

Biocompatible Synthetic Tracheal Matrices Based on Polymer Ultra-Fibrous Materials Colonized by Mesenchymal Multipotent Cells

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The aim of the investigation is to study biocompatibility and biodegradation of synthetic tracheal matrices based on ultra-fibrous polymer materials, colonized by mesenchymal multipotent stromal cells (MMSC) of recipient.

Materials and Methods. The study was performed on beagle dogs, which underwent heterotopic implantation of synthetic tracheal scaffolds made of a nonwoven material by electrospinning and colonized by the recipient MMSC. Implant biocompatibility was assessed by computed tomography and macro- and microstructure analysis of the extracted implant and adjacent tissues.

Results. It was established that the developed trachea matrix specimens implanted heterotopically to the dogs preserved biocompatibility, ensured colonization by recipient cells, did not cause local and systemic reactions of rejection, and had no general toxic effect. According to the data obtained there was made a conclusion on the biocompatibility of tracheal matrices made of the polymer ultra-fibrous materials and their prospective applications as bioimplants for trachea defect replacements.

Key words: tracheal matrix; heterotopic implantation; biocompatibility; mesenchymal multipotent cells.

Various defects of trachea due to malignant neoplasms, traumas and congenital abnormalities require sometimes its resection. Meanwhile, formation of primary “end-to-end” anastomosis is feasible less than in 50% patients with this pathology [1–3]. In other cases application of implants is required to replace significant tracheal defects. At present there are no prostheses in clinical practice, matching the native trachea by their biological, configurative and mechanical properties. The most perspective direction in creating trachea bioimplants is a tissue engineering approach [1–6]. The first successful transplantation of the bioengineering construction, made of decellularized donor trachea

colonized by recipient cells, was performed to the patient with a terminal stage of bronchomalacia in 2008 [7]. As an application of donor trachea has a number of limitations, and decellularization procedure may impair mechanical properties of implants and is rather labor-consuming, various synthetic matrices based on nanocomposite or ultra-fibrous materials capable to be populated by mesenchymal multipotent stromal cells (MMSC) of the bone marrow have recently appeared for this purpose [3, 8–10]. Despite the evident progress, the attempts to create a construction combining optimal mechanical properties, chemical stability and structure, providing colonization by recipient cells and

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intercellular interactions have not been a success so far. Therefore further investigations directed to the creation of hybrid synthetic and natural matrices, combining optimal mechanical properties and stability with biocompatibility.

The aim of the investigation is to assess biocompatibility, biodegradation and ability to colonize synthetic trachea matrices made of ultra-fibrous polymer materials by recipient cells.

Materials and Methods. Synthetic tracheal matrices for investigations on dogs were obtained from nonwoven material using proprietary pilot single capillary electrospinning system. Fluoroplastic F-42V (copolymer of tetrafluoroethylene with vinylidene fluoride) (ChimCombinat, Russia) was used as a polymer for the non-woven material. Rings, reinforcing the non-woven polymeric material, were produced by thermopressing, and polyurethane Elastillan 1195 A (Elastogran, Germany) was used as a basic material (Figure 1).

Mechanical properties of the specimens were tested using a universal testing machine Instron 5965 (Instron, USA) provided with a computer system for on-line data analysis.

The studies were conducted on 6 beagle dogs (1 female and 5 males) aging 4.0 ± 1.2 years, housed in vivarium and Department of Experimental Therapy of N.N. Blokhin Russian Cancer Research Center. All manipulations with the animals were performed in accordance with the ethical principles established by European Convention for the Protection of Vertebrata used for Experimental and other Scientific Purposes (the Convention was passed in Strasburg, March, 18, 1986, adopted in Strasburg, June, 15, 2006) and the requirements of the Declaration of Helsinki (the Declaration was passed in Helsinki, Finland, June, 1964, and revised in October, 2000, Edinburg, Scotland), and the World Medical Association (2000).

To improve biocompatibility and to boost matrix integration, tracheas were colonized with MMSC of dogs-recipients. MMSC were generated from the bone marrow cells, obtained by sternal puncture. The marrow cells were placed into sterile culture tubes with nutrition medium RPMI 1640 (PanEco, Russia), containing 10% of embryonal fetal serum (PanEco, Russia). Two passages were performed to remove nonadhesive cells.

The analysis of MMSC culture was performed with the help of inverted microscope Axiovert 40 (Carl Zeiss, Germany) in a transmitted-light or phase contrast mode. Histologic examination was done in compliance with the standard technique, sections were stained with hematoxylin and eosin (PanEco, Russia), preparations were examined and photographed using light microscope Axioplan 2 (Carl Zeiss, Germany). The level of superficial MMSC marker expression was determined by flow cytometry by means of cytofluorometer BD FACS Canto II (Becton Dickinson, USA) using antibodies

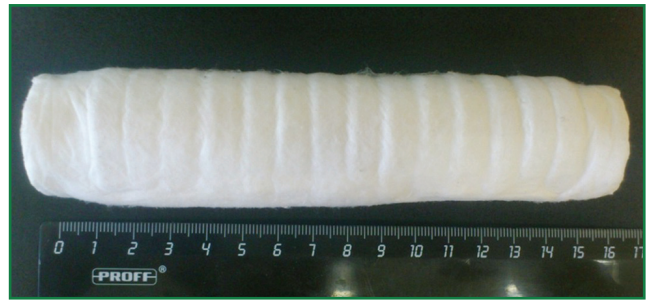


Figure 1. Tracheal matrix specimen based on polymer ultra-fibrous materials for the experiments on dogs

CD45, CD90, CD105 conjugated with fluorochromes (Becton Dickinson, USA).

Sterile matrices were populated by incubation with the suspension of MMSC in the cultural medium at 37°C and $4.5\% \text{CO}_2$ for 7 days, shaking periodically the cultural tube. Colonization of the matrix by MMSC was controlled by obtaining imprints from the surface of the implant fragments on the slide, covered by polylysine (Thermo Scientific, USA) with the following Romanovsky–Giemsa staining [11].

Heterotopic implantation of tracheal matrices, populated by autologous MMSC, was performed *ex vivo* on 6 beagle dogs: matrices were implanted in the intermuscular space of the thigh fold. For this purpose an intermuscular pocket, into which a matrix sample was placed, was formed in the dogs, then the wound edges were closed in layers by interrupted sutures, the external suture and adjacent skin area were treated with antiseptics. The operations were performed under general anesthesia observing all the requirements of aseptics and antiseptics in the operating unit of the Department of Experimental Therapy of N.N. Blokhin Russian Cancer Research Center.

Animals have been observed for a month. During this period a daily visual examination of the operative area was conducted, regular analysis of hematologic indices was performed using an analyzer (ProCyt Dx, The Netherlands), which has been especially calibrated to investigate the dog's blood. Dogs-recipients underwent computed tomography on SOMATOM Sensation 64 System (Siemens, Germany). Various planes of the lumbosacral region were analyzed in layers covering the area of xiphoid process. 30–35 days after the implantation specimens of the matrix were taken out under general anesthesia for microscopic and histologic examination.

To estimate the degree of significance of the revealed changes the analysis of a set of related individualized samples using Friedman test and “Nonparametric statistics” module of the Statistica 6.0 (StatSoft) program was performed. Differences were considered significant at $p < 0.05$.

Results and Discussion. The specimens of the

tracheal synthetic matrices had a tubular shape and did not essentially differ from the native tracheas of the dogs by their dimensions and mechanical properties. The differences did not exceed 30% (Tables 1 and 2).

Before the sterile matrices were implanted to the animals they were colonized *ex vivo* by MMSC, obtained from the bone marrow cells of the recipients by cultivation in the nutrition media. Target cells were presented mainly by large polygonal or spindle cells with long processes

being in contact with each other. The cell nuclei were moderately oxyphilic of the round or oval form with centrally located nucleoli. The whole area of the tube bottom was covered with a relatively uniform confluent cellular monolayer (Figure 2). Immunophenotype analysis of the 2nd passage cells established, that the subpopulation of CD45⁻CD90⁺CD105⁺ cells amounted to not less than 65%.

The morphological examinations and the assessment of the immunophenotype of the cells allowed us to characterize them as MMSC.

The analysis of the imprints from the matrix surfaces made it possible to characterize the revealed MMSC: they were distinguished by the enlarged nuclei and had colony-forming foci, indicating to the preservation of the proliferative potential of MMSC in the tracheal matrices (Figure 3).

Tracheal matrices populated by MMSC were implanted heterotopically to the dogs in the intermuscular pocket formed in the inguinal area (Figure 4).

Complete blood count was made periodically to

Table 1

Mean values of dimensional characteristics of the native dog trachea and matrices (mm)

Parameters of the semiring	Native trachea	Matrix
Width	4.0±0.3	4.0±0.2
Length	64.0±2.3	63.0±1.1
Thickness	2.0±0.3	2.0±0.1
Diameter	23.0±9.0	26.0±5.0

Table 2

Elasticity of matrix fragments, reinforced by polyurethane-based semirings, and the native trachea

Parameters of statistical analysis	Multiple compression – index of the developing load fall (%)		Single compression – index of the ring rigidity (kPa)	
	Matrix	Native trachea	Matrix	Native trachea
Median	8.2	7.0	43.4	37.2
Minimum	7.6	6.3	38.2	32.2
Maximum	8.6	8.2	47.2	41.0
First quartile (25 th percentile)	7.8	6.3	39.2	32.2
Third quartile (75 th percentile)	8.6	8.2	44.0	41.0
Comparison with the native trachea min–max (%)	6–20		4–28	

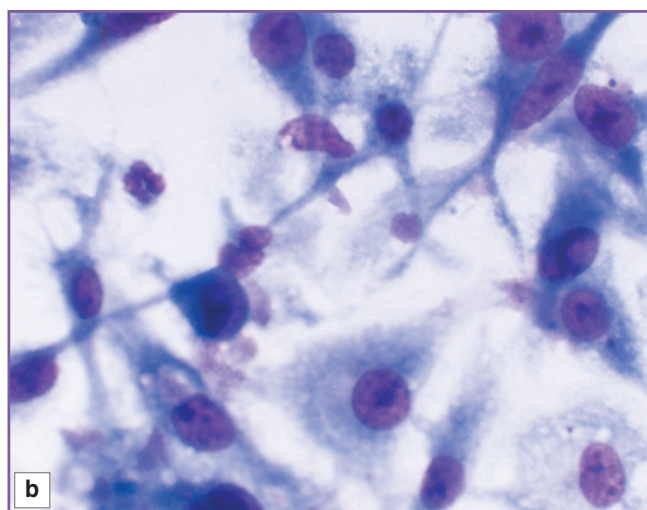
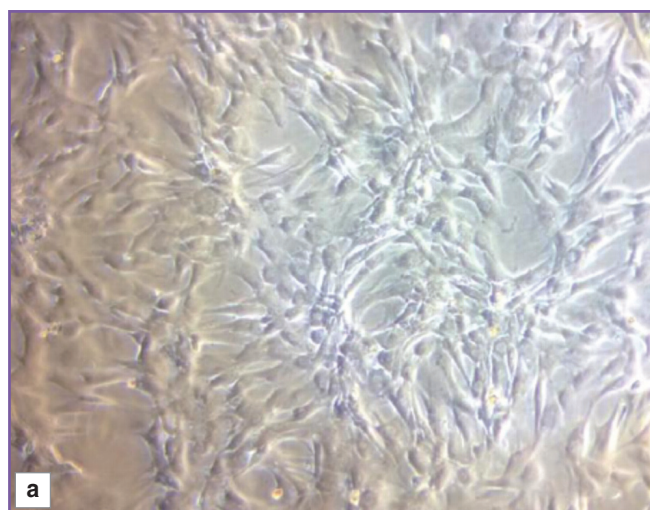


Figure 2. Morphology of dogs' mesenchymal multipotent stromal cells in the culture: (a) live culture, ×200; (b) Romanovsky–Giemsa cell staining, ×900

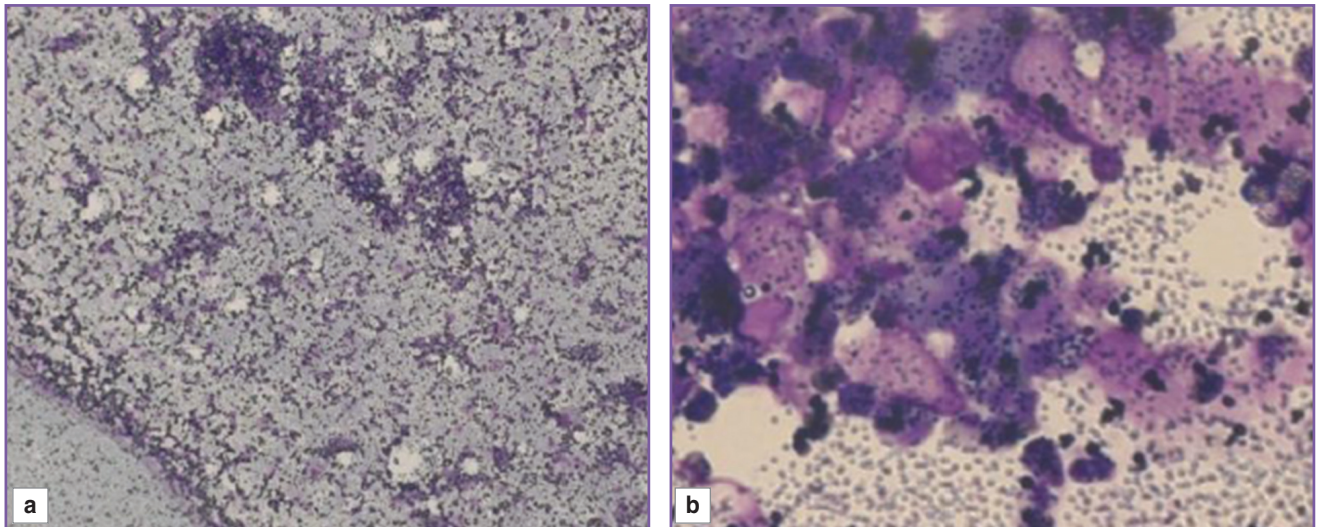


Figure 3. Cell composition of the matrix surface: (a) $\times 200$; (b) $\times 900$. Romanovsky–Giemsa cell staining

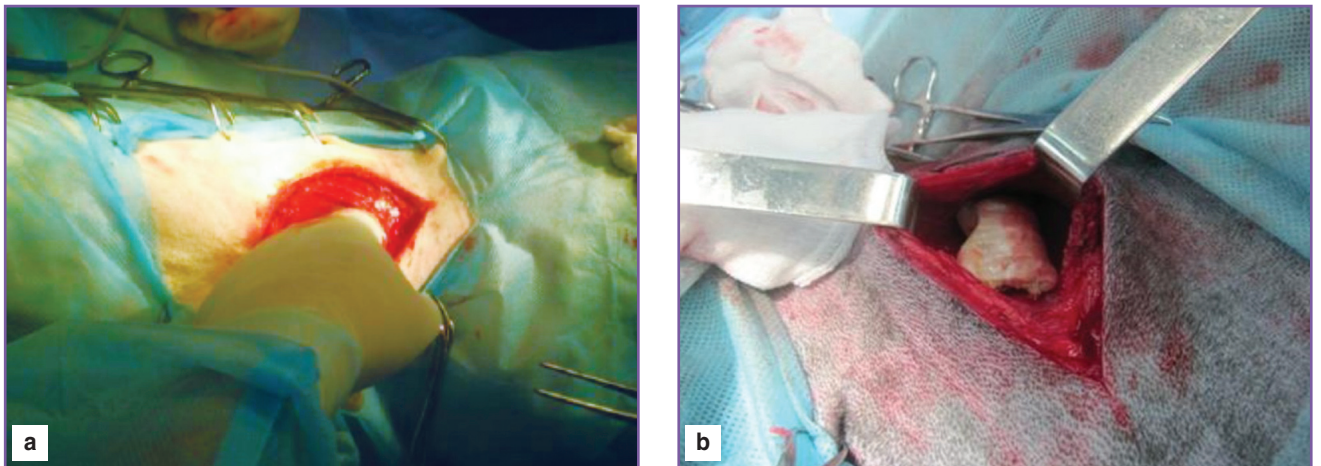


Figure 4. The main stages of heterotopic transplantation of matrix specimens loaded with mesenchymal multipotent stromal cells to the dogs: (a) intermuscular pocket formation; (b) implanted tracheal matrix specimen

monitor systemic reaction of implant rejection or inflammation following heterotopic implantation of the experimental specimens. Leukogram (WBC) and absolute leucocyte count were estimated, as changes in these hematological indices directly correlate with the development of the cell reaction cascade, implementing transplant rejection [12, 13]. Hematological indices of animals were shown to vary within the physiological norm during a month after the implantation (Table 3), i.e. no leukocytosis showing the signs of systemic reaction of rejection were observed.

20–25 days after the operation the animals underwent computed tomography under general anesthesia. The data obtained by computed tomography led to the conclusion that experimental specimens in the animal organisms did not lose such signs of functionality as preservation of matrix framework providing the inner lumen of almost a circular shape, and physical integrity (Figure 5).

Visual examination did not reveal any local reaction of rejection: edema, suppuration, reddening, fistulas, massive growth of fibrous tissue in the implantation area during the whole period of observation (Figure 6 (a)).

The tracheal matrix, removed from the inguinal fold a month after the implantation, preserved its shape and framework properties, and was covered with connective tissue capsule, intimately bound to the matrix tissue (Figure 6 (b)). Morphological investigations of the adjacent tissues revealed no inflammatory exudates in the implantation area. No signs of inflammatory leukocyte infiltration, which forms a cellular cascade of rejection in case of acute rejection, realizing cell destruction of the allograft, were found either [13].

A powerful colonization by the recipient cells with formation of vessels and connective tissue fibers extending from the surface deep into the implant was found in the extracted tracheal matrix alongside with

Table 3

Statistical analysis of the dogs' hematological index dynamics after heterotopic transplantation of tracheal matrix specimens, loaded with autologous mesenchymal multipotent stromal cells

Blood indices	Statistical values	Before the operation	After the operation		p (Friedman test)
			After 2 weeks	After 4 weeks	
Erythrocytes ($\times 10^{12}/L$)	Median	6.2	6.6	7.0	0.441
	Min	5.7	6.0	5.9	
	Max	8.1	8.0	8.1	
Hemoglobin (g/dl)	Median	14.7	14.6	15.9	0.052
	Min	13.1	11.7	13.8	
	Max	16.4	16.0	17.7	
Leukocytes ($\times 10^9/L$)	Median	9.5	10.2	11.2	0.097
	Min	8.5	9.8	9.4	
	Max	9.9	15.4	14.5	
Neutrophils (%)	Median	69.3	76.7	72.0	0.716
	Min	66.3	64.2	64.8	
	Max	71.5	79.8	77.3	
Lymphocytes (%)	Median	27.6	21.5	24.6	0.717
	Min	26.3	16.1	21.6	
	Max	31.5	34.6	32.1	
Monocytes (%)	Median	0.2	0.2	0.1	0.368
	Min	0.1	0.1	0.1	
	Max	0.2	0.2	0.2	
Eosinophils (%)	Median	2.0	1.5	3.0	0.497
	Min	2.0	1.0	1.0	
	Max	4.0	4.0	3.0	
Basophils (%)	Median	0	0.1	0.1	0.135
	Min	0	0	0	
	Max	0.1	0.2	0.3	



Figure 5. Computed tomography image of the tracheal matrix made of ultra-fibrous fluoroplastic on day 25 after heterotopic transplantation to the dog (craniocaudal projection)

the preservation of the ultra-fibrous structure (Figure 7). The data gained by CT, macro- and micromorphological examination showed that the shape, framework and constituent elements (undestroyed fibers of fluoroplastic and intact polyurethane rings) of the synthetic matrices were preserved and there were no signs of biodegradation of the tested experimental specimens.

Conclusion. Examination of the synthetic material specimens, colonized by mesenchymal multipotent stromal cells demonstrated preservation of physical properties (size, design, integrity), functional characteristics (framework, strength, elasticity), initial structure (regular fibers of the matrix base, reinforces by semirings), and absence of local and systemic signs of rejection within a month when implanted heterotopically. The specimens also preserved the integrity and lumen potency, provided their colonization by recipient cells, did not possess general toxic effect and were not

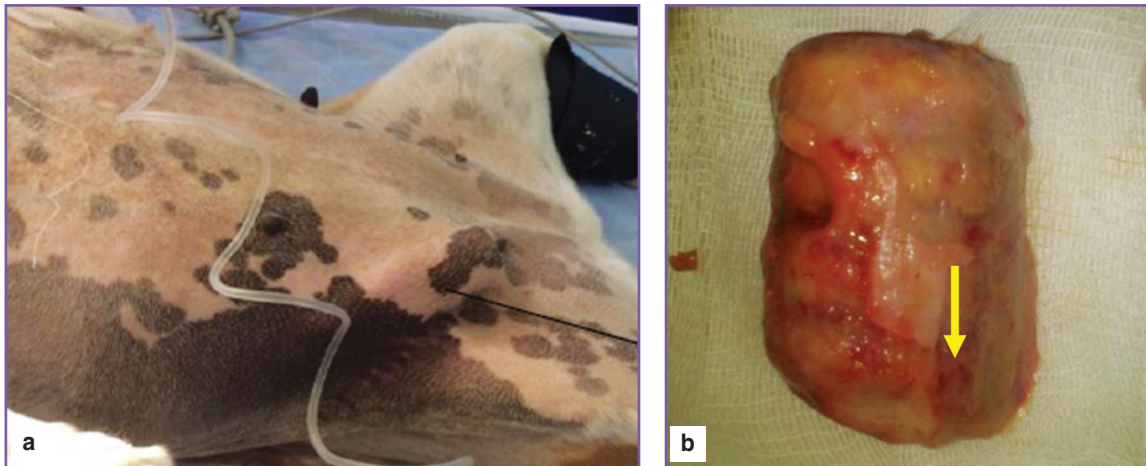


Figure 6. Synthetic tracheal matrix colonized by mesenchymal multipotent stromal cells 35 days after heterotopic transplantation: (a) inguinal area of the recipient dog after transplantation of the matrix specimen; (b) general view of the synthetic tracheal matrix after extraction from the recipient body tissues. Connective tissue capsule, intimately bound to the tracheal matrix, is indicated by the arrow

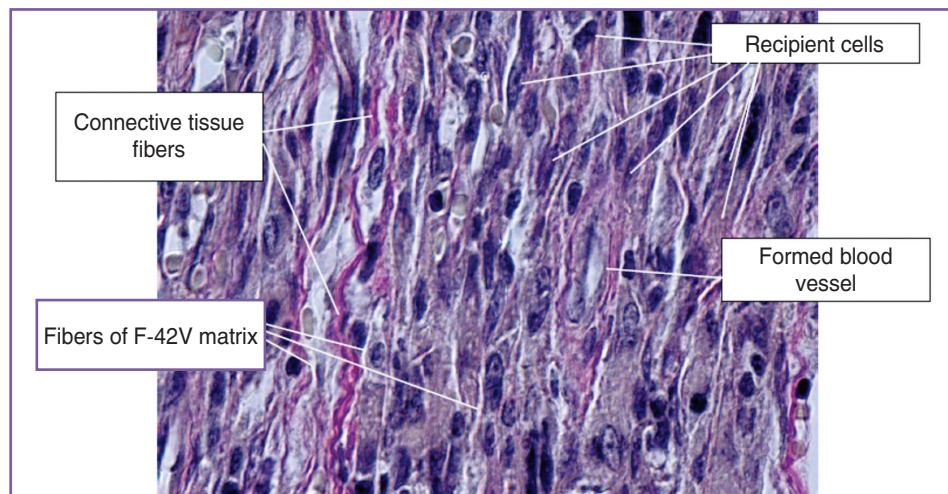


Figure 7. Active colonization of the fibers of the tracheal nonwoven matrix layer by the recipient cells

subject to biodegradation when exposed to the internal environment of the organism. These data allowed us to make a conclusion on the optimal compatibility of the developed matrices and their perspective application as bioimplants for the replacement of tracheal defects.

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Conflicts of Interest. The authors declare no conflicts of interest.

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