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HYPOPHARYNGEAL RECONSTRUCTION USING PRELAMINATED AUTOLOGOUS BIO-ENGINEERED PECTORALIS MAJOR FLAPS

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After removal of metastatic malignant tumors of the hypopharynx and larynx, hypopharyngeal defects are formed. To restore the hypopharynx, a mucosa and a muscular component are needed. The **objective** of this study is to develop a hypopharyngeal reconstruction technique using prelaminated pectoralis major flap with mucosal epithelium analogue from autologous epithelial layers. **Materials and methods.** Nine patients underwent reconstruction of the hypopharynx using bioengineered prelaminated pectoralis major flaps. The mucosa was restored by tissue-engineered autologous epithelial cell layers that were obtained by culturing in vitro cells isolated from skin biopsies that were previously obtained from patients. **Results.** Oral nutrition was restored in all cases. Pharyngeal stenosis was detected in one (11%) patient. A stratified squamous epithelium on the pectoral fascia was revealed in 67% of cases at week 2 after prelamination, in 89% of cases at week 4 after reconstruction and in 100% of cases at month 3, 6, 12 and 24 after reconstruction. **Conclusion.** Reconstruction using prelaminated bioengineered flaps allows recreating the anatomical integrity and function of the hypopharynx.

Keywords: hypopharyngeal defects, reconstruction, prelaminated pectoralis major flap, tissue engineering, epithelial layers, keratinocytes.

INTRODUCTION

Patients with advanced hypopharyngeal and laryngeal cancers (stages III and IV) are most often candidates for combined oncologic treatment, combining extensive surgical resection and reconstruction with adjuvant radiotherapy (RT) or chemoradiotherapy (CRT). Sometimes the surgery is limited to tumor removal without a reconstructive phase due to the presence of severe comorbidities or treatment in non-specialist hospitals.

Different variants of flaps are used for hypopharyngeal reconstruction, including local flaps from the neck area, displaced regional flaps from the chest area, displaced visceral flaps, microsurgical free fasciocutaneous flaps, musculocutaneous flaps, visceral flaps, etc. [1, 2]. The main disadvantage of skin flaps is hair growth in the pharyngeal lumen. Visceral flaps are inapplicable in somatically burdened patients; their use is limited by concomitant abdominal conditions and previous interventions on the abdominal cavity.

The ideal material for reconstruction is autologous identical tissue, whose formation involves minimally invasive procedures on the donor site. There are known attempts to solve these issues by tissue engineering. Mucosal epithelial fragments have been cultured for use as biomaterials [1]. The use of cultured epithelial cells to restore the mucosa of the upper GI tract has been described. After oral tumor resection and endoscopic resection of the esophagus, surgeons implanted tissue-engineered epithelial cell layers on the underlying tissue [3, 4, 5]. It is important to note that epithelial-stromal interaction is a key determinant of the phenotypic dynamics of the epithelium in homeostasis and injury. The epithelium and the direction of epithelial stem cell differentiation are influenced by the underlying stromal cells producing a complex of signaling molecules [6, 7].

Prelamination transforms a native axial flap into a stratified one by adding appropriate structures for composite reconstruction [8]. It is a process involving the implantation of tissues or structures into the blood supplying microenvironment before they are transferred directly to the defect area [8, 9, 10].

The objective of the present study is to develop a technique for hypopharyngeal reconstruction by prelaminated flaps with mucosal restoration with tissue-engineered autologous epithelial cell sheets.

MATERIALS AND METHODS

Ethics statement

A prospective study was carried out from January 2018 to December 2019 at the National Medical Re-

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search Radiological Centre, Moscow. The study was reviewed and approved by the local ethics committee. All patients signed an informed consent.

Patients

Nine patients with hypopharyngeal defects after surgical treatment for malignant laryngeal tumors were included in the study. They were not candidates for microsurgical reconstruction due to comorbidities. In all cases, the reconstruction was delayed.

Characteristics of the patients are shown in Table. There were 8 males and 1 female; their ages ranged from 57 to 82 years (median: 69.5). Histological examination revealed squamous cell carcinoma in all cases. Six patients had stage III and IVA primary tumors. Six patients with primary laryngeal tumors had postoperative staging according to the 8th edition of TNM classification (developed and adopted by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). The tumor process in these patients corresponded to the following indicators: T4a (n = 4), T3 (n = 2), N0 (n = 5), N2c (n = 1). Three patients had recurrent tumors after previous CRT. All patients underwent laryngectomy, hypopharyngeal resection, and unilateral and/or bilateral lymphadenectomy depending on the condition of the cervical nodes. Eight patients underwent RT or CRT according to the stages of antitumor treatment prior to the reconstructive phase. Most patients had comorbidities according to the adult comorbidity evaluation 27 (ACE-27) scoring system. Hypopharyngeal defect after tumor removal was partial with preservation of a fragment of the posterior pharyngeal wall in all cases (Fig. 1, a).

Fabrication of autologous cell sheets

To isolate autologous epithelial cells, a skin biopsy from the patient's scalp was used. The skin fragment was resected under local anesthesia; the wound was sutured with Vicryl® 3.0 sutures. After the biopsy was taken, it was placed in a special container with a transport medium (DMEM with 0.4 mg/ml gentamicin) and transported to the Koltzov Institute of Developmental Biology, Moscow. Epithelial cell isolation was performed on the day the biopsy was taken. All procedures for isolating, cultivating, and transferring cell cultures to biopolymer

Table

Demographic data, treatment modality, and follow-up periods

Patient	Age	Gender	Tumor location	Stage	TNM	Primary or recurrent	RT/ChRT (Gr)	Preoperative comorbidity (ACE-27)	Follow-up period (months)
1	65	M	Larynx	IVA	T4aN0M0	Primary	RT (40)	Severe	27
2	59	F	Larynx	IVA	T3N2cM0	Primary	RT (50)	Severe	25
3	57	M	Larynx	IVA	T4aN0M0	Primary	RT (46)	Moderate	24
4	61	M	Larynx	IVA	T4aN0M0	Primary	CRT (59, 6)	Severe	20
5	60	M	Larynx	IVA	T4aN0M0	Primary	RT (50)	Severe	27
6	65	M	Larynx	III	T3N0M0	Primary		Severe	22
7	63	M	Larynx		rT4aN0M0	Recurrent	CRT (66)	Moderate	27
8	70	M	Larynx		rT4aN0M0	Recurrent	RT (50)	Moderate	24
9	82	M	Larynx		rT4aN0M0	Recurrent	CRT (70)	Severe	10

Abbreviations: RT, radiation therapy; CRT, CRT; Gr, Gray.

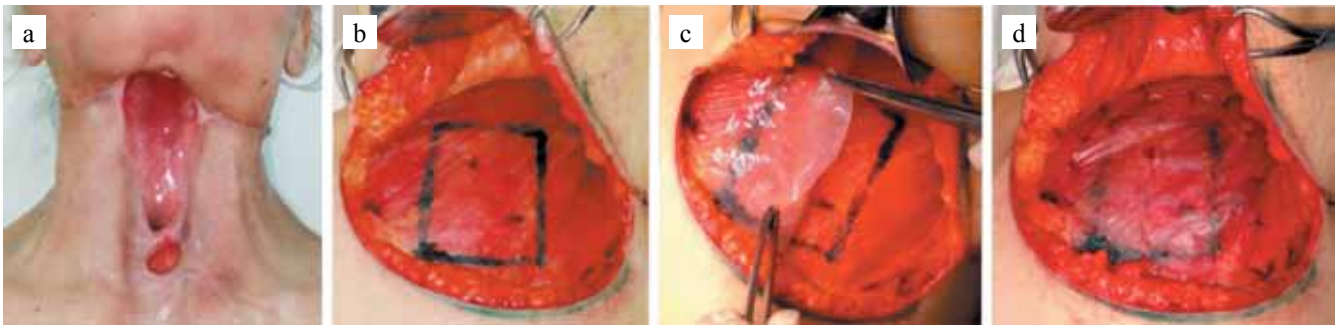

Fig. 1. The first stage of reconstructive surgery: (a) Patient with pharyngostomy after combined treatment of stage IVA laryngeal cancer; (b) surgical access for prelamination; (c) implantation of epithelial cell layer on the pectoral fascia; (d) isolation of epithelial cell sheet with film

matrices were performed strictly under aseptic conditions in the laboratory.

Isolation of epidermal keratinocytes

Skin biopsies obtained from the donor were cut into 3 × 10 mm wide strips, washed with phosphate-buffered saline (PBS) solution, placed in 0.2% dyspase solution (Sigma), and incubated at 4 °C for 18 hours. After fermentation, the epidermis was separated from the dermis along the basement membrane line, washed with PBS solution, and additional fermentation was performed with trypsin solution for 10 minutes to obtain a single-cell suspension. The suspension was centrifuged at 1000 rpm for 6 minutes, the supernatant was removed, and the precipitate was suspended in keratinocyte culture medium.

Cultivation of epidermal keratinocytes

A suspension of keratinocytes was seeded into 75 cm^2 cell culture vials (Costar) at a 2×10^5 cells/ml concentration, 15 ml each. Cells were cultured in a CO_2 incubator at 37 °C and saturation humidity in Keratinocyte-SFM medium (Gibco). The cells were grown with regular medium changes (every 2 days) for 3–6 weeks until the formation of a subconfluent layer. The date of pharyngoplasty was assigned depending on the growth rate of the patient's cells.

Immunohistochemistry and immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 minutes, followed by immunofluorescence staining for keratinocyte-specific marker using Abcam's first anti-cytokeratin 14 antibody (CK 14) (ab181595) and epithelial stem cell and early epidermal progenitor marker p63 (ab124762), using the method recommended by the manufacturer. After incubation, the preparations were washed with PBS, and then a solution of second antibodies conjugated with Alexa Fluor 555 and Alexa Fluor 488 (ThermoFisher) was added for visualization.

Immunohistochemical detection of keratinocyte-specific markers on the surface of the muscle flap was performed on histological preparations that were stained for total cytokeratin markers – PanCk (ab7753) and p63 (ab124762). Primary antibodies from Abcam were used according to the manufacturer's recommended technique; peroxisome method and second antibodies with streptovidin-DAB complex, followed by H&E staining, were used to visualize reaction products.

Planting and growing cell cultures on matrix

To form a cell layer, cultured keratinocytes were seeded on the surface of the matrix, which is a 0.3–0.5 mm thick plate consisting of hyaluronic acid and collagen. Epidermal keratinocytes were separated from the culture vial using a mixture of trypsin and Versene solutions (1 : 1). An epidermal keratinocyte suspension was seeded into a Petri dish with a 4×10^5 cell/cm^2 density matrix placed in it. The resulting stratum with keratinocytes was then incubated under standard conditions, at 37 °C, 5% CO_2. The bioengineered autologous stratum was ready for use after about 5–7 days, during which time the epidermal colonies formed a confluent layer on the matrix surface. The number of cells at the end of this period should be $80 \pm 20 \times 10^5$ cells/cm^2. The prepared epithelial layers on the matrix were packed in sterile packaging and used within 12 hours.

Assessment of cell viability on the surface of epithelial cell sheets

A fragment of the prepared cell layer was placed in a well of a 48-well plate, and 0.5 ml of Calcein AM working solution (BD Pharmingen) was added. After a 30-minute incubation, the cell sheet was visualized under an Olympus IX73 fluorescence microscope (CKP equipment, Koltzov Institute of Developmental Biology). The obtained micrographs made it possible to assess the morphology of viable cells and their distribution on the matrix surface.

Reconstructive stages

At the first stage, prelamination of the bioengineered muscle and epithelial flap was performed.

A graft grown from the patient's autologous epithelial cells on the matrix was implanted into the pectoral fascia and fixed with Vicryl 5.0 suture (Fig. 1, c). It was covered with a latex film and fixed with a Prolen 3.0 Prolen 3.0 suture (Fig. 1, d). The epithelial cell sheets were thus left to take root for 3 weeks.

At the second stage, the laryngopharynx was reconstructed. First, pharyngostoma edges were mobilized (Fig. 2, a). During the formation of the prelaminated pectoralis major epithelial flaps, the pectoralis major was mobilized from the chest wall and moved to the area of the defect, as in the traditional surgical technique (Fig. 2, b). The size of the muscle fragment of the pectoralis major and epithelial flap was determined according to the size of the soft tissue defect in the neck. The surface of the pectoralis major with a prelaminated cellular layer (bioengineered muscle and epithelial flap) was oriented such that it was directed into the hypopharyngeal lumen (Fig. 2, c). The bioengineered fragment was modeled according to the size of the defect in the hypopharyngeal mucosa and fixed using a Vicryl 2.0 suture to the mobilized edges of the hypopharyngeal mucosa along

the pharyngostoma perimeter (Fig. 2, d). The soft tissues were restored with the muscular part of the flap.

Postoperative period

Before starting oral feeding, all patients underwent imaging of the larynx, hypopharynx and cervical esophagus to determine the condition and patency of the newly formed organ and assess swallowing function. In the absence of signs that the contrast agent had exited the contours of the upper GI tract, the nasogastric tube was removed, and the patients began feeding by mouth. Results were assessed at weeks 2 and 4, and months 3, 6, 12, and 24 after pharyngoplasty. X-rays of the pharynx and esophagus with contrast agent and videolaryngoscopy with biopsy were performed. Biopsy of the bioengineered fragment of prelaminated flap with histological examination was performed at week 3 after prelamination, and week 2 and 4, month 3, 6, 12, and 24 after pharyngoplasty.

Statistical analysis

Only descriptive statistics were used to analyze treatment and outcomes. The authors had access to the data and reviewed and approved the final version of the manuscript.

RESULTS

All patients underwent hypopharyngeal reconstruction using a bioengineered prelaminated pectoralis major and epithelial flap as described above.

Cells obtained from patients undergoing antitumor therapy show low growth rate and high sensitivity to culture conditions in the first few days after isolation. Therefore, to increase the required cell mass, they were grown in culture vials on special low-calcium, proliferation-stimulating culture medium (Fig. 3, a, b). Immunofluorescence study at the stage of formation of stable monolayer culture cells revealed the presence of typical epithelial cells expressing ck14, a keratinocyte-specific marker, and a large number of cells positive for p63, the epithelial stem cell and early epidermal progenitor marker (Fig. 3, b). After the cells formed a normal epithelial layer, they were transferred to a biocompatible matrix. The base of the matrix used was collagens and hyaluronic acid, 0.5 mm thick. Thus, an epithelial tissue autograft of 60 to 80 cm^2 in area was created for each patient (Fig. 3, c). The presence of living cells in the graft was monitored using a fluorescent microscope, after staining a small area of the graft with viability dye Calcein AM (Fig. 3, d).

Histological examination at week 3 after prelamination revealed areas with keratinocyte colonies on the muscle flap surface (Fig. 4, a, b, c, d). Positive expression of cytokeratins (Fig. 4, b) and specific protein p63 characteristic of keratinocytes of basal skin layer (Fig. 4, d) indicates that these cells belong to epithelial tissue cells. This confirms that keratinocytes were present on the pectoralis major for 3 weeks after transplantation. Thus, after 3 weeks of preliminary preparation of the muscle and epithelial flap, we considered it ready for the reconstructive stage of surgery.

The median postoperative follow-up period was 23.1 months (range 10–27 months). Videolaryngoscopy of the pharynx and esophagus in the patients showed that the area of epithelial-cellular flap implantation at month 3, 6, 12 and 24 was macroscopically indistinguishable from the surrounding mucosa (Fig. 5, a, c).

In histological studies, we observed a stratified squamous epithelium on the pectoral fascia at week 2 after pharyngoplasty in 78% of cases, week 4 after pharyngoplasty in 89% of cases (Fig. 5, b), month 3, 6, 12 and 24 after pharyngoplasty in 100% of cases (Fig. 5, d).

Oral feeding was restored in all cases. The overall median time to resumption of feeding was 18 days after surgery (range, 14–22 days). The mean postoperative

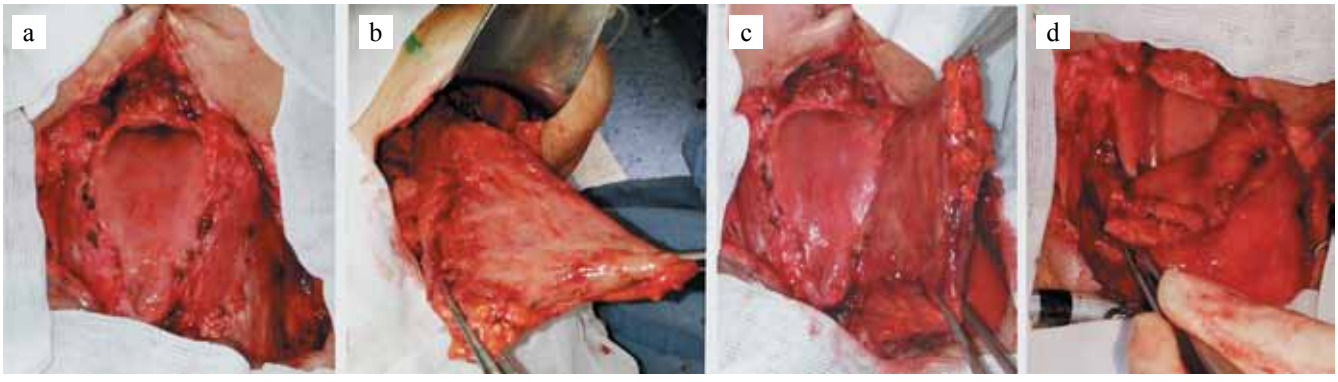

Fig. 2. Pharyngeal reconstruction using a prelaminated bioengineered pectoral flap: (a) mobilized edges of the pharyngostoma; (b) prelaminated bioengineered pectoral flap; (c) movement of the bioengineered pectoral flap to the neck; (d) fixation of the prelaminated flap section to the mobilized mucosa edges along the perimeter of the pharyngostoma

hospital stay was 17.5 days (range, 16 to 19 days). Voice function was restored by voice prosthesis implantation in 78% of cases.

No flap necrosis was noted. Fistula was observed in 4 patients (44%). The cause of delayed wound healing and formation of suture failure in the pharyngeal suture area could be RT/CRT performed before pharyngoplasty and nutritional deficiency. In all patients, fistulas were formed in the area of anastomosis between the upper edge of the flap and the base of the tongue, and were closed within 7–10 days after conservative therapy and dressings without the need for additional surgical intervention. One patient had hypopharyngeal stenosis in the lower edge of flap fixation. The stenosis area was subjected to bougienage dilation, and as a result, the hypopharyngeal lumen reached a diameter of 1 cm. This patient had difficulty swallowing only solid food. Suppuration of the donor area was observed in one patient; the wound healed after conservative therapy and dressings.

DISCUSSION

Reconstruction of the upper GI tract after laryngectomy with hypopharyngeal resection remains a major challenge for head and neck cancer surgeons, as in most cases it is performed after RT/CRT in somatically burdened patients [11]. Various reconstruction options are used to repair extensive defects after surgical treatment [12]. The reconstruction technique depends on the patient's health status, clinic options, size and composition of the defect, radiation history, and previous surgeries. Somatically burdened patients with a high risk of postoperative complications are rarely acceptable candidates for microsurgery. Because of these limiting factors, advanced flaps remain the preferred method [13].

In some cases, a bioengineered flap can be a good alternative to standard skin and muscle flaps. Bioengineering technologies facilitate the creation of tissue analogues of the mucosa used for restoration of the mucosa of the upper GI tract, oral cavity, urethra, bladder, vagina

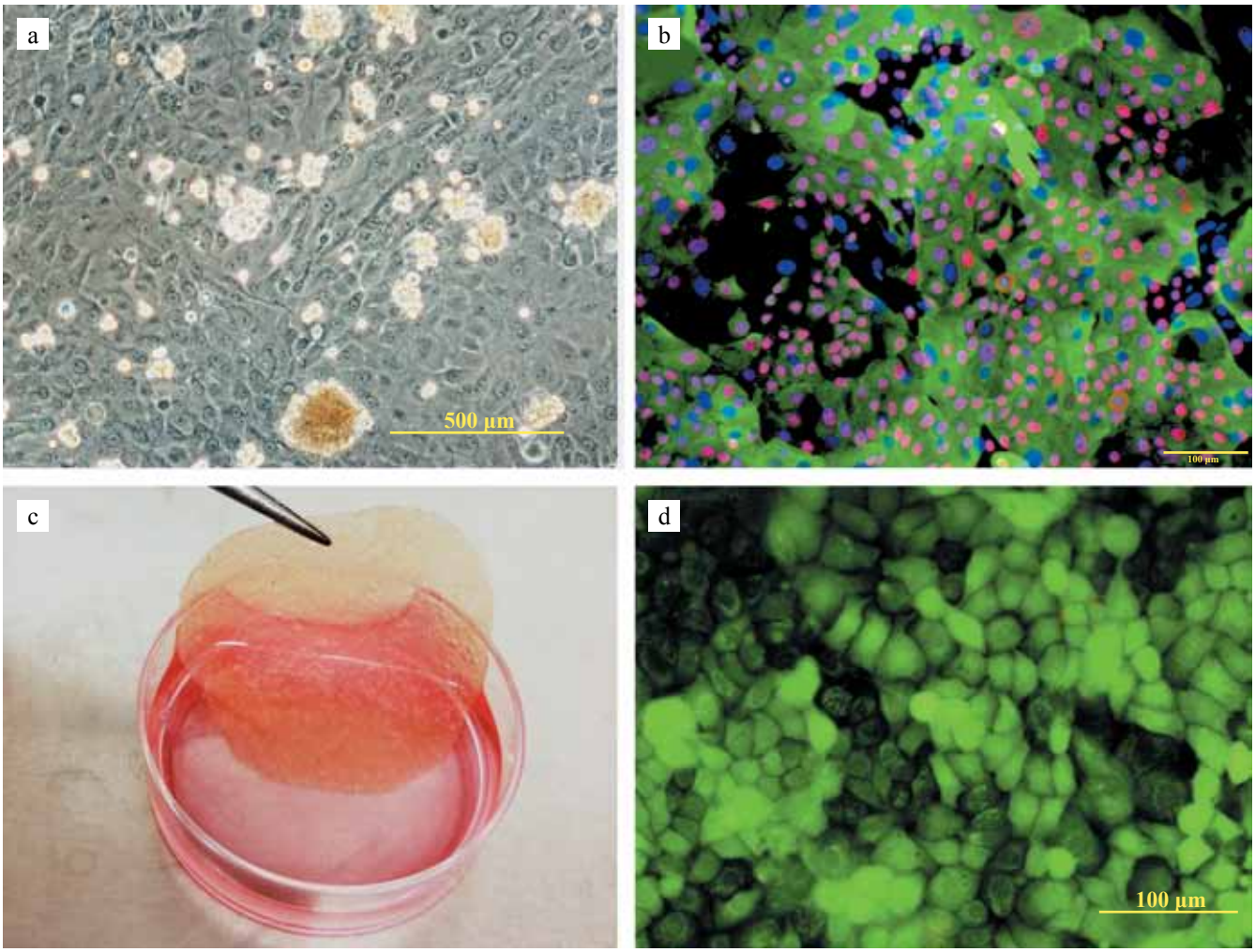

Fig. 3. Cell graft preparation: culture of patient's autologous keratinocytes at week 4 of cultivation: (a) phase-contrast; (b) immunofluorescence identification of keratinocyte-specific marker ck14 (green staining); specific epidermal transcription factor p63 (red staining), nuclei stained with DAPI (blue staining). Ready-to-use graft, appearance (c) Micrograph of human keratinocytes grown on matrix surface, detection of viable cells using viability dye Calcein AM (green staining) (d)

and cornea. Restoration of damaged epithelial tissue in this case occurs due to the fact that the graft contains autologous poorly differentiated cells of the basal layer of the epidermis, which can proliferate and integrate into the defect site. At the same time, the tissues surrounding the bioengineered construct influence the cells included in it. For instance, it was found that corneal cells can transdifferentiate into epidermal cells under the influence of signals from the embryonic dermis [14].

The plasticity of epidermal keratinocytes was also observed in experiments on cell transplantation into the urethra. It was shown that three weeks after transplantation of autologous EGFP-expressing rabbit skin keratinocytes into the urethra, they restored the urothelium, showing signs of specific marker expression [15].

Recently, the ability of esophageal cells to differentiate in the cutaneous direction under the influence of adult skin stroma has been demonstrated [16]. Since the hypopharynx itself is lined with non-keratinized, stratified squamous epithelium, it was reasonable to assume that the epidermis is highly suitable for its replacement. In the humidified microenvironment, epidermal keratinocytes lose their ability to keratinize as they do in culture conditions. Thus, they may well perform the functions of the pharyngeal epithelium. Previously, a study was published in which a displaced muscle graft consisting of the pectoralis major with a pre-implanted mucosal tissue equivalent, created on the basis of cultured donor keratinocytes, was used for hypopharyngeal reconstruction. Donor cells used as an epithelial layer in such constructions can temporarily act as a barrier epithelium, ensuring reliable engraftment of the graft in the recipient tissue area, and also modify the wound surface, stimulating the wound's own epithelization [5].

When eliminating hypopharyngeal defects after removal of locally advanced tumors, comprehensive restoration of soft tissues and mucosa, as well as normal functioning of the digestive tract and vocal function is necessary. Prelaminating the cultured cells onto a well-

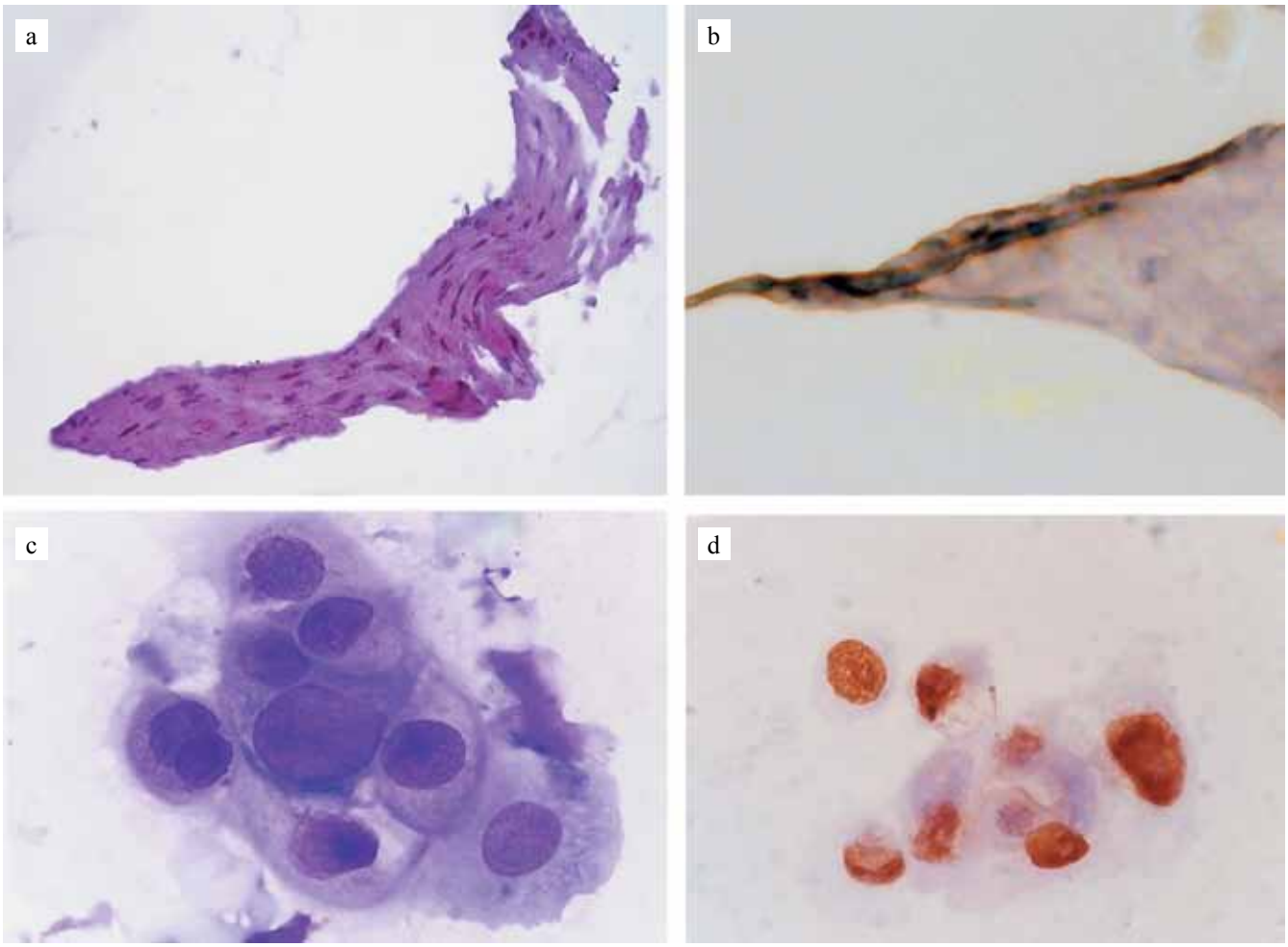

Fig. 4. Biopsy of muscle slice at week 3 of prelamination with epithelial tissue equivalent: a fragment of muscle tissue from the transplantation area (a) H&E staining (100× magnification); Immunohistochemistry. Expression of common cytokeratins (DAB staining, magnification ×100) (b). Cytological study of muscle flap surface with a ready-made analog: H&E staining (1000× magnification) (c); Immunohistochemical detection of epidermal transcription factor p63 (DAB, 1000× magnification) in cell nuclei (d)

perfused tissue of the pectoralis major allows creating flaps with the required properties: a layer of epithelial cells has time to form on the muscle surface, which together with the muscle tissue can be formed according to the size of the existing defect.

We used bioengineered prelaminated flaps to reconstruct extensive hypopharyngeal defects. The use of an epithelial layer grown from the patient's skin instead of a full-layer autodermal flap avoids such complications as hair ingrowth into the laryngeal lumen, stenosis of the upper GI tract lumen in the postoperative period. The percentage of fistula complications observed in patients in our study is 44%, which is comparable to the known rate of fistula in patients who received preoperative RT, which ranges from 13% to 50% [17]. After reconstructive surgery using skin and muscle flaps, 56.5% of patients mentioned difficulty swallowing only solid foods and 21.7% reported difficulty swallowing both solid and liquid food [17].

In our study, 1 (11%) patient had pharyngeal stenosis, which, after bougienage, caused problems with swallowing only solid food. Based on our postoperative follow-up, we can conclude that the technique we developed is able to restore the anatomy and functions of the laryngopharynx with identical tissues.

CONCLUSION

Hypopharyngeal reconstruction with a prelaminated bioengineered flap can was able to recreate the anatomical integrity and function of the hypopharynx in all the 9 clinical cases described.

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The authors declare no conflict of interest.

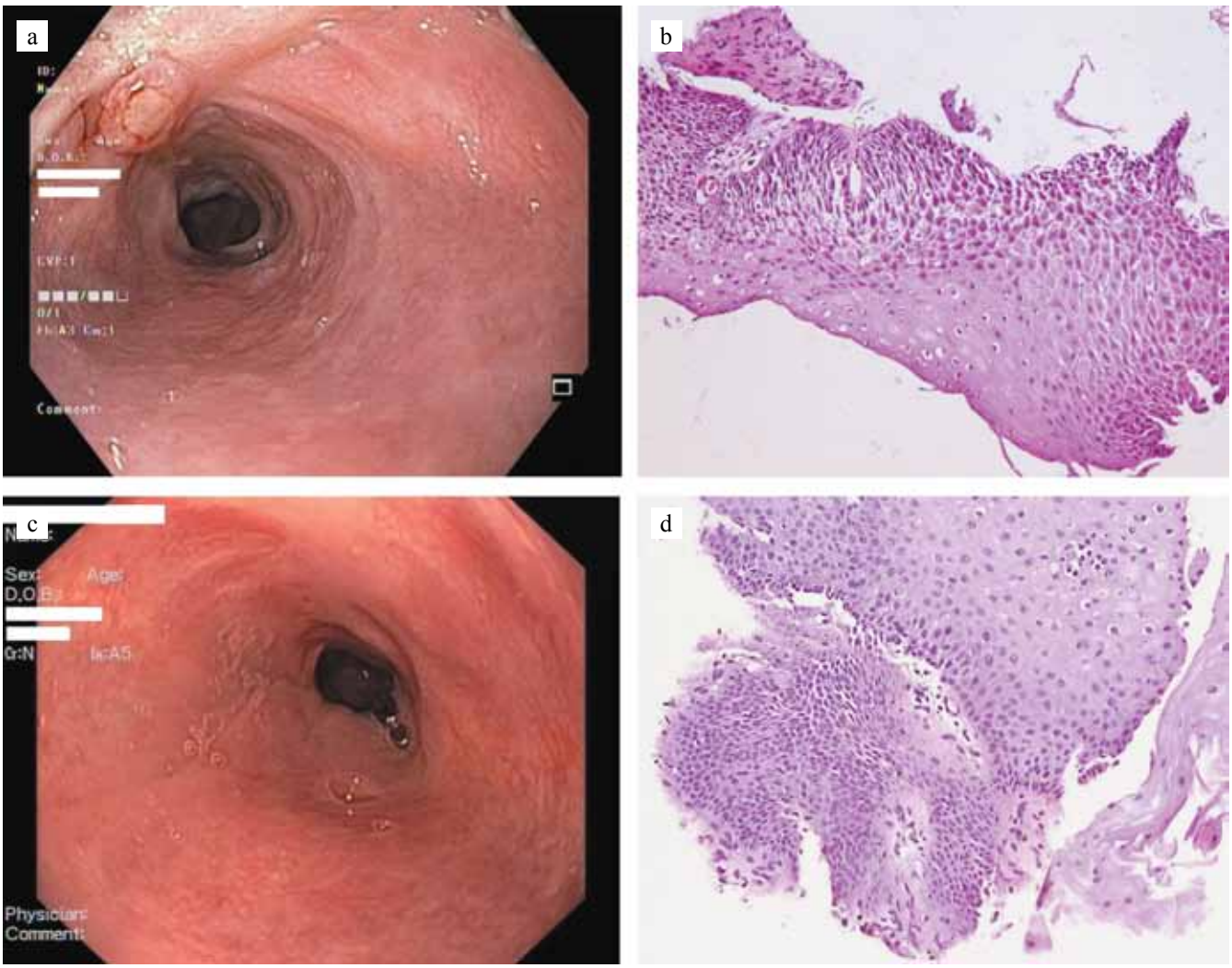

Fig. 5. Postoperative data: (a) Videolaryngoscopy at week 4 after pharyngoplasty; (b) H&E, at 100× magnification shows stratified squamous epithelium; (c) Videolaryngoscopy at month 24 after pharyngoplasty; (d) H&E, at 100× magnification shows stratified squamous epithelium with basal layer proliferation

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