CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Optimization of a Method for Preparation and Repopulation of the Tracheal Matrix for Allogenic Transplantation

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A method for decellularization of the trachea for subsequent repopulation with allogenic MSC was optimized. Tracheas from C57BL/6 mice were devitalized and repopulated with MSC from BALB/c mice. The tracheal matrix with devitalized mucosa and intact cartilaginous structure, fit for repopulation with allogenic MSC, was obtained by chemical treatment with $NaClO_4$. This approach seemed to be promising for transplantation of allogenic trachea.

Key Words: *transplantation; trachea; mesenchymal stem cells*

Stenosis of the trachea caused by malignant tumors, infectious diseases or injuries can necessitate resection of a significant portions of the organ. In some cases, the formation of primary anastomosis is impossible and the defects have to be replaced with a transplant [12]. Various prostheses and tissues were used for reconstruction of the trachea. Allotransplants of the trachea can be regarded as promising means for tracheal defect replacement. The epithelium and the connective tissue serve as the targets for rejection and hence, immunosuppressive therapy has to be carried out in order to prevent allotransplant rejection [13].

Cryopreservation reduces immunogenic activity of the tracheal transplant. However, the effect of cryopreservation on antigen-presenting cells remains disputable and is to be studied more profoundly [8-10].

An attempt at creating a cartilage by using chondrocytes and fibroblasts *in vitro* repopulating biodegraded films failed. The transplanted tissue degenerated and failed to support the mechanical properties needed for trachea functioning [5].

Studies of cadaveric trachea as a matrix were carried out. In order to reduce the immunogenic activity and eliminate the main histocompatibility complex (MHC), the trachea was decellularized by repeated cycles of detergent and enzyme treatment. This treatment led to virtually complete degradation of chondrocytes. The resultant matrix was then repopulated with recipient MSC and epithelial cells. That tissue transplant was then implanted to a recipient without using specific methods for transplanted tissue revascularization. Presumably, rapid revascularization of the allostransplant was promoted by MSC repopulating the biological prosthesis before transplantation [4,6]. However, the preparation of the allotransplant by this method took 6 weeks. This fact limited the potentialities of this technology, for example, in cancer patients with decompensated stenosis of the trachea in need of urgent intervention. In another study, a xenogenic decellularized trachea

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was transplanted to dogs [11]. A rapid method for decellularization of porcine trachea by exposure in 3% Triton X-100 for several days and subsequent irradiation was proposed. This treatment led to removal of the transplant mucosa cells without impairing chondrocyte structure. The xenograft revascularized 8 weeks after transplantation, its inner surface being lined with stratified ciliary epithelium. On the other

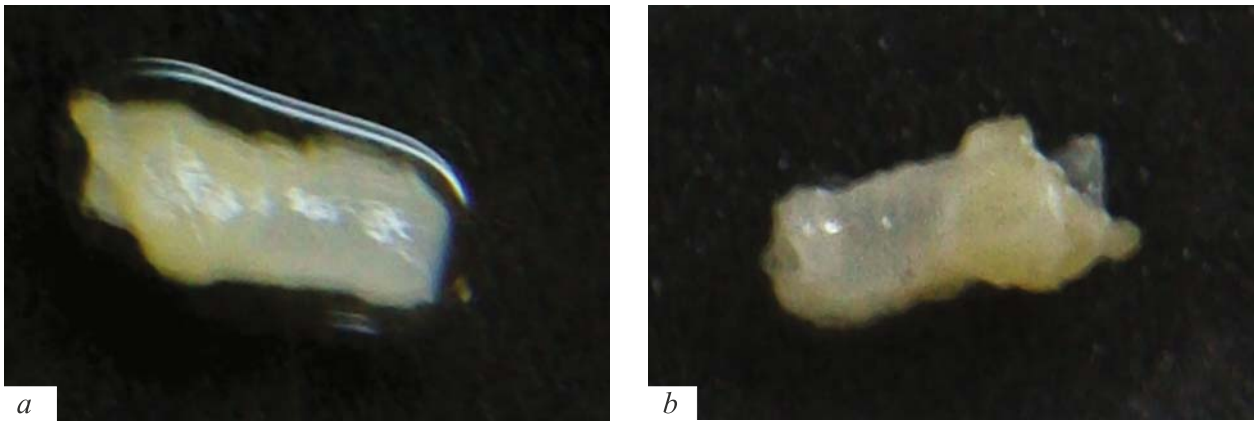

Fig. 1. Mouse trachea after freezing/defrosting (*a*) and $NaClO_4$ treatment (*b*).

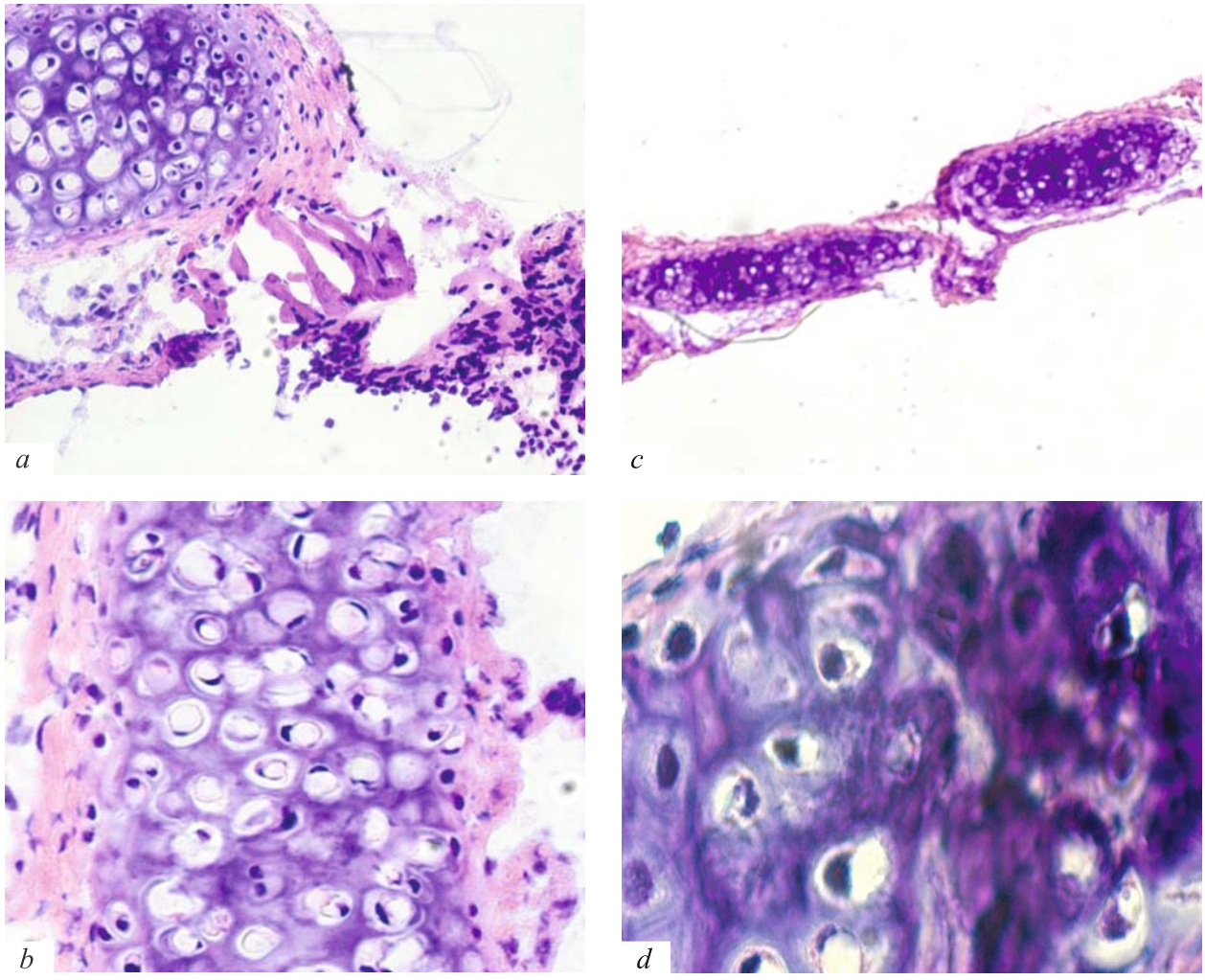

Fig. 2. Paraffin section of a mouse trachea after freezing/defrosting (*a*: ×400; *b*: ×900) and $NaClO_4$ treatment (*c*: ×100; *d*: ×900; hematoxylin and eosin staining).

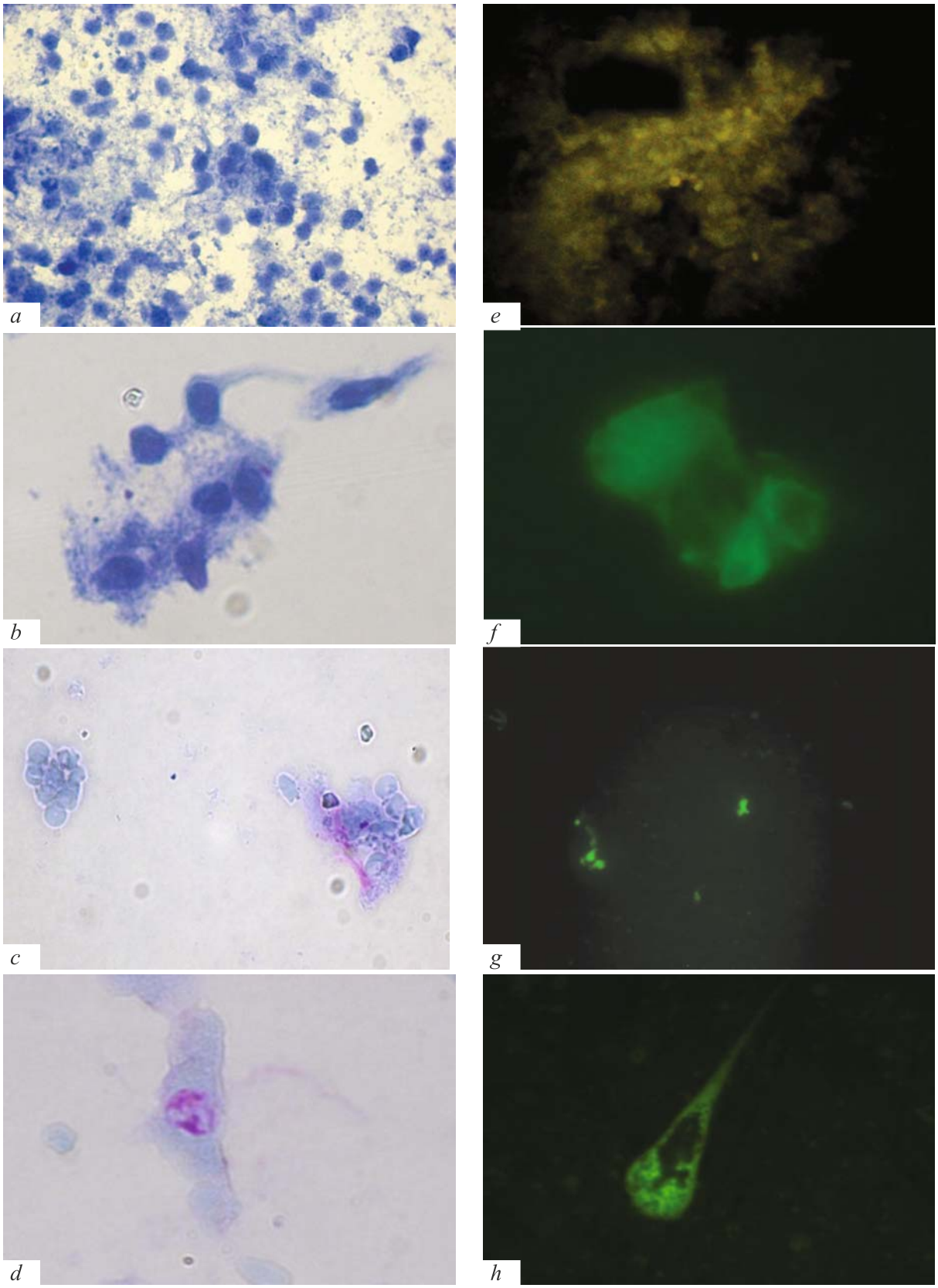

Fig. 3. Impression of mouse trachea after freezing/defrosting (*a*, *b*, *e*, *f*) and after $NaClO_4$ treatment (*c*, *d*, *g*, *h*). *a-d*: living cells (Romanowski–Giemsa staining); *a*, *c*: ×400; *b*, *d*: ×900. *e-h*: immunofluorescence; *e*: staining with MHC I-FITC and NK-PE (×400); *f*, *g*: staining with MHC II-FITC (×900); *h*: staining with MHC I-FITC.

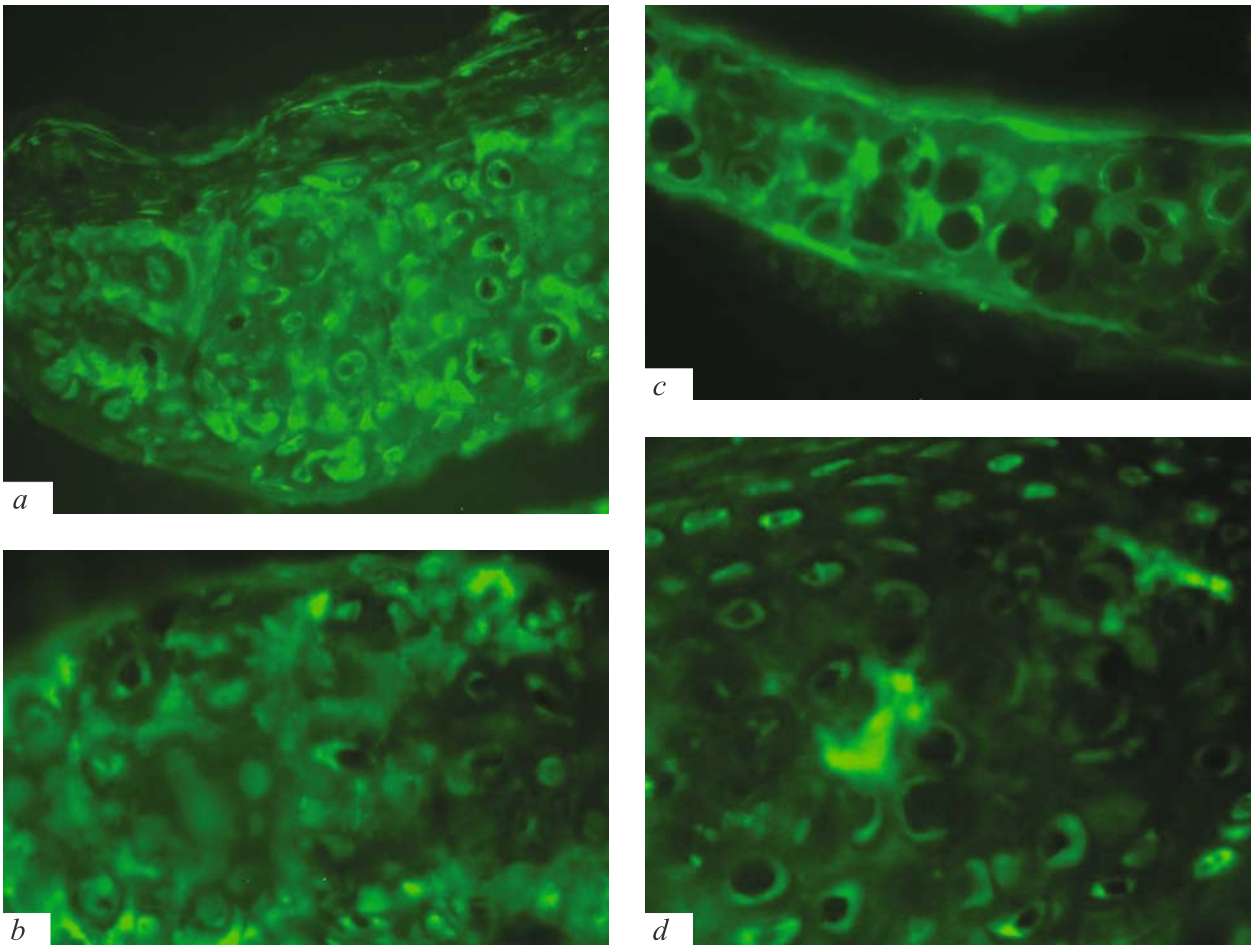

Fig. 4. Cryostat section of mouse trachea after freezing/defrosting (*a*, *b*) and $NaClO_4$ (*c*, *d*). Immunofluorescence (staining with MHC I-FITC antibodies). *a*: ×400; *b*, *c*, *d*: ×900.

hand, degradation of cartilaginous structures in the transplant was noted, which necessitates repopulation of the tracheal transplants with MSC.

Cell death could be attained by other, simpler methods, for example, by repeated freezing/defrosting or chemical treatment, *e.g.* with oxidants.

The aim of this study was optimization of a method for decellularization of the trachea for its subsequent repopulation with allogenic MSC.

MATERIALS AND METHODS

The study was carried out on C57Bl/6 and BALB/c mice (22-25 g).

The tracheas were removed from ether-narcotized C57Bl/6 mice and mechanically cleansed from mucus and muscle elements.

The tracheas were then mechanically cleansed from the epithelium and connective tissue and washed with distilled water. For decellularization, the tracheas were frozen 3 times at -70°C and defrosted (mode 1) or incubated in 5% $NaClO_4$ (72 h); the solution was replaced with fresh portions every 24 h (mode 2). The tracheas were then washed in saline and morphological and immunohistochemical studies were carried out.

MSC were isolated from the femoral bone marrow (BM) of BALB/c mice. Suspension of BM cells was resuspended in RPMI 1640 (PanEco) to a concentration of 10^6 cell/ml and incubated for 4 h in plastic flasks (Nunc). Free cells were then discarded, a medium for culturing of nonhemopoietic BM cells was added (NH Stem Cell Media; Miltenyi Biotec), and the cells were cultured for 72 h in a CO_2 incubator. The resultant MSC were mechanically removed from the culture flask and resuspended in RPMI 1640 (PanEco) to a concentration of 10^6 cell/ml. Decellularized specimens of the trachea were plunged in the resultant cell suspension and shaken on a shaker every 30 min for 4 h. Specimens of the trachea were incubated for 72 h in a CO_2 incubator, the medium with MSC suspension was replaced every 24 h and shaken.

The expression of classes I and II MHC antigens and natural killer (NK) differentiation antigens was evaluated in impressions and cryostat sections of tra-

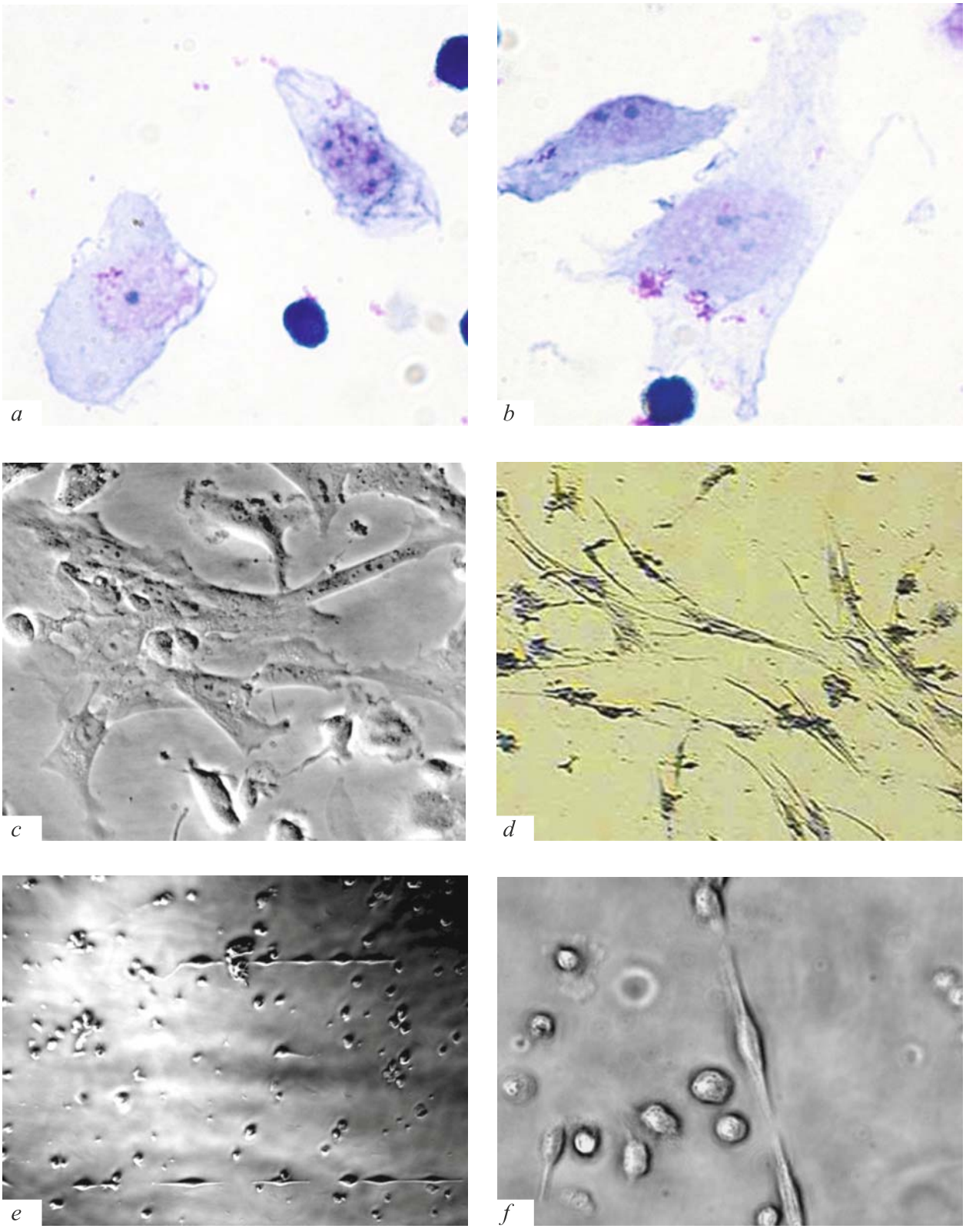

Fig. 5. Mouse BM stromal precursor cells on the bottom of a culture flask after 24 (*a*, *b*), 48 (*c*, *d*), and 72 h (*e*, *f*) of culturing. *a*, *b*: Romanowskii–Giemsa staining (×900); *e*, *f*: in clear field; *c*, *f*: ×400; *d*, *e*: ×200.

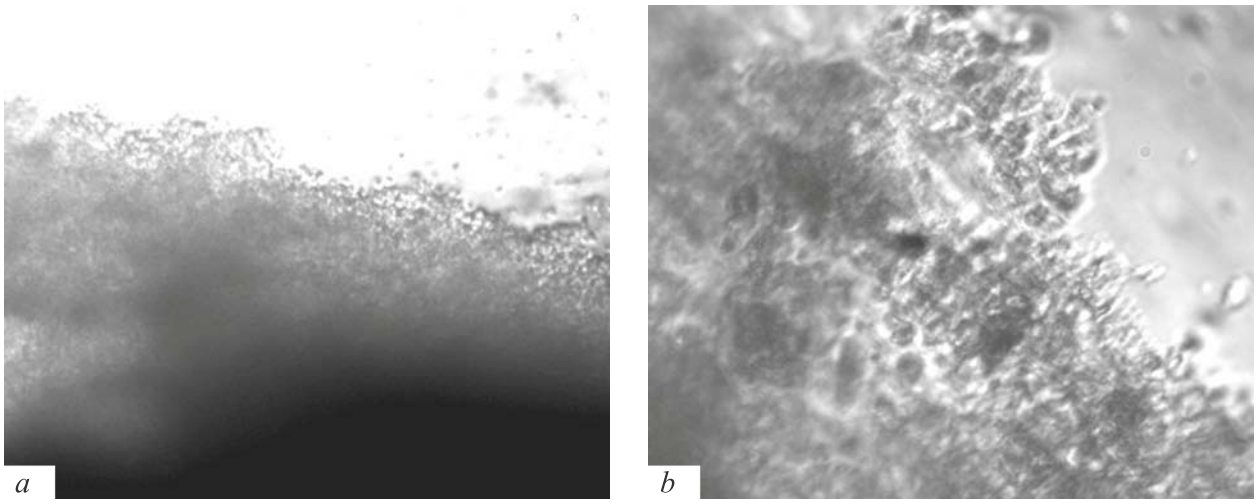

Fig. 6. Adhesion of mouse BM stromal precursor cells on the surface of decellularized tracheal matrix in a clear field: 48-h culturing (*a*: ×200; *b*: ×900).

cheal specimens using antibodies NK 1.1-PE, MHC 1-FITC, and MHC II-FITC (Coltag).

Photo- and fluorescent microscopy were carried out using Zeiss photovideosystem and Axiovision 2 software.

RESULTS

Specimens of the trachea retained their macrostructure and elasticity after treatment by both methods (Fig. 1).

Degradation of an appreciable number of chondrocytes and retention of some part of the mucosa and perichondrium were seen in histological preparations (tracheal sections) after mode 1 treatment (Fig. 2, *a*, *b*). By contrast, mode 2 treatment led to complete destruction of the mucosa, the cartilage tissue retain its structure, nonvacuolated cytoplasm and nuclei were seen in chondrocytes (Fig. 2, *c*, *d*).

On the other hand, the mucosa cells remained after preparation of the trachea were viable after mode 1 treatment, while after mode 2 treatment just solitary half-destroyed cells with vacuolated cytoplasm and nucleus-free cell fragments were found in the tracheal impressions (Fig. 3, *a*-*d*). Immunofluorescent analysis showed that the cells in tissue impressions of organs subjected to freezing/defrosting were characterized by high expression of classes I and II MHC molecules; in some cases, solitary lymphocytes, including $CD16^+$ natural killers, were visualized (Fig. 3, *e*-*h*). On the other hand, no specific reactions to these markers were seen after $NaClO_4$ treatment; just solitary MHC II-positive cells were detected (Fig. 3, *e*-*h*).

Positive reaction of class I MHC antigens on chondrocytes was registered on cryostat sections of tracheal specimens subjected to chemical treatment (mode 2) and freezing/defrosting (mode 1) (Fig. 4).

Hence, $NaClO_4$ treatment caused less severe changes in cartilaginous structures, but led to more pronounced death of mucosal cells than repeated freezing/defrosting.

In order to repopulate the decellularized tracheal scaffold, MSC were generated from allogenic BM cells. After 48-72-h incubation of BM cell suspension in conditioned medium, groups of elongated highly adherent cells and ordered structures (cell chains) were found in the culture. Staining of adherent cells revealed hypochromatic oval nuclei with nucleoli and slightly stained cytoplasm in them (Fig. 5).

After 48-h culturing of the tracheal scaffold with suspension of generated stromal precursor cells with periodical rotation of the specimen, adherent fibroblast-like cells were seen on the surface of decellularized trachea (Fig. 6). The studies showed that in contrast to 3-fold freezing/defrosting, a tracheal scaffold free from the mucosa, but retaining the structure of cartilage elements can be rapidly obtained by oxidant ($NaClO_4$) treatment. The study showed that to spare the cartilage in the tracheal allotransplant [13] was more effective than to use completely decellularized allogenic trachea for transplantation [6]. Studies in animals with transplanted tracheal xenograft showed that chondrocytes remained in the scaffold with devitalized mucosa did not induce graft rejection. Presumably, low immunogenic activity of chondrocytes was due to the intercellular matrix compactness, preventing the release of the antigen into recipient lymphoid tissue and providing their poor bioavailability for the transplantation immunity effectors [13]. Repopulation of the scaffold with donor SC (MSC) could reduce the formation of granulations at the site of anastomosis and promote more rapid epithelialization of the transplant. Stromal precursor cells could be derived from

BM cell suspension by using special culture media and used for repopulation of decellularized tracheal scaffold. Due to plasticity of MSC they can also participate in the formation of the endothelium, providing rapid revascularization of the transplant [1].

Hence, chemical treatment ($NaClO_4$) is a rapid method for preparation of the tracheal matrix with devitalized mucosa and intact cartilage structure, which can be repopulated with allogenic MSC. This approach can be effective in transplantation of allogenic trachea.

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