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FLUORESCENCE IMAGING IN EVALUATING THE REVASCULARIZATION OF HETEROTOPICALLY TRANSPLANTED PRIMATE TRACHEA SEGMENT

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Objective: to assess the potentials of using indocyanine green fluorescence angiography in evaluating revascularization of tissue-engineered construct that was obtained from the decellularized biological matrix of primate trachea, including using mesenchymal stem cells, after heterotopic tracheal allotransplantation. **Material and methods.** Tracheas were obtained from two male hamadryas baboons. After decellularization, 4 cm segments of tracheas were implanted under the lateral part of the latissimus dorsi in two healthy primates, one after recellularization with mesenchymal stem cells (animal 1), and the second without recellularization (animal 2). Immunosuppressive therapy was not performed. Blood flow in the transplanted segment of the trachea was evaluated 60 days after transplantation by surgical isolation of the flap of the latissimus dorsi with the transplanted segment of the trachea, while maintaining blood flow through the thoracodorsal artery. Indocyanine green near-infrared fluorescence angiography was visualized using a FLUM-808 multispectral fluorescence organoscope. **Results.** Sixty days after implantation, the tracheal cartilaginous framework macroscopically appeared to be intact in both animals, tightly integrated into the muscle tissue. The framework retained its natural color. After intravenous injection of indocyanine green, the tracheal vessels were visualized in both animals. Intercartilaginous vessels and portions of the cartilaginous semi-rings devoid of vessels were clearly distinguished. The entire implanted segment was almost uniformly vascularized. No local disruptions in blood supply were observed. The fluorescence brightness of the tracheal vessels was 193 ± 17 cu and 198 ± 10 cu in animals 1 and 2, respectively. The average muscle brightness in the implantation zone was 159 ± 9 cu and 116 ± 8 cu in animals 1 and 2, respectively. **Conclusion.** Indocyanine green fluorescence angiography is characterized by high-contrast images and high sensitivity. This facilitates vascular patency visualization and allows to assess the degree of neoangiogenesis after experimental transplantation of the tracheal segment, at different stages of experiment, without euthanizing the animal.

Keywords: trachea, tissue engineering, transplantation, angiography, fluorescence.

INTRODUCTION

Tracheal diseases, both tumor and benign, often require surgical treatment. Radical surgery is technically feasible where what is being resected is just less than 50% of the length of the trachea. This involves circular resection of the corresponding section of the trachea. Most surgeons believe that resection of a longer trachea comes with a significant risk of complications and is considered unrealizable [1]. A radical solution to this problem may be to replace the affected part or the entire trachea with a cadaveric donor organ or tissue-engineered construct (TEC) [2]. Regenerative medicine is a promising new interdisciplinary field of research and clinical practice. Its methods avoid the need for life-long postoperative immunosuppressive treatment. Tissue engineering involves the modeling and creation of biological or synthetic scaffolds of the trachea in order to replace the affected organ. It is crucial to ensure that the matrix of the bioengineered organ could repeat the mechanical and biological properties of the extracellular matrix of the native organ, could have a three-dimensional structure that facilitates attachment, growth and reproduction of the corresponding cell type, could ensure cell migration and influx of growth factors, could support neoangiogenesis and adequate reinnervation. Unfortunately, there is currently negative experience in the clinical use of tissue-engineered trachea based on both synthetic and decellularized matrices, including in the case of repopulation with the recipient's stem cell. Most attempts at one-time allotransplant of donor organs have also ended unsatisfactorily. This was associated with the difficulty of ensuring adequate graft vascularization in the postoperative period [3]. Attempts at ensuring revascularization of the transplanted trachea with the

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greater omental flap, transplanting it together with the thyroid gland, and applying microvascular anastomosis, were mostly accompanied by major complications and lysis of the donor trachea [4, 5].

Existing limited clinical experience shows that the main reasons for the failure of the tracheal graft is the lack of sufficient formation of an epithelial lining on its inner surface, as well as the loss of frame function due to lysis, primarily tracheal cartilage lysis. To prevent such complications, it is necessary at least to achieve adequate revascularization of the transplanted organ. Some researchers believe that the most reliable way to achieve higher positive outcomes in tracheal transplantation is to base the method on preliminary heterotopic implantation of the donor organ in the recipient's well-vascularized tissue (greater omentum, muscles) perfused by a vascular pedicle [6]. This method allows prefabrication of the TEC to increase its survival rate and reduce the risk of complications.

At the same time, common visual inspection will not be able to prove whether in vivo revascularization was achieved. This problem can be solved via indocyanine angiography, based on systemic injection of indocyanine green dye (ICG) into the bloodstream, followed by observation of the zone of interest in an infrared fluorescence light [7–9].

Objective: to assess the potentials of using indocyanine green fluorescein angiography in evaluating revascularization of a tissue-engineered trachea obtained from decellularized biological matrix of primate trachea, including using mesenchymal stem cells, after heterotopic tracheal allotransplantation.

MATERIALS AND METHODS

Procurement of donor trachea, recellularization of tracheal acellular matrix, and surgical interventions on hamadryas baboons were carried out at the Research Institute of Medical Primatology, Sochi. Tracheal implantation and explantation, as well as methodological and hardware support for infrared fluorescein angiography, were carried out by employees of Pavlov First St. Petersburg State Medical University. Donor tracheal decellularization was carried out at the regenerative medicine fundamental research laboratory of Kuban State Medical University, Krasnodar. The study protocol was approved by the local ethics committee of the Research Institute of Medical Primatology, Sochi.

Tracheas were procured from two male hamadryas baboons that died from natural causes at the age of 6 and 7 years; the animals weighed 9 and 10 kg, respectively. Within 60 minutes after their death, pathological and anatomical examination, excision and explantation of the cervical and thoracic trachea were performed under aseptic conditions. The material was transported to the laboratory for decellularization within 24 hours of explantation in sterile containers containing phosphate-buffered saline with addition of a 2% solution of antibiotic and antimycotic at a temperature not exceeding 4 °C.

Both tracheas were decellularized according to a single protocol: after removal of connective tissue and washing with phosphate-buffered saline, the trachea was placed in a specialized bioreactor ORCA (Harvard Apparatus, USA) on a rotating platform. The procedure included 3 cycles of treatment with detergents and enzymes for 24 hours each: 4% sodium deoxycholate solution in combination with 0.002 M Na2-EDTA solution (Sigma Aldrich, USA), then 1% sodium dodecyl sulfate solution and porcine pancreatic DNase-I 2000 IU/200 mL of phosphate buffer with calcium and magnesium (Sigma Aldrich, USA; Gibco, Life Technologies, USA). Decellularization was completed by washing the trachea in a 10% solution of chlorhexidine digluconate in a phosphate buffer with a three-fold change of solution every 8 hours.

After completion of decellularization, the biological scaffold was transported to the laboratory of the Research Institute of Medical Primatology within 12 hours in a sterile container with a phosphate buffer containing an antibiotic-antimycotic. Two healthy male hamadryas baboons, aged 1 year and weighing about 5 kg, were selected as recipients of the tissue-engineered trachea. Sections of donor trachea, 4 cm each, were implanted under the lateral portion of the latissimus dorsi. A tracheal section was implanted in one primate (animal 1) after recellularization with mesenchymal stem cells, the other primate (animal 2) was implanted without recellularization. Stem cell preparation and the recellularization process were carried out according to the protocol described earlier [3].

All operations were carried out according to the general plan: after epilation of the right lateral thoracic wall, under general intravenous anesthesia (ulnar vein), about a 7 cm long skin incision was made, the outer surface of the latissimus dorsi was isolated and a donor trachea section was sutured using three Prolen 4.0 ligatures, and the implant was completely circularly enveloped with the muscle (Fig. 1). The wound was sutured without leaving drainage tightly. Within 5 days after operation, the animals received antibiotic prophylaxis with ceftriaxone – 300,000–500,000 IU/day (depending on body weight); 2 mL of ketorol was also intramuscularly injected over 3 days. No immunosuppressive therapy or corticosteroid therapy was administered in the postoperative period.

The presence of blood flow in the transplanted segment of the trachea was assessed 60 days after transplantation. Under general anesthesia, a latissimus dorsi flap with the transplanted segment of the trachea was isolated with preservation of blood flow through the thoracodorsal artery (Fig. 2); the zone of interest was determined intraoperatively by palpation, as well as by localization of the filaments that fixed the tracheal areas. Then, an ICG solution (1 mg in 10 mL of water for injection) was

injected intravenously. Infrared fluorescein angiography was visualized using multispectral fluorescence organoscope FLUM-808 [8, 9], which included a fiber-coupled infrared diode laser (λ = 808 nm, 5W power) to excite ICG fluorescence, and a small-sized multispectral television system mounted on a tripod that records images in four parts of the spectrum, including in a 820–850 nm infrared range in which ICG is emitted. The camera is connected via a USB 3.0 port to a computer running the specialized RSScam program. This system can record photo and video images with a 960 × 960 pixels resolution at 25 frames per second and quantify the intensity of infrared fluorescence in the zone of interest.

RESULTS

All surgical interventions were performed based on a predetermined plan. No intra- or postoperative complications were noted. None of the animals died during the chronic experiment.

Tracheal decellularization quality, determined during routine histological examinations and in the quantitative determination of residual DNA, was found to be satisfactory and sufficient to continue the experiment. Hematoxylin and eosin staining of the decellularized trachea did not reveal the presence of intact nuclei and cells both in the mucous membrane and in the submucosal layer. However, single cells with significantly damaged nuclei remained in the cartilaginous part.

60 days after heterotopic implantation, the implanted tracheal fragments were found in both animals, the cartilaginous tracheal framework appeared to be intact macroscopically, tightly integrated into the muscle tissue, and of native color (Fig. 3, a); at the same time, the membranous wall of the trachea, and, accordingly, the lumen of the trachea, were absent. In animal 1, the extent of cicatricial changes in muscle tissue was minimally expressed, tracheal contours were even. In animal 2, the cicatricial process was expressed in the implantation zone, there were adhesions with neighboring muscles, the edges of the implanted trachea were not clearly defined palpatorically. In both animals, about half the length of the implanted trachea was separated from the latissimus dorsi, these areas were the areas of interest during fluorescein angiography imaging.

Immediately after introduction of ICG, a strong glow in the operating wound was detected in the infrared mode, while localization of the glow corresponded to the selected area of the latissimus muscle. In both animals, the tracheal vessels were visualized; inter-cartilage vessels and sections of cartilaginous half-rings devoid of vessels were clearly distinguished (Fig. 3, b). The entire implanted segment is almost evenly vascularized, there were no local blood supply disturbances. Fluorescence intensities of the tracheal vessels in animal 1 and animal 2 were 193 ± 17 and 198 ± 10 standard units, respectively. The average muscle brightness in the implantation zone of

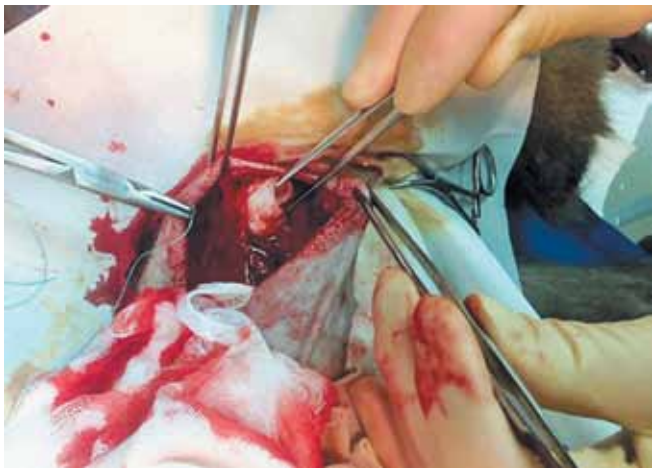

Fig. 1. Implantation of a decellularized trachea

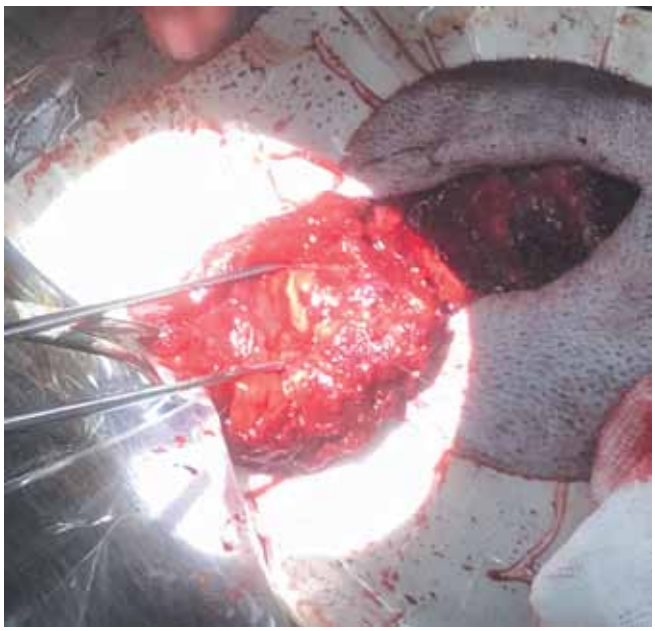

Fig. 2. Dissection of the latissimus dorsi flap with a transplanted segment of the trachea 60 days after heterotopic transplantation

animal 1 and animal 2 was 159 ± 9 and 116 ± 8 IU standard units, respectively; that is, the fluorescence intensity of the implanted trachea was the same in both animals, and noticeably higher compared to the brightness of the surrounding muscle areas. Subsequently, within approximately 60 seconds, fluorescence intensity decreased both in the muscle and in the implanted trachea.

DISCUSSION

Regenerative medicine offers a method for replacing a totally damaged trachea with a tissue-engineered organ for those patients who were previously considered incurable. The advantage of this approach is that immunosuppressive therapy is completely avoided in the postoperative period [2]. However, it should be noted that to date, the mechanisms of regulation of regenera-

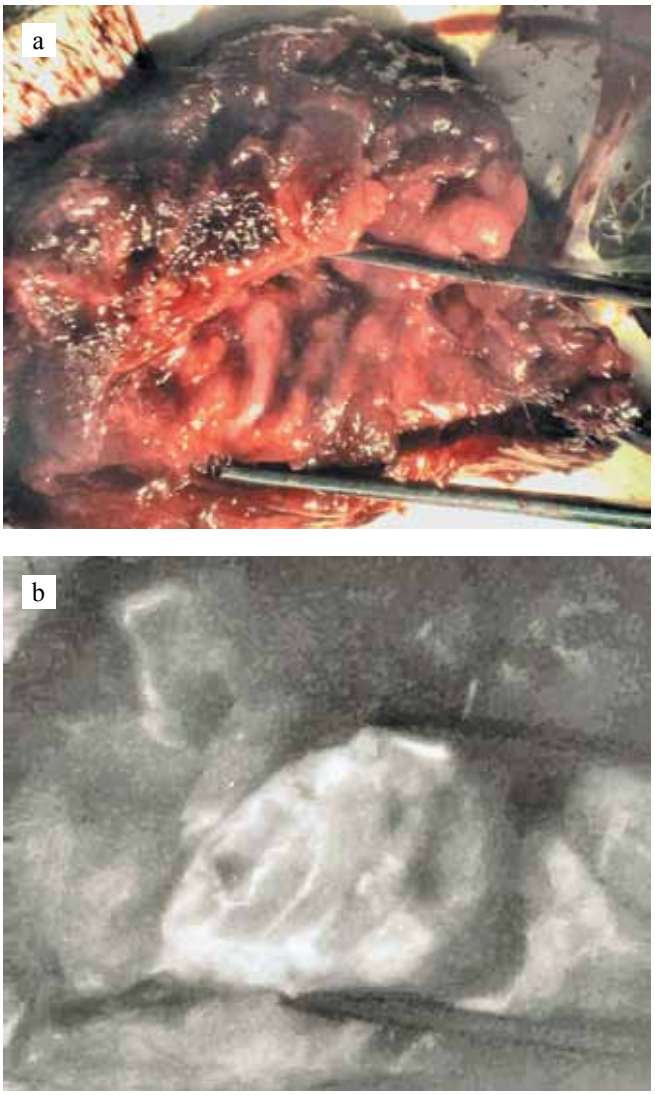

Fig. 3. Macroscopic evaluation of the implanted segment of the trachea (animal 1): a – in white light; b – in the light of ICG infrared fluorescence

tive processes in the body itself are not fully understood yet. This essentially complicates improving therapeutic approaches, which in itself is a very difficult task and requires more than one decade of scientific research and observation. The authors of the study planned to get an answer to the question about the possibility of prefabricating a tissue-engineered trachea during heterotopic transplantation in order to restore blood flow in the transplanted organ. This is one of the most important aspects of restoring organ function. They also had plans to explore the possibilities of a relatively new method of vascular imaging using indocyanine green fluorescence angiography. Existing vascular bed imaging methods using classical dyes with tissue tropicity (e.g. trypan blue), unfortunately, do not allow for a lifetime assessment of vascular patency. Moreover, the animal would need to be removed from the experiment at each stage of the study. This does not allow us to track neoangiogenesis process over time during TEC transplantation. These shortcomings are absent in infrared fluorescence imaging of hidden (from the eyes) features of the state of tissues in functioning organs. This imaging is based on the use of the fluorescence properties of ICG [9].

The results obtained allow us to suggest that implantation of a recellularized and unrecellularized tissue-engineered trachea is accompanied by inclusion of the transplanted segment of the trachea into the bloodstream 60 days after implantation. The pronounced fibrotic changes in the implantation zone in animal 2 may indicate a more pronounced reaction of the recipient's immune system to the implantation of a donor trachea in the absence of recellularization. Blood flow restoration mechanism in a transplanted trachea is quite complex and consists of many factors [10]. It is fundamentally important that fluorescein angiography is an informative method for assessing tracheal revascularization [11]. The fluorescence intensity of muscles and transplanted segments was almost the same.

It can be stated that in both animals there was neoangiogenesis stimulation regardless of the recellularization of mesenchymal stem cells. Of course, this is not enough for formation of a complete organ structure for the implanted trachea, but the indocyanine green angiography technique can be used to monitor blood flow restoration and an orthotopically transplanted organ, including during a tracheoscopy. The recently developed video endoscopic system for bronchoscopy, which provides a fluorescence imaging regime in the infrared region of the spectrum, can serve as an instrumental basis for such studies, including in clinical conditions [5].

CONCLUSION

Indocyanine green fluorescence angiography is characterized by high image contrast and high sensitivity. Therefore, visualizing the patency of the vasculature and assessing the extent of neoangiogenesis after experimental transplantation of a tracheal segment at different stages of the experiment can be possible without animal euthanasia.

The authors declare no conflict of interest.

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