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CULTIVATED AUTOLOGOUS ORAL MUCOSAL EPITHELIAL TRANSPLANTATION

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According to the World Health Organization, corneal blindness is the fourth most common cause of blindness and visual impairment worldwide. In Russia, up to 18% of blindness is caused by corneal damage. Limbal stem cell deficiency (LSCD) is one of the causes of corneal blindness and visual impairment due to anterior epithelial replacement with fibrovascular pannus. Bilateral LSCD may develop in patients with aniridia, Steven–Jones syndrome, and severe corneal burns of both eyes, leading to severe decrease in visual acuity in both eyes and, as a consequence, physical disability associated with blindness. In such cases, cell therapy, based on autologous oral epithelial culture as an alternative to allogeneic limbus transplants, is proposed for reconstruction of the anterior corneal epithelium. This new treatment method promotes corneal reepithelization, better visual acuity, reduced nonspecific ocular complaints and improved quality of life of patients. The effectiveness and significant increase in the frequency of transparent engraftment of donor corneas after cell therapy drives huge interest in this topic all over the world. This review presents literature data on the features of histotopography and methods for obtaining a cultured autologous oral mucosal epithelium, on cell markers that are used to identify epithelial cells, and on methods for creating cell grafts for subsequent transplantation to the corneal surface in LSCD patients.

Keywords: oral mucosal epithelial cells, oral epithelium, stem cell transplantation, cornea, corneal epithelium, limbal stem cell deficiency.

BACKGROUND

The World Health Organization states that corneal blindness is the fourth (5.1%) most common cause of blindness and low vision in the world [1]. Reports have it that as of 2015, there were 23 million unilaterally corneal blind people and 4.9 million bilaterally corneal blind people in the world due to corneal disease [2]. In Russia, up to 18% of blindness is caused by corneal disease [3]. Shortage of donor material, as well as pathogenesis of several corneal diseases resulting in ineffective keratoplasty for one reason or the other have led to exceptionally high demand for new directions of treatment for these conditions to be developed and introduced into clinical practice.

Limbal stem cell deficiency (LSCD) is one of the causes of corneal blindness and visual impairment in corneal pathology. It is known that the anterior corneal epithelium is renewed during life due to local unipotent progenitors located in the limbus called limbal epithelial stem cells (LESC) [4]. In acute extensive alteration or in the case of a chronic process, LESC damage can be irreversible. At the same time, deficiency in function and/or a lack of these cells lead to disruption of the natural epithelial renewal process and is classified as LSCD. In LSCD, the physiological barrier with the conjunctiva is destroyed, which leads to migration of fibrovascular tissue to the surface of the corneal stroma, and causes severe persistent decrease in visual acuity [5].

With progression, LSCD leads to corneal punctuate epitheliopathy and, often, appearance of persistent epithelial defect, significantly increasing the risk of corneal ulceration and perforation [6]. With unilateral lesion, if the paired eye is healthy, the possibilities of social adaptation in such a patient are not considered limited. However, depending on the severity of symptoms, the quality of life can be significantly reduced. In aniridia, Stevens-Johnson syndrome, and corneal burns in both eyes, bilateral LSCD develops, causing a marked decrease in visual acuity in both eyes and, consequently, disability due to blindness. Because of superficial and/or deep corneal neovascularization, keratoplasty in any patient with LSCD is classified as "high risk" due to an unsatisfactory prognosis for transparent engraftment [7]. According to J.S. Friedenwald [8], LSCD is considered as a trigger mechanism for superficial corneal neovascularization. Hence, epithelial reconstruction in this syndrome is a pathophysiologically grounded and justified procedure.

Currently, there is no unified approach to the treatment of LSCD. Many groups of researchers propose various methods and surgical techniques depending on the extent of the process (the extent of limbal corneal zone

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lesion along its circumference), involvement of one or both eyes, and the tear production level [9, 10]. Several clinical studies in bilateral LSCD have investigated the effectiveness of surgical methods of allogeneic corneal limbus transplantation from a deceased donor [11] or from a living relative donor [12]. However, the protocol of pharmacological support for this operation is long-term systemic immunosuppression [13]. To solve this problem, a number of research groups suggest using cell therapy based on cultured oral mucosal epithelial cells [14]. Cell culture for transplantation is obtained under laboratory conditions from a biopsy specimen of the oral mucosa [15]. Early works on this topic suggested the use of cultivated autologous oral mucosal epithelium [16, 17]. The rationality of choosing this type of cells was due to its morphological properties similar to the anterior corneal epithelium [18]. It is non-keratinized, stratified squamous and is in contact with air. According to reports, successful corneal re-epithelialization based on these cells was observed in 72% of cases, with follow-up periods from 1 to 7.5 years [14]. The barrier between the corneal and conjunctival epithelium was restored, chronic inflammation regressed, and visual acuity increased in 68% of patients. Y. Satake et al. showed that engraftment of such an epithelial graft according to the Kaplan–Meier analysis is relatively stable over time, and is 64.8% in the first year, 59.0% in the second and 53.1% in the third. [19]. In the work of A. Baradaran-Rafii et al. [20], after transplantation of cultivated autologous buccal epithelial cells, penetrating keratoplasty was performed for optical purposes. Kaplan–Meier analysis revealed that the corneal graft retained transparency in 92.9% of cases after the first year of observation and in 69.2% at 3.3 years.

Thus, clinical studies show that cultured autologous oral epithelium can be considered as the main method of repairing the corneal epithelium in bilateral LSCD, as well as an alternative treatment option in unilateral LSCD. The prospect of *de novo* epithelialization in severe corneal diseases by transplantation of a cultured autologous oral epithelium is responsible for the increased interest in this topic worldwide.

Objective: to analyze literature data on experimental methods of obtaining a cultured autologous oral mucosal epithelium and to identify the most relevant directions in the development of the technology for transplanting these cells.

FEATURES OF THE STRUCTURE OF THE ORAL EPITHELIUM

By embryogenesis, the epithelial tissues of the oral cavity have heterogeneous origin, which is reflected in their structure and physiological properties [21]. Based on histological studies with staining for specific markers in the oral cavity, it is possible to detect areas of both keratinized and non-keratinized stratified epithelium [22]. Specifically, the epithelium of masticatory surfaces, such as the hard palate and gingiva, is considered to be the keratinized type. The epithelium lining the lower surface of the tongue, soft palate and floor of the mouth, as well as the mucosa of the lips and cheeks (buccal) is classified as non-keratinized [22]. According to literature, buccal epithelium may contain areas of parakeratinization, and may be presented as keratinized along the teeth clamping line [23]. In contrast, the mucosal surface of the lip is lined with a histologically more homogeneous non-keratinized epithelium, which has fewer stratified layers [23]. It is generally known that the corneal epithelium is non-keratinized stratified squamous epithelium [24]; hence, transplantation of cultured cells with keratinization and/or parakeratinization properties for its reconstruction is not an optimal solution.

METHODS FOR OBTAINING AN ORAL EPITHELIAL CULTURE

One of the key issues in the application of cell technologies in clinical practice is the standardization of the culture medium and conditions. It is important to note that for clinical use, it is recommended to use culture media that have no animal components [25], while supplements used to stimulate the growth of a certain cell type (insulin, hydrocortisone, and others) must have a GMP (Good Manufacturing Practice) certificate [26]. It has also been shown that autologous patient serum can be used as a common mitogen in cell transplant production process [27].

Among the culture media used in the clinic as the base for obtaining the buccal epithelium culture, the following were used: DMEM/F12 medium (1:1–1:3) containing 1.05–1.425 mM calcium and medium for keratinocyte growth with a low calcium content: 0.06–0.07 mM [28]. Low calcium content in the medium is a method of culture selection of the epithelium, due to morpho-functional transformation and elimination of fibroblast-like cells [29]. High calcium content, in turn, causes stratification of epithelial cells [30] and can reduce the overall regenerative potential of the future cell preparation.

According to reports, the most common group of culture medium supplements used to stimulate oral epithelial growth includes factors such as insulin, hydrocortisone, human epidermal growth factor (hEGF), triiodothyronine, and cholera toxin [31]. According to our data, the last two factors are supplied as research reagents and are not GMP certified.

Primary oral epithelial cell culture can be obtained by cultivating explants or by treating tissue with enzymes [26]. The first method is relevant if the mucosal biopsy is small (2–4 mm), and enzymatic treatment can lead to

the death of progenitor epithelial cells. The disadvantage of this method is slow growth and potential possibility of culture contamination by fibroblast-like cells from the submucosa of the biopsy specimen. For larger tissue samples, the enzyme treatment technique, which is in two stages, is applicable [26]. The first uses a dispase solution in DMEM medium (1.8 mM calcium) to split the basement membrane. For this, the mucosal tissue is placed in a solution with a 2.4 U/mL dispase concentration for 18 hours at +4 °C (cold version) or at +37 °C for 2 hours (accelerated version). At the second stage, the split-off epithelium is treated with trypsin-versene (0.25–0.02%) to obtain a cell suspension for seeding. According to some authors, the concentration of 4–5 × 10^5 cells per cm^2 is the most optimal for seeding buccal epithelial cells [32]. The primary culture and its passaging are carried out in standard conditions under phase-contrast light microscopy with a change of medium after 1 day. The buccal epithelial cell culture is distinguished by its high proliferative potential and ability to maintain the population during subculturing [31].

IDENTIFICATION OF ORAL EPITHELIAL CELLS IN CULTURE AND TISSUE

The most common technique for identifying oral epithelial cells in culture is immunofluorescent staining of cultured cells. Proliferation markers are among the most important ones, as they make it possible to identify progenitor cells both by the general marker of dividing cells Ki67 [33] and by the more specific ones for oral epithelium p75 [33] and p63 [34]. The cell phenotype is confirmed by staining for epithelium-specific integrin β1 (basement epithelium) [35], vimentin (intermediate filaments) [36], ZO-1 (Zonula occludens-1) (dense intercellular contact protein type 1) [33], connexin-43 (gap junction protein) [36]. Staining for cytokeratin markers detects keratinized (CK 1 and 10) and non-keratinized (CK 4 and 13) epithelium [37, 36]. Cytokeratin 8 and 18 staining can be used to detect cells expressing markers characteristic of leukoplakia and squamous cell carcinoma in situ [38]. Additional staining for CD 44 and 73 markers allows identification of fibroblast-like cells in culture [28]. Oral mucosal epithelium in biopsy specimen can be routinely identified on cross sections using hematoxylin-eosin paraffin staining. For a more detailed characterization of the epithelium in the tissue, immunohistochemical staining for the above markers on cryosections is used.

METHODS FOR CREATING CELL GRAFTS BASED ON CULTURED ORAL EPITHELIAL CELLS

Cell therapy in the context of corneal surface reconstruction cannot be accomplished by simply instilling a suspension of cultured cells. Therefore, carriers or matrices are needed to anchor the cultured cells and create a tight contact between the graft and the cornea [39]. Based on the properties of the cornea in general and its epithelium in particular, it should be understood that they should be transparent, easy to manipulate both during cultivation and in the process of transplantation. It is necessary that the matrix maintains the proliferation of cultured cells and maintains their high viability [31]. In experimental clinical studies, the amniotic membrane (amnion), fibrin gel, and cell layer creation technology were used for transplantation of autologous cultured buccal epithelium [31].

The amnion is a flat membrane that mimics the basement membrane, upon cultivation on which buccal epithelial cells spread horizontally, forming a planar structure [40]. When used as a substrate for the growth and transfer of cultured cells, their quantity and quality (immunophenotype) before transplantation is extremely difficult to assess.

Fibrin glue makes it possible to encapsulate cells in a bulk tissue-engineered construct by sequentially mixing a suspension of cultured cells and glue components [41]. Fibrin glue, which has a registration certificate in Russia (Ivisel®, Johnson & Johnson), has never been studied before as a carrier of oral epithelial cells. Unlike its counterpart (Tisseel®, Baxter), this glue has a shorter biodegradation period due to the absence of antiproteoletic enzyme aprotinin in its formulation. Both adhesives are not autologous products, although they have a high safety profile. Their widespread use is limited due to the complexity of their delivery and storage, which are carried out at temperatures below –20 °C.

The technology of obtaining cell layers was proposed, among others, to create a buccal epithelial cell graft [16]. For this, special laboratory glassware was used, with a heat-sensitive polymer applied to the culture surface [42]. When transferred from an incubator (+37 °C) to room temperature (+20…24 °C), the polymer changes its properties to hydrophobic and allows the separation of the cultured cell layer as a thin film without using enzymes. The resulting cell sheet, however, is a fragile object and also requires fixation to the cornea during transplantation.

Thus, today there are a variety of methods for cultivating the oral mucosal epithelium and methods for obtaining a graft. Almost every stage is variable, from choosing a biopsy site to determining a substrate for cultivation. For the reader's convenience, a clarifying characteristic of the methods is presented (Table).

CONCLUSION

Due to the disabling nature of the diseases causing LSCD, reconstruction of partially or completely lost corneal epithelial cover has been a challenging issue in oph-

thalmology for many decades. Published reports on cell therapy based on cultivated autologous oral mucosal epithelium in patients with bilateral LSCD indicate that it is highly efficient. The application of the new method contributes to corneal re-epithelialization, improved visual acuity, reduced basic nonspecific complaints and better quality of life in patients, most of whom are disabled due to corneal blindness. However, in the literature there are various and often contradictory data on the methods of isolation and cultivation of oral epithelial cells, as well as on the methods of cell graft construction. This may be the reason for obtaining heterogeneous cell populations, and, consequently, incomparable results. The question also remains open as to what determines the best corneal re-epithelialization outcome – cell transplantation, depending on the type of tissue-engineered construct, or the quality of the resulting cells in terms of the ratio of markers. Finally, due to the heterogeneity of the oral mucosa, the properties of an autologous epithelial cell transplant may differ.

Thus, a critical analysis of scientific publications on the problem of therapy with cultured oral epithelial cells in limbal stem cell deficiency allowed us to conclude that today there are some general rules and guidelines underlying this experimental approach. But, at the same time, the cardinal difference in points of view on several key issues requires further research in this direction.

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Table

Methodological approaches used to obtain oral mucosal epithelial cell culture and graft

Methodology	Types	Brief description
Source of nonkeratinized epithelium in the oral cavity [22, 23]	Lips and cheek mucosa, Inferior surface of the tongue, Soft palate, Floor of mouth	The most accessible for biopsy are lips and cheek mucosal surfaces
Method for obtaining primary epithelial culture [26] Cell substrate [40, 41, 42]	Enzymes (dispase, collagenase, trypsin)	Enzymes promote rapid production of cells with reduced viability and are usually used in combination with a feeder layer
	Explants cultivation	Slower cell yield, the niche of local stem cells and the surrounding matrix are preserved
Feeder layer [15, 32] Culture medium [28, 29, 30] Serum [27, 28]	Amniotic membrane	Used in many protocols as a substrate for epithelial cell culturing; it is a transparent membrane, composed mainly of type 4 collagen
	Fibrin gel	Transparent hydrogel obtained from commercial fibrin gel (Tisseel®, Baxter; Evicel®, Jonson)
	No substrate	Cells are cultured on the surface of the culture dish
	Thermo-responsive polymers	When the temperature drops to +20…24 °C, it becomes hydrophobic and separates cells from the culture surface
Source of nonkeratinized epithelium in the oral cavity [22, 23] Method for obtaining primary epithelial culture [26]	3T3 mouse fibroblasts	A confluent fibroblast monolayer inactivated by cytostatic agents or irradiation; enriches the culture medium with growth factors
	No feeder layer	Cultivation without this layer requires the addition of epithelial cell mitogenic stimulants
Cell substrate [40, 41, 42] Feeder layer [15, 32]	“High calcium” (≥1.0 mM)	Activates epithelial cell maturation, promotes active migration and attachment of fibroblasts
	“Low calcium” (≤0.1 mM)	Retains the immature state of the epithelial cell population; prevents migration and attachment of fibroblasts; the basis for selective culture media
Culture medium [28, 29, 30] Serum [27, 28]	Xenogenic	There is a risk of transmission of known and unknown pathogens; batch-to-batch variability
	Autogenous	The disadvantages of xenogeneic serum are eliminated; a cryobanking stock can be created
Specific epithelial growth factors [31]	GMP: insulin, hydrocortisone, human epidermal growth factor (hEGF)	These three growth factors are produced as GMP certified products
	Non GMP: triiodothyronine and cholera toxin	Not released with GMP certification, additional regulatory approval required

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