue-engineered graft (TEG) based on a devitalized tracheal scaffold (DTS) seeded with mesenchymal stromal and epithelial cells in an experiment on rabbits with assessment of cytocompatibility and biocompatibility *in vivo*. **Materials and methods.** Syngeneic mesenchymal stromal bone marrow cells (MSBMCs) and syngeneic lung epithelial cells of rabbit were obtained. The morphology and phenotype of the MSBMC culture were confirmed via immunofluorescence staining for CD90 and CD271 markers. Pulmonary epithelial cells obtained by enzymatic treatment of minced rabbit lung tissue were stained with CKPan, CK8/18 and CK14 markers characteristic of epithelial cells. The donor trachea was devitalized in three successive freeze-thawing cycles. Double-layer cell seeding of DTS was performed under static and dynamic culturing. Orthotopic implantation of TEGs was performed at the site of the anterolateral wall defect in the rabbit that was formed as a result of tracheal resection over four rings. Results were evaluated by computed tomography, histological and immunohistochemical analyzes. **Results.** A TEG implant, based on DTS, with bilayer colonization by cell cultures of rabbit MSBMC and epithelial cells was obtained. Three months after implantation, TEG engraftment was noted, no tracheal wall stenosis was observed. However, slight narrowing of the lumen in the implantation site was noted. Six months after implantation, viability of TEG was confirmed by histological method. Epithelialization and vascularization of the tracheal wall, absence of signs of purulent inflammation and aseptic necrosis were shown. The small narrowing of the lumen of trachea was found to have been caused by chronic inflammation due to irritation of the mucous membrane with suture material. **Conclusion.** A new model for assessing the viability of a tissue engineering implant when closing a critical airway defect was created. The developed TEG – based on DTS seeded (bilayer) by lung epithelial cells and BMSCs – was successfully used to replace non-extended tracheal defects in an *in vivo* experiment. The use of tracheal tissue-engineered graft for orthotopic implantation showed biocompatibility with minimal tissue response.

Keywords: devitalization, implant, cell- and tissue-based therapy, tissue engineering, tissue therapy, thoracic surgery, transplant, trachea.

INTRODUCTION

Chronic trachea stenosis caused by oncological and non-oncological processes, is usually difficult to treat when widely spread. The frequency of stenotic tracheal lesions is up to 1% of all intubations [1] and reaches 20–25% with prolonged intubation [2]. Endoscopic stenting with porous endoprostheses does not solve the problem due to the germination of the mesh structure of the stent by the patient's own tissues or the formation of marginal restenosis. Radical surgeries like the circular resection of the affected area is significantly limited by, for example, the extent of the defect, and also carry a high risk of complications [3]. The most promising technique for treating such defects may be the use of tissue-engineering grafts (TEGs). However, the clinical use of tissue-engineering structures of the trachea is accompanied by many problems, such as the need to ensure sufficient blood supply, the formation of a respiratory epithelium and preventing softening of the tracheal wall [4, 5].

At present, an acellular scaffold of the tracheal cartilage was obtained in the experiment under the combined action of detergents and DNase I [6], the airway was recellularized by seeding mesenchymal stromal cells (MSC) [7], and the stimulating effect of the bone marrow mesenchymal stem cells (BM MSC) was shown for epithelialization and formation of cartilage tissue [8]. The combination of these techniques was used to obtain the trachea TEG.

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Go et al. (2010) evaluated the effect of epithelial cells and chondrocytes obtained during differentiation from MSC on the effectiveness of TEG in airway regeneration in porcines [9]. The scaffolds were obtained by detergent-enzymatic decellularization of allogeneic trachea. Autologous MSCs were obtained from bone marrow and differentiated into chondrocytes. Autologous epithelial cells were isolated from the trachea mucous membrane. Both types of cells were plated on a scaffold using a two-chamber bioreactor under dynamic cultivation. Substitution of a 6 cm trachea was performed by TEG engraftment. The seeding of scaffold with epithelium and chondrocytes obtained from MSC was shown to lead to tissue regeneration of the tracheal wall.

In Jungebluth et al. (2012) TEG was studied on the basis of porcine decellularized trachea seeded by autologous mononuclear cells from the bone marrow from the outside and epithelial cells from a biopsy of the oral mucosa from the inside [10]. The resulting grafts were implanted into pigs in a 6 cm tracheal defect. By the 60th day after engraftment, there were no differences in mechanical properties compared to native tissue, and the inner surface of the trachea was lined with epithelium, without signs of the graft rejection or any pronounced inflammatory response.

To repair the rabbit tracheal defect, Shin et al. (2015) used BM MSC – based TEGs and a scaffold formed from crushed decellularized articular porcine cartilage [11]. Histological examination showed that cartilage was formed with minimal inflammation or tissue granulation.

However, the viability of TEG based on a devitalized tracheal wall with a large residual content of exogenous markers at seeding of such a scaffold with a combination of two cell types was not previously evaluated in *in vivo* experiments. Besides, the literature does not describe the use of a full-fledged experimental model to assess the viability of a tissue-engineering graft when closing critical airway defects which would allow drawing conclusions about the biological and physiological compatibility and stability of the used design.

Purpose. To study the viability of a tissue engineering graft (TEG) based on devitalized tracheal scaffold (DTS) seeded by mesenchymal stromal and epithelial cells, on a model for assessing the viability of a tissue engineering graft at closing a critical airway defect in rabbits and to assess the TEG potential to maintain a stable trachea lumen in the area of engraftment.

MATERIALS AND METHODS

Laboratory animals

The experiments were performed on linear Gray Giant breed rabbits (n = 3) with 3.5–4.5 kg initial body weight, 0.5–1 years old, grown in the nursery of the Biomedical Technologies Research Center of the Federal Biomedical Agency of Russia (Svetlyie Gory settlement, Moscow Oblast). The animals were kept in the Central Vivarium of the I.M. Sechenov First Moscow State Medical University of the Ministry of Healthcare of the Russian Federation with free access to food and water.

All manipulations with laboratory animals were approved by the Local Ethics Committee of the FGAOU VO I.M. Sechenov First Moscow State Medical University of the Ministry of Healthcare of the Russian Federation (Sechenovsky University) and carried out in compliance with the Rules of bioethics approved by the European Convention for the Protection of Vertebrate Animals used for experiments or other scientific purposes (2005), and in accordance with the Laboratory Practice Rules approved by Order of the Ministry of Health of Russia No. 708 of 23.08.2010.

BM MSC culture

BM MSC was obtained in accordance with the protocol used in the CCP "Regenerative Medicine" of the Sechenovsky University. Syngeneic rabbit MSCs were isolated from bone marrow obtained by the femur perfusion. A bone marrow aspirate was placed in a sterile tube containing 50 IU/ml heparin and 0.25 mg/l gentamicin in PBS and delivered to the laboratory at +20–24 °C. Cells isolation and handling were carried out in the 5th grade clean zone according to ISO. A 10 ml bone marrow aspirate was placed in a centrifuge tube and precipitated at 350 g for 5 min. The supernatant was removed, the cell pellet was resuspended in 20 ml of lysis buffer (114 mM NH4Cl, 7.5 mM KHCO3, 0.1 mM EDTA) for 3–5 min, then centrifuged again; the cell pellet was resuspended in BM MSC culture medium based on DMEM/F12 culture medium (Invitrogen, USA) containing 10% FBS (Invitrogen, USA), 0.4 μM insulin, 20 ng/ml bFGF, 10 nM dexamethasone, 100 u/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). The cell suspension was seeded in an amount of 1.0–1.5 × 10^6 cells/ml in culture flasks and placed in a CO_2 incubator with a 5% CO_2, atmospheric air 95% at +37 °C with high humidity. The culture medium was changed every 72 h. When 90% confluency was reached, the cells were washed with DPBS without Ca^{2+} and Mg^{2+} ions, then removed from the culture plastic with TrypLe solution (Invitrogen, USA), centrifuged at 350 g for 5 min; the supernatant was taken and the precipitate resuspended in a nutrient medium, and $^1/_3$ of the cell suspension was placed on a new culture dish.

Epithelial cell culture

Syngeneic epithelial cells were isolated from a native rabbit lung that was washed from the blood components by prolonged perfusion of the pulmonary circulation with

physiological saline with a continuous supply of the latter into the right heart ventricle of a donor rabbit with a roller pump. The purified lung tissue was cleaned to white. The lung was mobilized, separated from the surrounding tissues and, after vascular junction, was placed in a container with a transport medium of DMEM/F12 with the addition of 100 U/ml penicillin and 100 μg/ml streptomycin, and delivered to the laboratory at +20…24 °C. In a clean zone, the lung was crushed into 4 = 2 mm^3 pieces and treated with a dispase solution of 5 Г/ml for 45 min at +37 °C. The resulting suspension was filtered through a 70 μm sieve to separate lung tissue fragments from the cell suspension. The filtrate was centrifuged at 1200 rpm and the pellet was resuspended in complete KSFM culture medium (Invitrogen, USA) with the complement of Keratinocytes Supplements (Invitrogen, USA), 100 U/ ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA). The resulting suspension was seeded to the bottom of a 24-well culture plate previously coated with type I collagen. The cells were cultured under conditions of CO_2 incubator with the culture medium replaced every 72 h.

Assessment of BM MSC phenotype and tracheal epithelium

The preparation of BM MSC cultures and rabbit tracheal epithelium was confirmed by TIPS with antibodies: CD90, CD271, CDH, SOX2, COL1, Vimentin for BM MSC and CK8/18, CK14, CKPan for epithelium, respectively. For this, BM MSC of the first passage and the zero-passage tracheal epithelium was washed three times in PBS and fixed in 4% paraformaldehyde solution in PBS (pH 7.4) for 10 min at 25 °C. Then, the cells were washed three times in PBS with 0.05% Tween 20 (PBS-T) for 5 min, permeabilized with methanol at –20 °C, and nonspecific antibody binding was blocked with a solution of 2% BSA in PBS-T for 20 min at +25 °C. The solution was removed and the primary monoclonal antibodies were applied at a dilution of 1:100 anti-CDH, anti-COL2, anti-CD90, anti-SOX2, anti-Vimentin for MSC culture and anti-CK8/18, anti-CK14, anti-CKPan for culture tracheal epithelial cells in a solution of 2% BSA in PBS-T and incubated for 12 h at +4 °C. Then the cells were washed three times in PBS for 5 min and secondary polyclonal antibodies at a dilution of 1:500 was applied conjugated with biotin to primary antibodies CD90, CD271, SOX2, CKPan, diluted in PBS-T with the addition of 2% BSA. Incubation was carried out for 60 min at +25 °C. Then the cells were again washed with PBS three times for 5 min and applied to PBS-T with the addition of 2% BSA: streptavidin-Alexa Fluor 488 to secondary polyclonal antibodies conjugated with biotin; secondary polyclonal antibodies Alexa Fluor 594 to primary antibodies CDH, Vimentin and biotinylated antibodies to CD90, CD271, SOX2, CKPan, CK-8/18, CK-14. As a negative control of secondary antibodies to the test cultures, no primary antibodies were added. Incubation was carried out for 30 min at +25 °C in the dark. Cells were washed with PBS three times for 5 min. The cores were stained with Hoechst-33342 at 1 μg/ml.

Rabbit trachea devitalization

A donor rabbit trachea sample isolated from the second tracheal ring to the bifurcation was used as a native material to obtain a cell-free host scaffold. By devitalization we understand the death of living cells in tissue while maintaining cellular contents and intercellular scaffold in the material. Prior to devitalization, the trachea sample was cleaned of surrounding tissues and washed with PBS buffer to remove blood clots. Then the sample was placed in a test tube with PBS buffer and frozen for 15 min in liquid nitrogen. After freezing, the samples were thawed in a water bath at +37 °C for 30 min, the PBS buffer was replaced, and then the freeze/thaw cycles were repeated twice. The process of devitalization was completed by washing the trachea samples in a 70% solution of ethyl alcohol for 1.5 h. Then the samples were placed in PBS with the addition of 100 units/ml penicillin and 100 μg/ml streptomycin for 72 h at +4 °C to remove residual amounts of ethyl alcohol and prevent the possibility of contamination.

Scaffold devitalization quality assessment

To qualitatively assess devitalization, the DTS samples were fixed in 10% formalin in PBS, washed with running water for one hour, dehydrated (Microm STR 120), and enclosed in paraffin blocks according to the standard protocol. 4 microns thick slices (Microm HM 355s) were made from the blocks, placed on uncoated glass slides, then the sections were adhered at +56 °C for 30 min. The resulting sections were dewaxed, hematoxylin and eosin stained, dehydrated and clarified in xylene (Microm HMS 70). Sections were put under coverslips in a synthetic mounting medium. The resulting preparations were digitized with Pannoramic DESK followed by a morphological study.

DTS cytotoxicity assessment

DTS cytotoxicity was evaluated by extraction on a rabbit BM MSC culture. Rabbit DTS extracts were prepared as follows: rabbit DTS samples with 3 cm^2 surface area were placed in 1 ml of BM MSC culture medium and incubated for 72 h at +37 °C. The fourth passage BM MSC were seeded in a 96-well plate at a concentration of 5×10^3 cells/well and cultured in a CO_2 incubator. Cells were counted with Countess II FL Automated Cell Counter. In 24 h, obtained DTS extracts were added to

the cells, titrated six times by successive dilutions (n = 3) in BM MSC growth medium, and cultured for 48 h.

The cytotoxicity of DTS extracts was assessed by evaluating the metabolic activity of cells with PrestoBlue reagent [12]. To conduct a cytotoxicity test, after 48 h of cultivation, the medium was changed to 90 µl/well of HBSS with Ca^{2+} and Mg^{2+} adding PrestoBlue (10 µl/well) and incubated for 30 min in a CO_2 incubator. Then, to stop the reaction, SDS solution was added to 1% final concentration in the well. The absorption was measured at 540 nm wavelength with 630 nm reference by Multiskan FC flatbed photometer. As a control, BM MSC was used in a nutrient medium without additives (n = 3). The background value was considered by adding a reagent to HBSS with Ca^{2+} and Mg^{2+} (n = 3).

TEG creation by seeding of cell cultures

TEG was produced through sequential seeding mesenchymal cells over the entire surface of rabbit DTS samples and epithelial cells of the DTS inner surface. For this, the second passage rabbit BM MSC were seeded on the surface of tracheal samples at a rotation of 3–4 rpm for 48 h at a concentration of 0.25 million cells in 10 µl of BM MSC culture medium per 1 mm^2 of DTS surface. Then, 24 h before engraftment, the inner TEG surface was seeded with epithelial cells of the rabbit trachea of the first passage at 60 × 103 cells/cm^2 and cultivated by the static method in complete KSFM nutrient medium.

Assessment of the viability of cells seeded on DTS

The confocal microscopy with TIPS of cells and the Live/Dead technique was implied to determine the viability of BM MSCs seeded on the DTS surface. For this, the cells seeded on DTS were calcein-AM and ethidium homodimer solutions stained in an incubator for 20 min in a culture medium for BM MSC cultivation. Visualization was performed by Zeiss LSM 710 confocal microscope with Plan-Apochromat 10x/0.45 M27 lens.

TEG orthotopic engraftment

Surgery was performed in aseptic conditions under combined anesthesia (intramuscular injection of a combination of Xila preparations (0.1 ml/kg) and Zoletil-100 (0.1 ml/kg) supplemented with local anesthesia of the surgical field with a 0.5% novocaine solution. For orthotopic engraftment, TEGs received access to the cervical trachea through a longitudinal section along the midline of the neck up to 1 cm long dividing the muscles and fascia of the neck by blunt and sharp dissections. The front and side walls of the trachea were mobilized by blunt dissection. The anterolateral tracheal wall was resected over four rings below the second tracheal ring to form a window. The TEG sizes were corrected intraoperatively for the defect size, fixed in the engraftment area with the inert non-resorbable Proline 6-0 material with the closure of the anterolateral wall defect, the tightness of the tracheal suture being checked, and the wound sutured in layers. The animal was monitored until anesthesia withdrawal and antibiotic therapy for 5 days after the operation. Ketanov (30 mg/ml) was administered 0.5 ml per day.

MSCT

In three months after TEG engraftment, multispiral computed tomography (MSCT) under sedation (intramuscular injection of tiletamine and zolazepam solutions) was performed for an intermediate assessment of TEG engraftment and tracheal lumen in the engraftment area. CT was performed on Siemens Magnetom Verio tomograph with DICOM imaging. To visually assess the condition of the airways and the trachea lumen, a 3D model was created of the airways in STL format by the volumetric segmentation in 3D Slicer.

Morphological analysis

Animals were removed from the experiment after 6 months with Xila (1.0 ml per 1 kg of animal body weight). Autopsy material was taken within the boundaries of the intact trachea, at least 5 mm from the edges of the implant. The trachea with the TEG area was removed and cut into two parts lengthwise in the sagittal projection.

To morphologically evaluate the results of TEG engraftment, the trachea samples were fixed in a 10% neutral buffered formalin solution for 24 h, histologically prepared and enclosed in paraffin blocks by the standard protocol. 4 µm thick slices (Microm HM 355s) were placed on slides and glasses coated with poly-L-lysine, and then adhered at +56 °C for 30 min. The obtained sections were deparaffinized, hematoxylin and eosin stained and placed under coverslips in a mounting medium (Shandon-Mount). The resulting preparations were digitized with Pannoramic DESK, and then analyzed morphologically.

The sections coated with poly-L-lysine were prepared for TIPS to further confirm TEG epithelization. For this, dewaxing was first performed and then the antigen was unmasked in Tris-EDTA (pH 10.0) buffer in a microwave oven at 100 W for 10 min, cooled to room temperature and washed in PBS. Blocked the non-specific binding of antibodies with a solution of 2% BSA in PBS-T for 20 min at +25 °C. The solution was removed and the primary anti-CDH, anti-CKPan and anti-Ki67 monoclonal antibodies were applied in 2% BSA solution in PBS-T and incubated for 12 h at +4 °C. Then the cells

were washed three times in PBS for 5 min and secondary polyclonal antibodies applied with biotin to primary CDH, CKPan antibodies, diluted in PBS-T with the addition of 2% BSA. Incubation was carried out for 30 min at +3 °C. The cells were again washed with PBS three times for 5 min and PBS-T was applied with the addition of 2% BSA: streptavidin-Alexa Fluor 488 to secondary polyclonal antibodies with biotin; secondary polyclonal antibodies Alexa Fluor 594 to Ki67 primary antibodies. The sections without addition of primary antibodies were used as a negative control. Incubation was carried out for 30 min at +32 °C. the sections were washed with PBS three times for 5 min. The cores were Hoechst-33342 stained in 1 μg/ml. The preparations were enclosed under coverslip in 80% glycerol in PBS. Microscopy was performed with Nikon Eclipse TE-2000 fluorescence microscope.

Statistical analysis of results

Statistical analysis was performed in GraphPad Prism version 8.0 with a Gaussian distribution test with Shapiro–Wilk's test and the D'Agostino and Pearson omnibus normality test. The differences between the control group without the extract addition and the analyzed dilutions were checked by One-way ANOVA test and post hoc Dunnett test. Differences was considered significant at $p < 0.05$.

RESULTS

Phenotyping primary cell culture

At BM MSC TIPS it was shown that according to cell morphology, the cells were large, flattened, fibroblast-like. Cells expressed CD90 and CD271surface markers; their concurrent presence is characteristic of MSC. Cells also expressed type II collagen, which is necessary for the synthesis of extracellular scaffold, especially cartilage.

Morphology showed small lung cells with a large number of intercellular contacts, densely located on the plastic. All cells expressed a cytoskeleton protein, total cytokeratin (CKPan), the main epithelial cells marker. At this, CKPan-negative and Vimentin-positive cells were not observed in the culture, indicating the purity of the obtained culture from fibroblast-like cells. Cells were also stained for cytokeratins CK8/18 and CK14, which confirmed the epithelial phenotype [13].

Tracheal tissue devitalization

DTS was compared with native tracheal tissue histologically and with hematoxylin and eosin staining (Fig. 1). In Fig. 1, b, the tissue of the cartilaginous ring of the trachea is seen, with the destroyed tissues of the perichondria, submucosal membrane and epithelium around, especially clear in comparison with native tissue (Fig. 1, a). The structure of the cartilage tissue is preserved. In the depths of the tissue, the remaining chondrocytes are observed in gaps. The architectonics of the cartilaginous tissue has not altered, meaning the preservation of its mechanical strength.

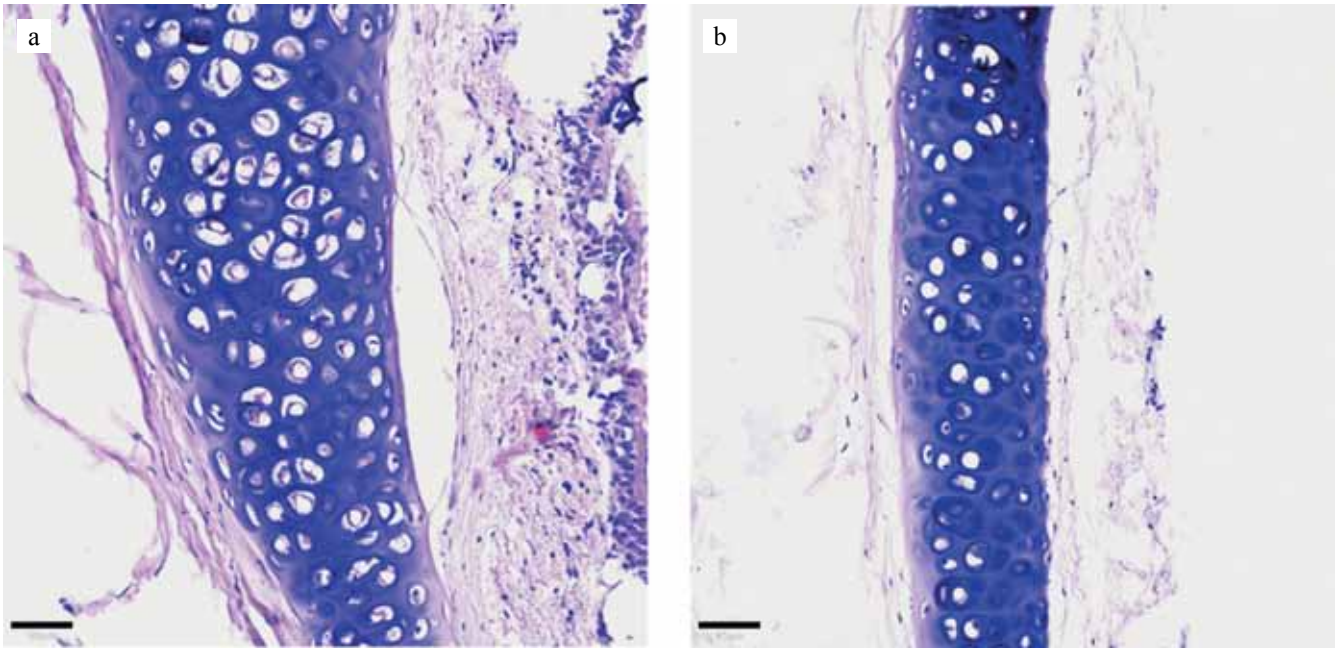

Fig. 1. Tracheal cartilage sample after devitalization: a – native tracheal tissue; b – devitalized tracheal scaffold. Hematoxylin-eosin staining. The scale bar is 50 μm. ×100

DTS cytotoxicity and seeded cell viability

BM MSC cellular activity after DTS extract addition in comparison with the control group was 144.3 = 3.8% ($p < 0.05$).

DTS vitalization created the trachea TEG with completely seeded BM MSC on the surface, most of which were calcein-AM-stained viable cells.

TEG engraftment after implantation

In the 3rd month after implantation, for an interim assessment of wound healing and the condition of the trachea lumen, CT was performed under general anesthesia. The set of slices was converted into 3D image where images were taken in three planes: from above (Fig. 2, a), from the side (Fig. 2, b) and from the front (Fig. 2, c).

A slight narrowing was noted in the area of implantation of not more than $^1/_3$ of the lumen, which is seen above and below the green line (plane of cut sections of photographs) in Fig. 2, a. To fully assess the magnitude of the narrowing relative to the entire lumen of the trachea, a volume model was created, the segmentation area is shown in green in Fig. 2, c.

Fig. 3 shows a calculated model of the respiratory tract which includes soft tissue of the rabbit's pharynx and nasopharynx, larynx, trachea before bifurcation, bronchi and soft lung tissue.

Fig. 3, d shows the area of engraftment is shown; a slight narrowing of the lumen is seen. No stenosis was observed in the area of engraftment.

Morphological analysis

In the areas of TEG engraftment neither granular leukocytes nor lymphocytes were detected, which meant the absence of signs of purulent inflammation or aseptic necrosis around TEG material (Fig. 4, a, c). This indicates the absence of complications in the animal during the experiment associated with tissue response to the graft and the development of infectious diseases of the respiratory tract.

The reason for the narrowing of the lumen revealed in the 3rd month was found by CT. On the right side of the trachea (Fig. 4, a), a chronic inflammatory reaction developed around the suture material which led to partial destruction of the graft in this area, replacement by connective tissue and hypertrophy of the epithelial layer with an increase in the content of goblet cells producing mucus. This is clearly visible on the posterior wall of the trachea (Fig. 4, b), where the epithelium became thickened and polyps formed. On the opposite side of the incision, the area of inflammation decreased in size, which implies that the reaction to the suture material was local in nature. The tissue at a distance from the described suture material had normal morphology. As can be seen in Fig. 4, d, the epithelium on the implant

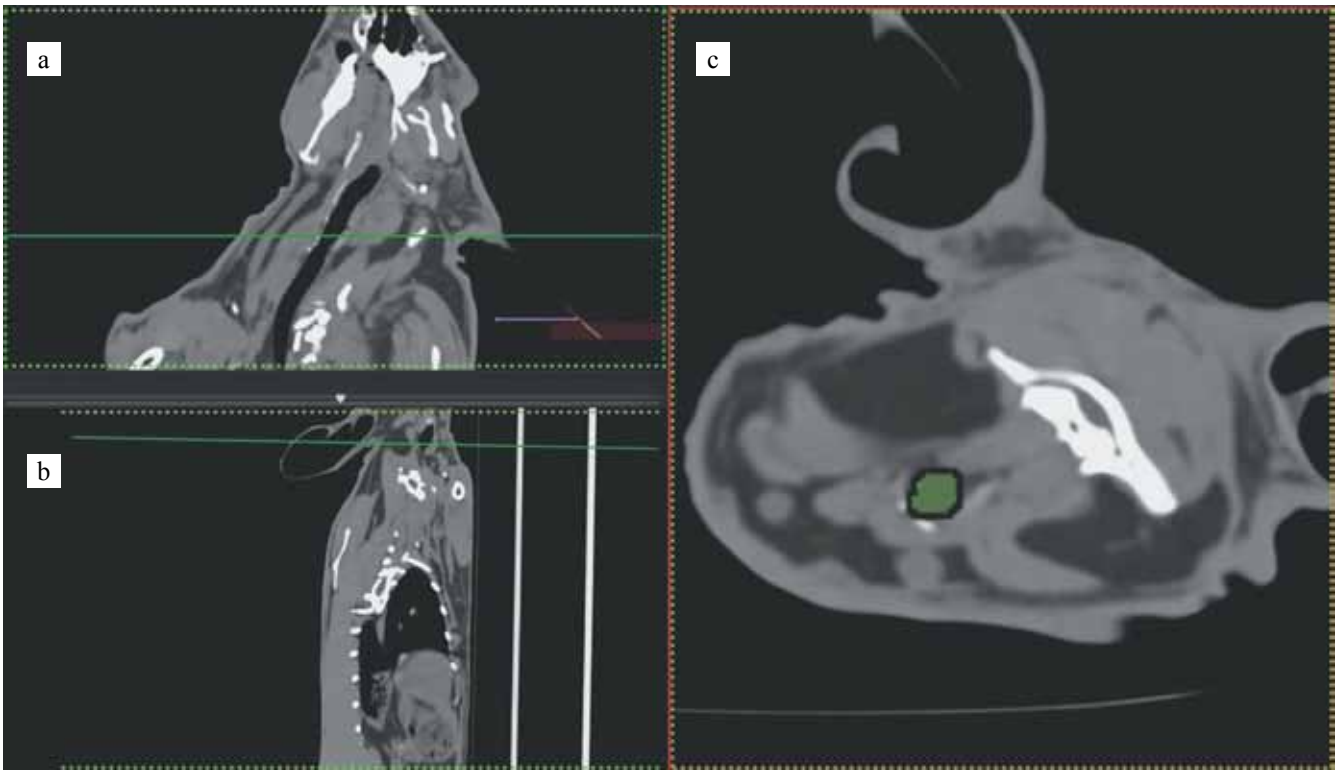

Fig. 2. CT scan of the rabbit neck and thorax, the image planes: a – from above, b – from the side and c – from the front. The green line (a, b) – is the image cut-off plane. The green area (c) – the area of automatic segmentation of the respiratory tract

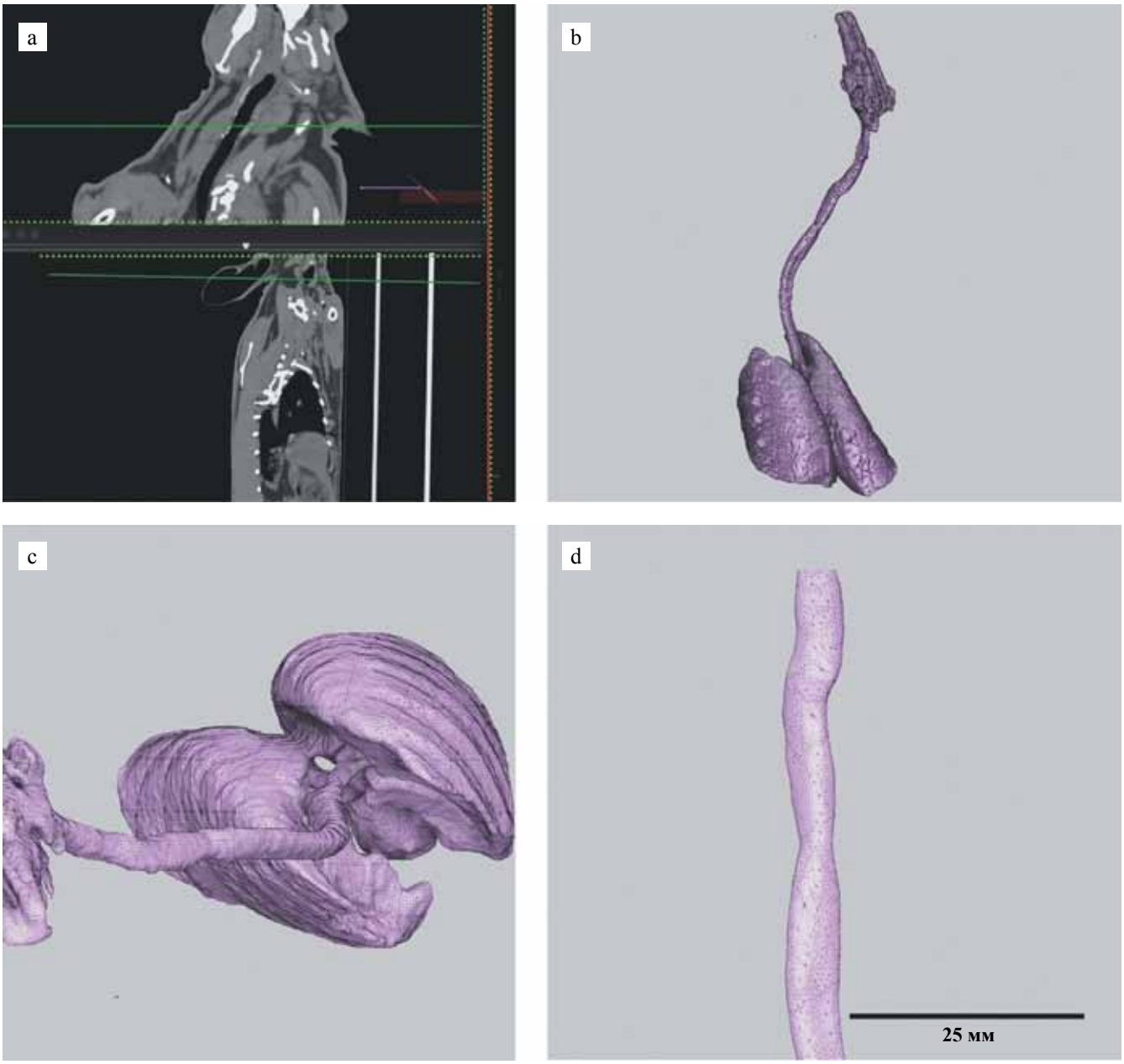

Fig. 3. The rabbit respiratory tract model: a – CT scan of the tissue-engineered graft engraftment area; b–c – general view of the respiratory tract model; d – tracheal lumen model in the area of engraftment

was not hyperplastic; tracheal and implant tissues fused, two rows of cartilaginous rings were observed: large – native, smaller – TEG sections. The submucosal layer was represented by multidirectional bundles of collagen fibers that surrounded the sites of resorption of the cartilage structures of the implant destroyed by macrophages.

Fig. 5 shows the results of immunofluorescence staining for CKPan (Fig. 5, a–c), intercellular interactions – cadherins (CDH) (Fig. 5, d), and Ki-67 cell proliferation marker (Fig. 5, a, b).

The TEG area without signs of inflammation from the suture material is shown in Fig. 5, a. The epithelium of the normal morphology is seen, while the proliferating cells (Ki-67) are located mainly in the submucosal layer. In the area of inflammation (Fig. 5, b) Ki-67 positive cells are observed both in the submucosal and epithelial layers, which indicates continuous cell proliferation. The epithelium in the area of inflammation is also hypertrophied, as can be seen in Fig. 5, c and d.

DISCUSSION

Evaluation of the quality of obtaining biologically and physiologically compatible scaffolds for creating TEG is based on experiments both *in vitro* and *in vivo*. At the native trachea devitalization, most of the cells from the volume of the tracheal scaffold were removed in the freeze/thaw cycles, which, most likely, favorably affected the decrease in the immune response to the

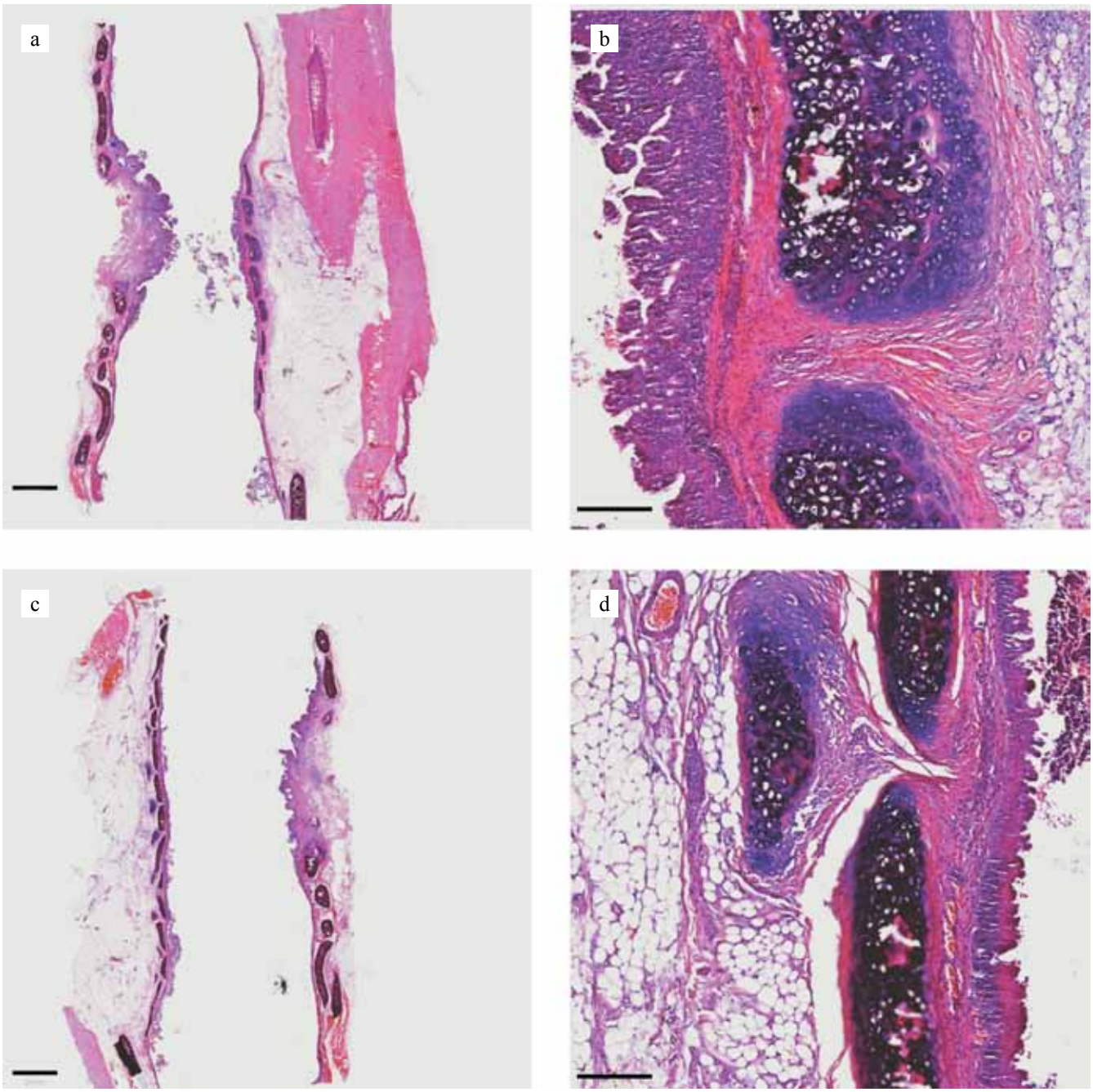

Fig. 4. Sample of rabbit trachea tissue at 6 months after the graft engraftment, hematoxylin-eosin staining: a, c – overview of tracheal tissues from two sides relative to the incision, the scale bar is 2 mm; b, d – areas of the mucous membrane at a distance from the source of inflammation, the scale bar is 200 μm

implant. Moreover, if previously protocols with 5-fold freeze-thaw cycles were used to get the devitalized TEG scaffold [14, 15], then our use of a 3-fold cycle was also suitable for obtaining DTS. Moreover, the method demonstrated satisfactory results of DTS vitalization with a known high level of presence of syngeneic cells preserved after freezing and thawing cycles in the material. Accordingly, this devitalization method may be suitable for producing TEG scaffolds.

Earlier techniques for the efficient seeding of TEG scaffolds in the regimes of static and dynamic cultivation [16] have shown their suitability for creating TEG trachea with a two-layer cell coating.

A model was proposed and developed for assessing the viability of a tissue-engineering graft when closing a critical airway defect using MSCT, histology, and immunohistochemistry.

It can be concluded, by the absence of neutrophils and eosinophils in the areas of the epithelium and submucosal layer of the surrounding TEG tissues, that the graft did not have toxic effects on the recipient's tissue. In the absence of chronic inflammatory reaction, a specific

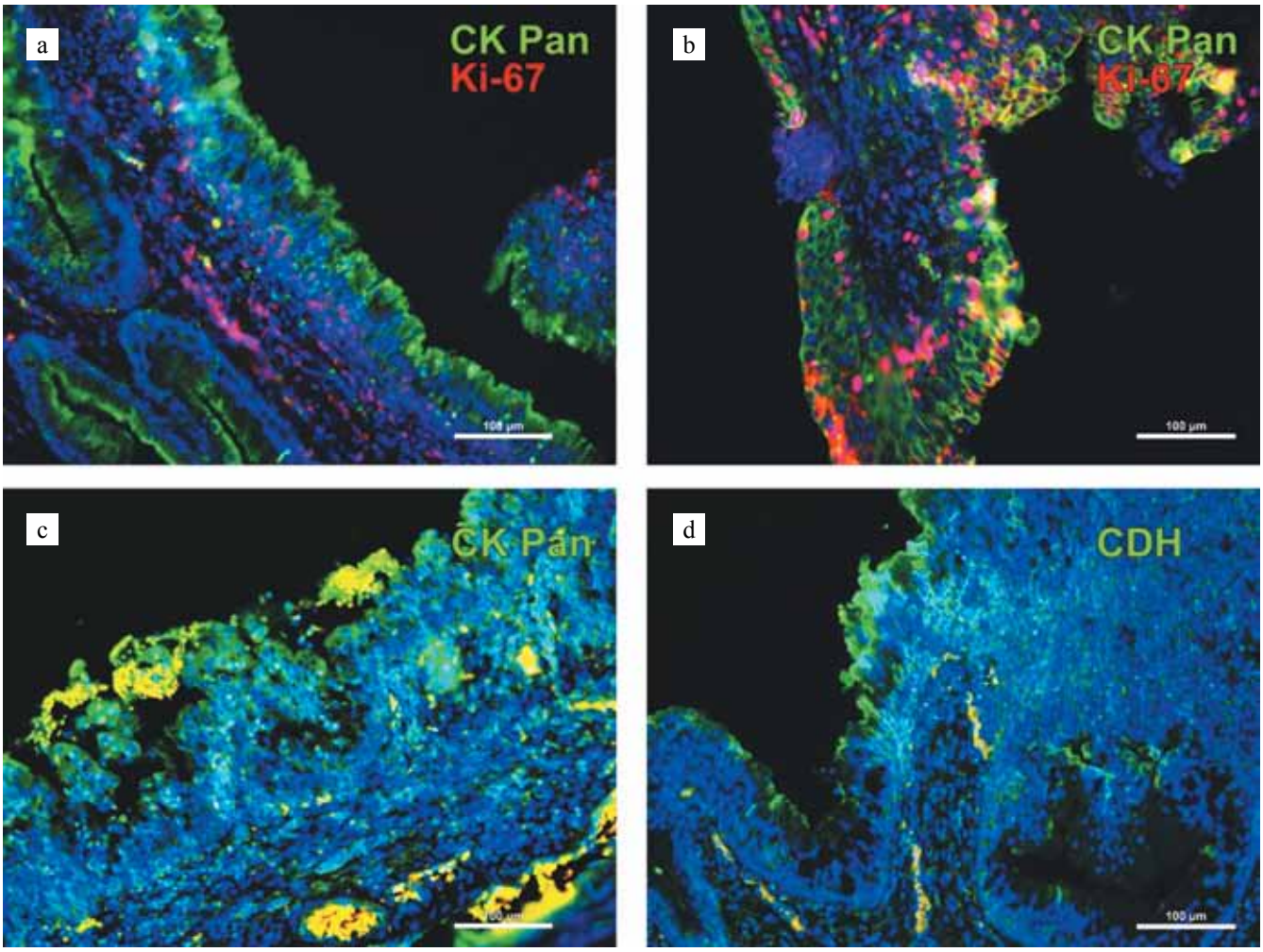

Fig. 5. The wall of the rabbit trachea in the engraftment area. Immunofluorescent staining of cell nuclei for Hoechst 33342 (blue fluorescence). The scale bar is 100 μm. ×200

immune response to TEG also did not develop. A large number of vessels and capillaries were observed in the submucosal layer, which indicates good vascularization of the structure.

Based on the results of a histological examination of TEG and the tissues surrounding the implant, it can be concluded that the graft is viable, well epithelized, vascularized and integrated into the structure of the recipient's trachea, despite the development of complications associated with inflammation due to suture material.

CONCLUSION

The creation and evaluation in *in vitro* and *in vivo* surgical experiment of a tissue-engineering graft on the basis of the devitalized rabbit tracheal scaffold seeded by bone marrow mesenchymal stromal cells and epithelial cells confirmed the cytological and biological compatibility of the graft. The minimal narrowing of the trachea in the engraftment area in the absence of stenosis indicates the TEG ability to maintain a constant lumen of the recipient's trachea. Further research is needed on improving the biologically and physiologically compatible tissue-engineering trachea based on the devitalized tracheal scaffold retaining tissue microarchitectonics.

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The authors declare no conflict of interest.

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