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INTERLEUKIN IL-1β STIMULATES REVITALIZATION OF CARTILAGE MATRIX *IN VITRO* WITH HUMAN NASAL CHONDROCYTES

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Revitalization of decellularized or devitalized matrix scaffolds in tracheal tissue engineering typically involves seeding the autologous recipient cells or allogeneic cells under long-term cultivation. **Objective:** to study the capability of human nasal chondrocytes for colonization of devitalized scaffolds based on native human tracheal cartilage, with proinflammatory stimulation (cytokine) by adding Interleukin-1-beta (IL-1β) to the culture medium. **Materials and methods.** Scaffolds for tracheal tissue engineering were obtained from native human tracheal cartilage through devitalization and laser etching. The scaffold was revitalized by seeding the human nasal chondrocytes. Histological examination was performed after staining with hematoxylin and safranin-O, with further microscopy using a Nikon Eclipse L200 light microscope. X-ray microtomography was performed on a Phoenix nanotom m apparatus. Electron microscopy was performed on a Nova NanoSEM 230 setup. **Results.** There was statistically significant increase in the intensity of colonization ($p = 0.0008$) with nasal chondrocytes and stimulation of their migration activity ($p < 0.0001$) in the presence of IL-1β compared with the control groups. **Conclusion.** Addition of proinflammatory cytokine IL-1β (1 μg/ml) to the culture medium enhances volumetric seeding of devitalized cartilage scaffold with human nasal chondrocytes, allowing to create highly revitalized materials for tracheal tissue engineering.

Keywords: vitalization, inflammation, interleukin-1-beta (IL-1β), cultured cells, laser etching, revitalization, tissue engineering, physiological relevance, chondrocytes, cartilage matrix.

INTRODUCTION

Obtaining tissue-engineering constructions involves the revitalization of decellularized host scaffolds before implantation through colonization with recultured cells [1, 2]. The immobilization of autologous recipient cells to the scaffold further reduces its immunogenicity and increases biocompatibility.

There are various ways to stimulate cell migration to the host scaffold, e.g. the use of chemoattractants and cell adhesion molecules at various concentrations and gradients, as well as dynamic cultivation in the bioreactor conditions [3, 4]. In this case, body reactions in response to implantation will affect the viability and differentiation of cells necessary for the maturation of physiologically compatible cartilage tissue [5].

Any surgical intervention is accompanied by aseptic inflammatory response, known as a surgical inflammation [6], and the implantation area of the finished tissue-engineering constructs is usually represented by inflamed tissues of the affected organ of the recipient. Moreover, the effect of factors and products of the inflammatory response on the behavior of populated cells after implantation of such a construct is difficult to predict; it remains an understudied area, which makes studying the effect of pro-inflammatory cytokines on the process of scaffold colonization *in vitro* quite promising.

To simulate inflammation *in vitro*, various pro-inflammatory factors, such as interleukins, tumor necrosis and interferons, can be added to the medium. Interleukin-1-beta (IL-1β) is known to inhibit the proliferation of rabbit articular chondrocytes [7]. IL-1β is also involved in the cellular regulation of chondrocytes, stimulating proliferation and inhibiting differentiation [8]. IL-1β effect on chondrogenesis of MMSC CM [9], the regulation of chondrocyte cytoskeleton components [10] and the protective physiological responses of chondrocytes during mechanical stimulation in the presence of IL-1β [11] has been shown, suggesting the effects in IL-1β that promote scaffold colonization with chondrocytes [12].

At present, it has been established that the natural IL-1β concentration in human physiological fluids reaches 200 to 300 ng/ml [13], while at inflammatory processes due to exogenous materials implantation, its value in interstitial fluid increases to 750 ng/ml or over, depending on the implantation site and individual characteristics of the recipient [13, 14]. Therefore, as a model medium, it was decided to use a solution of IL-1β at a concentration of 1 μg/ml, adopted physiologically relevant to the active inflammatory process at the implantation site.

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The ***purpose*** of the present study was to investigate the efficiency of colonization of devitalized scaffolds with nasal chondrocytes when adding IL-1β in a physiologically relevant inflammation concentration to the culture medium during scaffold revitalization in a static culture *in vitro*.

MATERIALS AND METHODS

Ethics Compliance

Donor tissues have been sampled in accordance with the scientific study protocol approved by the ethics committee of Sechenov University (decision No. 07-15 of July 15, 2015).

Preparing a tracheal scaffold based on natural human cartilage

Native human cartilage tissue devitalized through three freeze/thaw cycles and laser engraving with the formation of blind holes of 150–200 μm diameter and up to 300 μm depth was used as a scaffold. Laser engraving of material samples of 2×4 mm and 1 mm thickness was used to ensure bulk colonization.

To devitalize the native tissue, the cadaver donor trachea was separated in a laminar cabinet from the surrounding tissues, placed in a sterile PBS solution at room temperature and frozen for 15 minutes in liquid nitrogen. After freezing, the sample was thawed in a water bath at 37.5 °C for 30 minutes. After the cycle, the PBS solution was removed, the sample was washed 3 times with a sterile solution and placed in a PBS tube again. Then the freeze/thaw cycles were repeated 2 more times, and then the devitalized cartilage was separated from the perichondrium and cut into plates no more than 1 mm thick.

Next, the cartilage tissue was laser-engraved with BD-01 CO_2 laser at 10.6 μm wavelength. Laser engraving resulted in the blind holes formation of 150–200 microns diameter, up to 300 microns depth and the density of about 4 holes per 1 mm^2. The obtained samples were washed 3 times with a cold sterile PBS solution and sent for sterilization with gamma radiation by iridium-192 isotopes with 1.3 kGy absorbed dose.

Isolation and cultivation of chondrocytes for scaffold revitalization

The cartilaginous part of the human nasal septum was used as a source of chondrocytes for primary culture. The resulting cartilage of the nasoseptal septum was checked for bacterial and fungal contamination and then fermented with 0.15% Collagenase Type II solution for 12 h at 37 °C with 5% CO_2. After that, the fermented cartilage was filtered with a sterile strainer of 100 μm pore size and the filtrate was centrifuged for 4 minutes at 1,300 rpm. Cells in the obtained suspension of chondrocytes were counted, placed in a culture dish and cultured for 21 days until the first passage at 37 °C of 5% CO_2 in DMEM medium (Gibco, USA) with the addition of 5% FBS, TGF and FGF-2, changing the medium every third day. Next, the nasal chondrocytes of the first passage were cryopreserved and stored in a cryobank at –196 °C.

Scaffold revitalization with human nasal chondrocytes

To revitalize the scaffolds, human nasal chondrocytes of the second passage were used, cultured from previously cryopreserved cells of the first passage. Cell expansion was performed in DMEM medium with a high glucose content (4.5 g/L) and 5% FBS, TGF and FGF-2 at 37 °C 5% CO_2 with the medium changed every third day.

The scaffolds to be revitalized (n = 6) were laid on semipermeable membranes of Transwell plates (Corning, USA) and colonized with cells at the rate of 0.25 million nasal chondrocytes per 1 mm^2 of the scaffold surface. All samples thus revitalized were cultured under incubator conditions at 37 °C and 5% CO_2. The complete DMEM medium with a high glucose content (4.5 g/l) and the addition of 5% FBS, insulin and ascorbic acid was used as a nutrient medium.

After the first day of cultivation, all samples were divided into control (n = 3) and experimental (n = 3) groups. The culturing conditions of the control group samples remained unchanged until the end of the experiment. For the experimental group samples, the proinflammatory cytokine of human recombinant interleukin IL-1β (Sigma, art. SRP3083) was added to the culture medium at 1 μg/ml concentration. All samples were removed for histological examination after the next 6 days of cultivation.

Histological examination and X-ray microtomography

Histological examination was performed after all samples were preserved in 4% formaldehyde solution for 24 h, dehydrated with ascending ethanol concentrations and paraffinized by a standard protocol. 5 μm thick sections were sequentially hematoxylin and safranin-O stained to visualize the state of intercellular substance and populated cells. Safranin-O stains cartilaginous glycosaminoglycans in red orange, thus making it possible to qualitatively assess the saturation of cartilage tissue by color intensity [15].

For a comparative qualitative and quantitative assessment of scaffold revitalization, an original four-point scale (table) was developed based on the principles of the Bern score for evaluating pellet cultures of cartilage cells [16].

With the original scale for an independent assessment of three regions for each sample, the degree of colonization of the wells and the degree of destruction of the microarchitectonics of the scaffold were evaluated when

Table

The original four-point scale for quantitative evaluation of scaffold revitalization

Category	Value
A. Well colonization	
Complete absence of cells or single cells	0
Parietal colonization	1
Multilayer colonization with free space in wells	2
Total colonization (no visible space in wells)	3
B. Cell migration	
The shape and boundaries of the pores are clearly visible while maintaining the distance between wells	0
Single local well merging	1
Multiple well merging	2
Complete fusion of wells (no visible boundaries of individual wells)	3

the cells migrated into the scaffold thickness outside the wells.

Histological studies were performed at the Institute of Surgical Research, University of Basel, using with microtomes (Thermo Fisher Scientific, USA) and Nikon Eclipse L200 light microscope (Nikon, Japan).

Surface colonization of the samples by nasal chondrocytes was evaluated by scanning electron microscopy with gold sputtering (Nova NanoSEM 230, FEI, USA).

X-ray microtomography of tissue-engineering structures for assessing the preservation of wells in the 3D structure of the sample after revitalization was made with Phoenix nanotom (General Electric, USA).

Statistical data processing

Statistical data processing was performed using the Mann–Whitney U test with the GraphPad Prism 7 software (GraphPad Software, Inc). Differences were considered significant at $p < 0.05$.

RESULTS

Scanning electron microscopy of tracheal cartilage tissue samples after laser engraving and revitalization allowed visualizing a dense coating of the sample surface with nasal chondrocyte cells and a loose intercellular substance synthesized *de novo* (Fig. 1).

Microcomputer tomography of host scaffolds after revitalization allowed identifying the location and confirm the integrity of the wells in the sample structure in the control group; however, the microtomograph resolution was insufficient for imaging cells. The histological examination was the most informative, revealing a high degree of colonization of the wells. The results com-

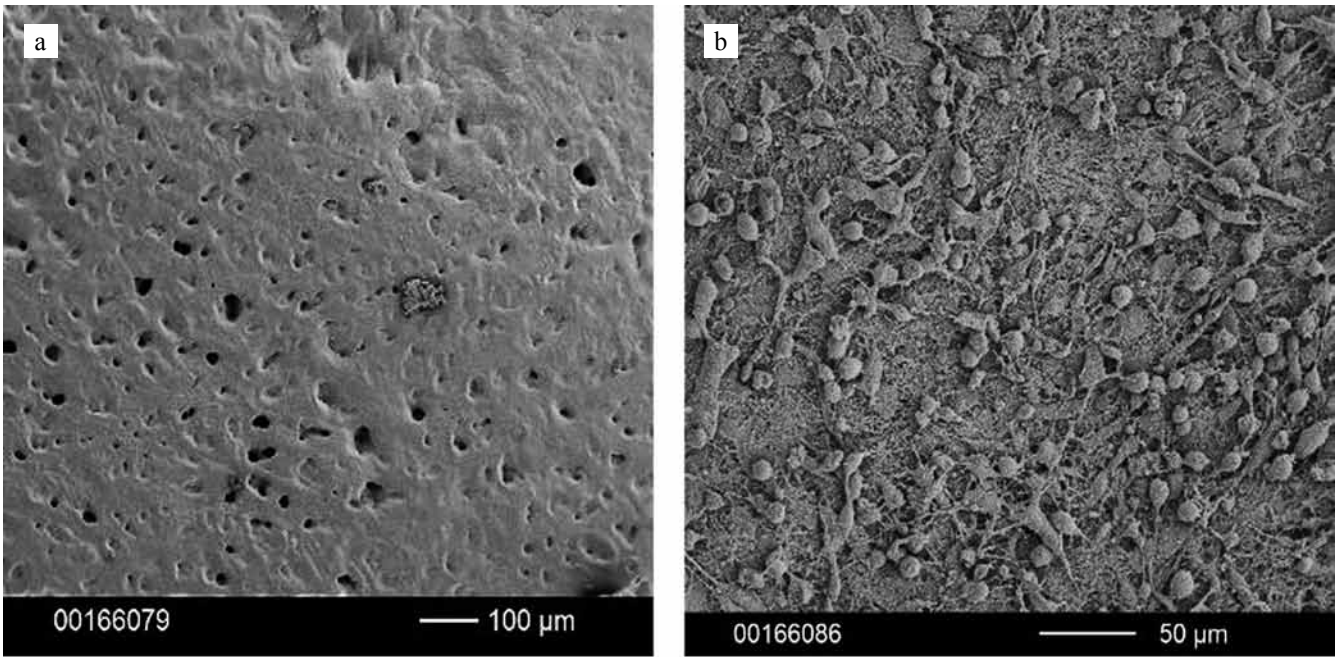

Fig. 1. High-density scaffold prior to laser engraving: a – devitalized scaffold: empty lacunas surrounded by high-dense extracellular scaffold are visible on the cartilage surface, b – laser-engraved scaffold revitalized by nasal chondrocytes, densely colonizing the surface of the material. Scanning electron microscopy

parison of histological examination and microcomputer tomography for samples of the control group is shown in Fig. 2.

The exposure to IL-1β during revitalization was shown to lead to a denser and more intensive colonization of the wells. At the same time, the migration of cells beyond the depths of the wells deep into the scaffold led to a pronounced wells and channels fusion and ultimately to the destruction of the microarchitectonics of the tissue-engineering structure (Fig. 3).

The results of a quantitative assessment of the scaffold colonization intensity and cell migration beyond the wells on the original four-point scale for structured experimental samples (when exposed to IL-1β) and the control group after colonization with nasal chondrocytes are shown in Fig. 4.

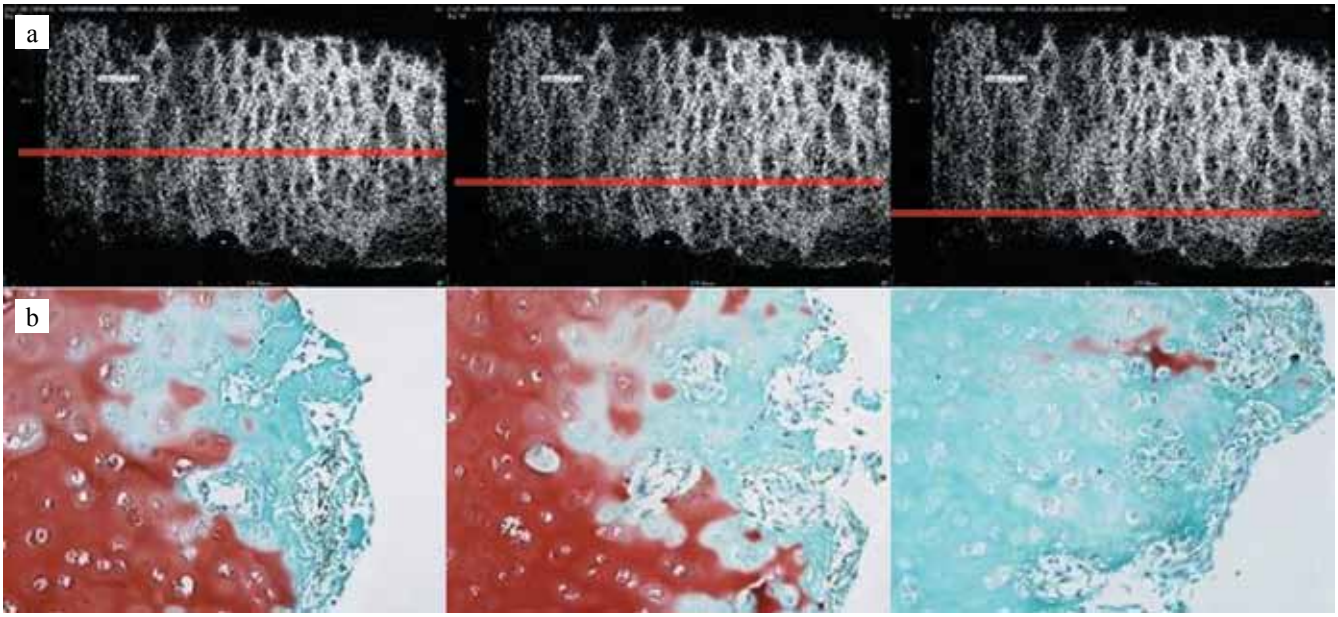

Fig. 2. Tissue-engineered graft generated by revitalization of the laser engraved tracheal cartilage with human nasal chondrocytes: a – tissue engineered graft, X-ray microtomography, red lines showing the cross-section levels; b – histological examination of transects (perpendicular to the direction of engraving). Safranin-O & Hematoxylin staining. Light microscopy, ×100

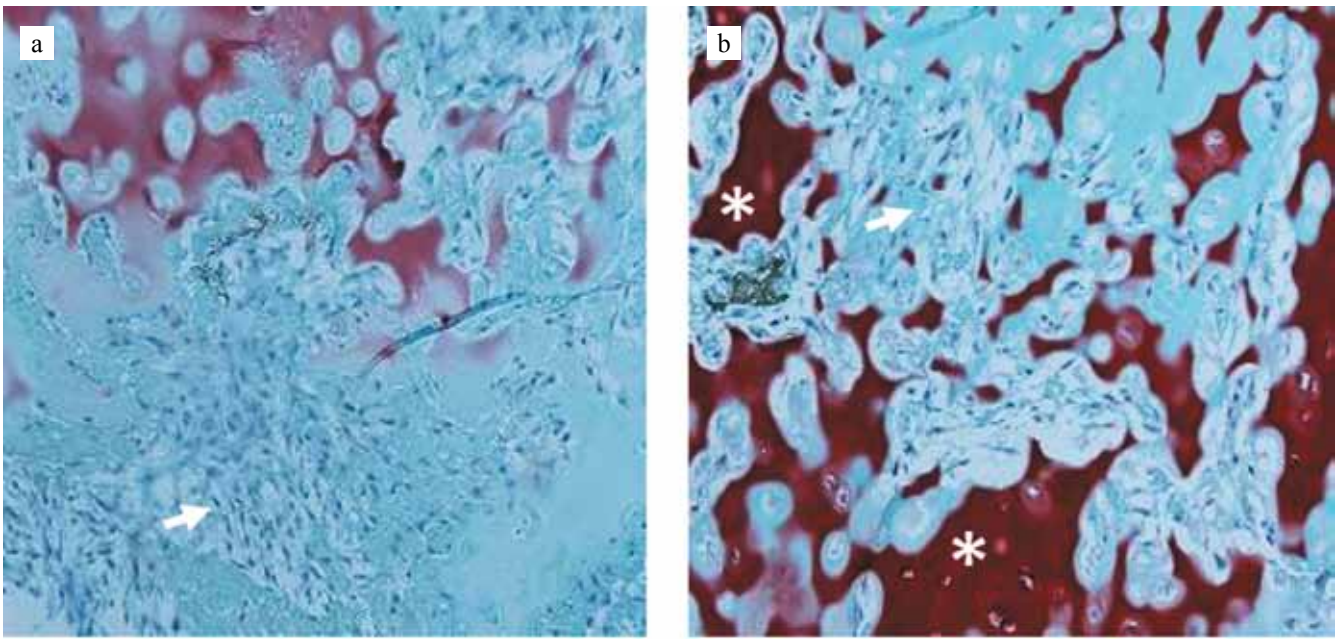

Fig. 3. Tissue-engineered graft obtained by revitalization of the laser engraved scaffold with human nasal chondrocytes under continuous exposure to IL-1β in the culture medium: the cartilage scaffold marked by asterisks; the nasal chondrocytes marked by arrows. Histological study of transverse sections (perpendicular to the direction of the engraving). Safranin-O & Hematoxylin staining. Light microscopy, ×100

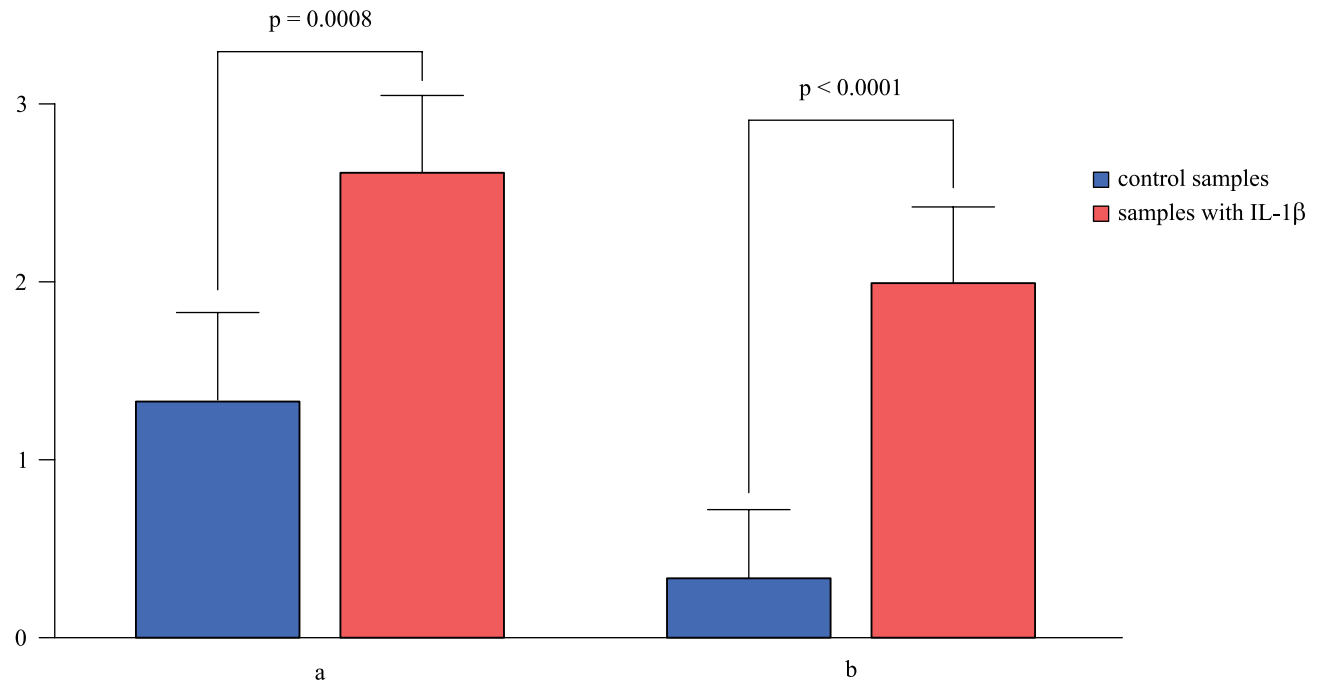

Fig. 4. Assessment of the values of intensity of scaffold colonization with human nasal chondrocytes (a) and migration of nasal chondrocytes outside the lacunae (b) under control conditions and in the presence of IL-1β. Values are means ± SD

Statistically significant differences in the intensity of colonization by nasal chondrocytes ($p = 0{,}0008$) and their migration beyond the borders of the wells ($p < 0{,}0001$) in the presence of IL-1β compared with control groups are shown.

DISCUSSION

Earlier, our experiments have showed that increasing the population density to 0.5 million cells per 1 mm^2 did not increase the density of colonization of the wells but increased the number of cells remaining on the surface of the sample. However, a lower population density does not allow to colonize all available wells in the allotted time period (up to 7 days). Besides, the use of dynamic cultivation under bioreactor conditions does not always allow volumetric population of the material *in vitro*.

It was shown that the appearance of a pro-inflammatory cytokine in the culture medium makes it possible to increase the density of the revitalization of the host scaffold, though having an ambiguous effect on the quality of the formed tissue-engineering structure. The addition of IL-1β qualitatively improves the colonization of the scaffold wells with chondrocytes, increases the population density of the construct by recipient cells, among other reasons due to increased migration of cells into the intercellular substance, which is well correlated with published data on the role of IL-1β in the regulation of cell proliferation, differentiation and apoptosis [17, 18]. A chronic aseptic proliferative (productive) inflammatory process in the field of implantation of a revitalized tissue-engineering construct can be assumed to also further contribute to the colonization of the scaffold by previously implanted recipient cells. At the same time, histological studies demonstrate partial or almost complete destruction of the microarchitectonics of the scaffold at its colonization under the influence of IL-1β: the boundaries of individual wells are erased, and the volume of intercellular substance decreases. The latter may adversely affect the mechanical characteristics of the tissue engineering structure. The effect may result from the increased synthesis of metalloproteinases by chondrocytes under the action of IL-1β [19]. Comparative studies of the mechanical characteristics of the finished structure may be the subject of further research.

Thus, the possibility of effective revitalization of laser engraved scaffolds with the colonization of scaffold wells by human nasal chondrocytes while maintaining the bulk of the initial intercellular substance of cartilage tissue was shown. It was proved that the addition of the pro-inflammatory cytokine IL-1β to the culture medium stimulates the colonization of the host scaffold with chondrocytes, being one of the necessary conditions for obtaining complete revitalized materials intended for tissue engineering of the trachea.

CONCLUSION

The addition of the pro-inflammatory cytokine IL-1β in in concentration physiologically relevant to inflammation (1 μg/ml) to a complete DMEM nutrient medium with a high glucose content of 4.5 g/l, 5% FBS, insulin and ascorbic acid, promotes volumetric colonization of the cartilage-based devitalized scaffold on nasal chondrocytes tracheal tissue, but at the same time it can cause a violation

of the complex microarchitectonics of the natural intercellular substance of cartilage. Stimulation of the population of the native scaffold cells can be used to produce highly effective revitalized materials for tissue engineering of the trachea.

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The authors declare no conflict of interest.

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