DIFFERENTIATION OF iPSCs INTO CORNEAL EPITHELIAL PRECURSORS IN THREE-DIMENSIONAL *IN VITRO* CULTURE

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Diseases associated with limbal stem cell deficiency, such as chronic epithelial erosion and corneal scarring, require new therapeutic approaches rooted in regenerative medicine. This study aimed to develop a protocol for obtaining progenitor cells of the epithelium of the limb and cornea from induced pluripotent stem cells (iPSCs). We differentiated iPSCs toward eye organoids to obtain three-dimensional heterogeneous structures within three weeks. The resulting organoids contain corneal epithelial progenitor cells expressing keratin 3 and collagen 7, which confirms the possibility of generating functional epithelium in vitro. The protocol enables the generation of isogenic, patient-specific cell lines for treating limbal insufficiency and dystrophic epidermolysis bullosa, including applications following preliminary genome editing of iPSCs.

Keywords: iPSCs, spheroid, differentiation, eye organoid, epithelium, limbus, cornea, collagen 7

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ДИФФЕРЕНЦИРОВКА иПСК В ПРЕДШЕСТВЕННИКИ ЭПИТЕЛИЯ РОГОВИЦЫ В ТРЕХМЕРНЫХ УСЛОВИЯХ *IN VITRO*

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Заболевания, связанные с дефицитом лимбальных стволовых клеток (хронические эрозии, рубцевание роговицы), требуют новых подходов регенеративной медицины. Цель исследования — разработать протокол получения клеток-предшественников эпителия лимба и роговицы из индуцированных плюрипотентных стволовых клеток (иПСК). Использовали метод дифференцировки иПСК в направлении органоида глаза для получения трехмерных гетерогенных структур за три недели. Полученные органоиды содержат клетки-предшественники эпителия роговицы, экспрессирующие кератин 3 и коллаген 7, что подтверждает возможность генерации функционального эпителия *in vitro*. Протокол позволяет создавать изогенные пациент-специфичные линии для терапии лимбальной недостаточности и дистрофического буллезного эпидермолиза, в том числе после предварительного редактирования генома иПСК.

Ключевые слова: иПСК, сфероид, дифференцировка, органоид глаза, эпителий, лимб, роговица, коллаген 7

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Blindness and eyesight disorders are a global health problem affecting at least 2.2 billion people worldwide (according to WHO), of whom 6.17 million have severe visual impairments caused by corneal pathologies [1]. Corneal transplantation (keratoplasty) restores vision in such cases by replacing the damaged area with a donor transplant. This is one of the most common and successful transplantation surgeries in the world: over 180,000 such operations are performed annually [2]. However, the demand for donor corneas far exceeds their availability, particularly in countries with limited resources. Therefore, the scientific community looks for alternative corneal allotransplantation solutions, from the use of decellularized corneal xenografts to the introduction of tissue engineering and regenerative medicine methods using differentiated stem cells [3, 4].

The eye is a complex organ consisting of highly specialized tissues originating from different rudiments [5]. The retina develops from the neuroectoderm through the optic vesicle, the

corneal epithelium originates from the superficial ectoderm, and the iris and corneal stroma originate from the neural crest. The development of methods that enable the differentiation of stem cells into specialized eye cells provides a promising resource for new studies of morphogenesis and eye development.

Induced pluripotent human stem cells (iPSCs) are a unique model that allows using the advantages of regenerative medicine. iPSCs are similar in properties to embryonic stem cells; they can differentiate into a wide variety of cell types. There are various methods for obtaining iPSCs, and there are many sources of somatic cells for their reprogramming to ultimately produce an autologous material, from keratinocytes and dermal fibroblasts obtained during skin biopsy to peripheral blood mononuclear cells or donor urine cells.

iPSCs differentiation protocols can be conditionally divided into two groups: those stemming from standard protocols for suspended cell culture management and those based on three-dimensional models [6, 7].

According to a report [8], the number of clinical studies on retinal pigment epithelium (RPE) transplants derived from iPSC differentiation has increased significantly in recent years. The mammalian retina is devoid of significant regenerative potential in adulthood, it does not restore its function when degenerative processes begin. Congenital and acquired degenerative diseases of the retina are accompanied by the loss of photoreceptors in it, which leads to severe and irreversible vision loss. Against the background of significant progress in understanding the pathogenesis of degenerative retinal diseases, the task becoming especially important is that of the development of methods of cell generation (RPE) from progenitor cells. Promising sources are allogeneic cells and autologous cells obtained from patient-specific progenitor cells of RPE, which can potentially compensate for functional defects [6].

Unlike the retina, the anterior corneal epithelium of the adult body is capable of reparative regeneration, although the rate and type of replacement tissue depend on the severity of traumatic injuries. With deep defects, not only the epithelium is affected, but also the Bowman's membrane, which consists of collagen fibers, as well as the underlying part of the corneal stroma. During the repair of lesions, the epithelium covering the wound multiplies, and the resulting epithelial layer is often thicker than normal. The defects of the underlying layers are replaced by coarse fibrous (scar) tissue [9]. In addition to injury, various diseases and genetic disorders can lead to damage and degenerative changes in the cornea. Among the diseases affecting the functioning of the limb and cornea, those deserving a special mention are the limbal insufficiency syndrome (LIS), bullous keratopathy (endothelial-epithelial dystrophy of the cornea), and symblepharon, in which the fusion of the bulbar conjunctiva (from the eye) with the palpebral conjunctiva (from the inner surface of the eyelid) leads to scarring of the cornea. For example, ophthalmological complications are common in patients with epidermolysis bullosa (EB) in general, but their frequency varies significantly depending on the subtype of the disease. The most severe manifestations are observed in recessive dystrophic epidermolysis bullosa (RDEB) and borderline subtypes. As a result of impaired synthesis of type VII collagen, patients with RDEB experience, inter alia, recurrent corneal erosions, blisters, and scarring, which may lead to complications such as exposure keratitis and symblepharon [10].

This study aimed to develop a protocol that combines the advantages of various approaches for generating eye organoids and corneal epithelium, particularly through the three-dimensional culture of differentiable cells.

METHODS

Culturing

iPSCs (KYOU-DXR0109B) were cultured at 37 °C (5% CO_2) in mTESR medium (85850, STEMCELL Technologies Inc., Canada) on plastic Petri dishes coated with matrigel (356234, Corning, USA). For individual iPSC colonies, we seeded 3,800 cells per cm^2. After 5 days, the iPSC colonies were collected following treatment with 0.4 mg/ml dispase (17105041, Gibco, Thermo Fisher Scientific, USA), which detaches the colonies from the culture surface without dissociating them into single cells.

For the subsequent cultivation of non-adhesive cultures, we used the ultra low attachment Petri dishes (3261, Corning, USA).

The transition between different types of culture media was always done in stages over 4–5 days, as follows:

– 1st day: –1 ml/+1 ml;

– 2nd day: –1.5 ml/+1.5 ml;

– 3rd day: –2 ml/+2 ml, etc.

where "–" indicates the removed volume of the current culture medium, and "+" indicates the added volume of the differentiation medium of the next stage.

The medium for embryoid body (EB) formation consisted of DMEM/F12 (C470p, PanEco, Russia) supplemented with 20% fetal bovine serum (F800820, GlobeKang, China), 100 μM β-mercaptoethanol (21985023, Thermo Fisher Scientific, USA), 100 μM MEM non-essential amino acids (F115/100, PanEco, Russia), 1 mM sodium pyruvate (11360070, Thermo Fisher Scientific, USA), 50 U/mL penicillin-streptomycin (15140122, Thermo Fisher Scientific, USA), and 2 mM GlutaMAX (35050061, Thermo Fisher Scientific, USA) [11].

XF-KO-SR (xeno-free KnockOut serum medium) consisted of DMEM (C455p, PanEco, Russia) supplemented with 15% KnockOut SR XenoFree CTS (12618012, Thermo Fisher Scientific, USA), 100 μM β-mercaptoethanol (21985023, Thermo Fisher Scientific, USA), 100 μM MEM non-essential amino acids (F115/100, PanEco, Russia), 2 mM GlutaMAX (35050061, Thermo Fisher Scientific, USA), 50 U/mL penicillin-streptomycin (15140122, Thermo Fisher Scientific, USA), and 10 μM ROCK inhibitor (ab120129, Abcam, USA) [12].

At subsequent stages of the differentiation protocol, 50 ng/mL Noggin4 (PSG100-10, SciStore, Russia), 50 ng/mL bFGF (PSG060-10, SciStore, Russia), and then 25 ng/mL BMP4 (PCH9534, Thermo Fisher Scientific, USA) were added to the XF-KO-SR medium.

Human corneal epithelial cells (hCEpCs) were cultured in DMEM/F12 medium (C470p, PanEco, Russia) supplemented with 10% KnockOut SR XenoFree CTS (12618012, Thermo Fisher Scientific, USA), 2 mM GlutaMAX (35050061, Thermo Fisher Scientific, USA), 10 μM ROCK inhibitor (ab120129, Abcam, USA), 0.4 μg/mL hydrocortisone (H0888, Sigma-Aldrich, USA), 5 μg/mL insulin (F062, PanEco, Russia), 1.4 ng/mL 3,3′,5-triiodo-L-thyronine (100-0548, STEMCELL Technologies Inc., Canada), 24 μg/mL adenine sulfate (A0013500100, SUVCHEM, India), 10 μM forskolin (F6886, Sigma-Aldrich, USA), 10 ng/mL EGF (PSG130, SciStore, Russia), 10 ng/mL KGF (PSG230-10, SciStore, Russia), and 50 U/mL penicillin-streptomycin (15140122, Thermo Fisher Scientific, USA) [13].

The composition of these media was developed based on the referenced literature sources with partial modifications due to the unavailability of some components. The replacement substances, according to the literature, had similar mechanisms of action.

Photographs of whole organoids were taken with a Leica M205 stereomicroscope.

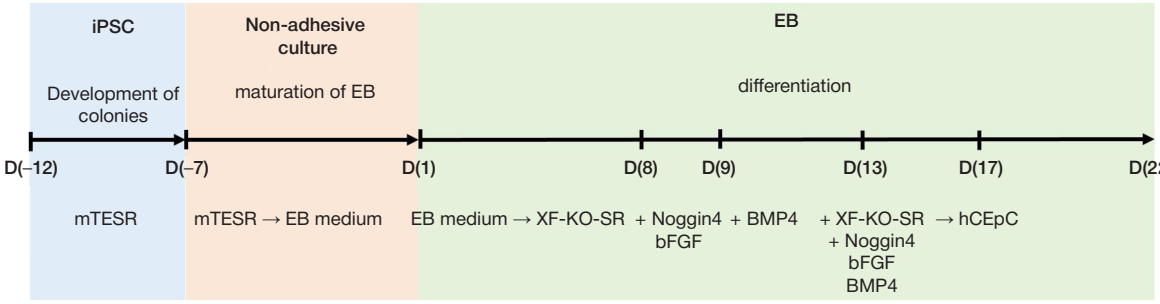

Fig. 1. Protocol for generating EBs and their subsequent differentiation into corneal epithelial cells. D(–12) — the beginning of the experiment. D(1) — the first day of the differentiation protocol. D(22) — the last day of differentiation; collection of organoids for subsequent analysis

Histological examination

The organoids were fixed in 10% neutral buffered formalin (00020709, Histoline, Russia) for 30 minutes, washed with DPBS (P060p-1, PanEco, Russia) to remove the fixative, and then incubated for 1 hour in DPBS containing sucrose solutions (170764, Lenreactive, Russia) of increasing concentrations (10%, 20%, and 30%) to prevent deformation of the organoids. The organoids were collected, drained of excess solution, embedded in Tissue-Tek O.C.T. Compound (4583, Electron Microscopy Sciences, USA), and snap-frozen in liquid nitrogen.

We prepared 10 µm-thick sections using a Thermo Scientific Microm HM 525 cryostat, dried them, and further fixed them in 10% neutral buffered formalin (00020709, Histoline, Russia) for 15 minutes at room temperature. The sections were then washed three times in DPBS (5 minutes each), followed by incubation in DPBS containing 1% hydrogen peroxide (153142, Lenreactive, Russia) for 30 minutes, and subsequently in DPBS containing 2.5% BSA (A7906, Sigma-Aldrich, USA).

Immunohistochemistry

For subsequent immunohistochemical staining, the sections were incubated overnight at +4 °C with primary antibodies against keratin 3 (PAA490Hu01, Cloud-Clone, USA) and collagen VII (pA5-18390, Invitrogen, Thermo Fisher Scientific, USA), diluted 1:100 and 1:300, respectively, in DPBS blocking solution containing 2% BSA (A7906, Sigma-Aldrich, USA), 1% Triton X-100 (1001776062, Sigma-Aldrich, USA), and 1% Tween 20 (P7949, Sigma-Aldrich, USA). The next day, the sections were washed in DPBS and incubated overnight at +4 °C with secondary antibodies: Alexa 594–conjugated anti–rabbit IgG (A11012, Invitrogen, Thermo Fisher Scientific, USA) and Alexa 488–conjugated anti–goat IgG (A32814, Invitrogen, Thermo Fisher Scientific, USA), both diluted 1 : 1000 in blocking solution. The next day, the sections were washed with DPBS and mounted in VectaSHIELD Antifade medium containing DAPI (H-1200-10, Vector Laboratories, USA).

Using a Nikon Eclipse Ni upright microscope equipped with a DS-Ri2 camera (Nikon Corporation, Japan), we analyzed the sections and acquired a series of images using the microscope's set of optical filters, which enabled detection of fluorescence emission. The images were processed using the ImageJ software package (NIH, USA) and converted into microphotographs.

RESULTS

There were two stages to the formation of the initial EB (Fig. 1). At the first stage, which began on D(–12), iPSC colonies were cultured under adhesive conditions; on D(–7), they were transitioned to a non-adhesive form. After 7 days of cultivation under non-adhesive conditions, the EBs matured, and the colonies acquired a more rounded shape with smooth edges. Subsequently, we switched to the differentiation protocol for corneal epithelial cells (D(1)).

From day 8 of the differentiation protocol, Noggin4 and bFGF factors were injected into the XF-KO-SR medium, blocking TGFß and Wnt signaling and activating FGF signaling to induce ectodermal differentiation. Additionally, we introduced BMP4, as it was shown that this factor promotes the differentiation of iPSCs into corneal epithelial cells [14].

On day 13, we began to gradually replace the XF-KO-SR medium with a medium for culturing human corneal epithelial cells (hCEpCs).

By day 22, both monospheres (Fig. 2A) and complex formations were observed among the organoids, likely resulting from the aggregation of several individual elements (Fig. 2B, C). Most of the organoids became optically translucent in transmitted light, but some objects had pigmented areas (Fig. 2).

To analyze the expression patterns of differentiation markers, we prepared cryosections of the organoids and examined them

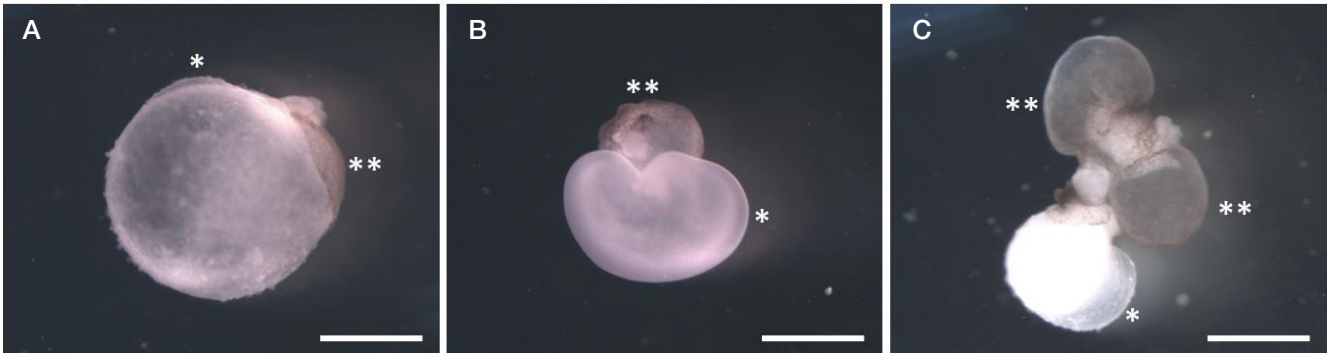

Fig. 2. Darkfield photo of organoids on the 22nd day of differentiation. There are both translucent spheres and pigmented areas. * — translucent zones at the periphery of the organoid, presumably corresponding to corneal regions; ** — pigmented areas of the organoid, presumably corresponding to retinal pigment epithelium (RPE) regions. Each scale division is 1 mm

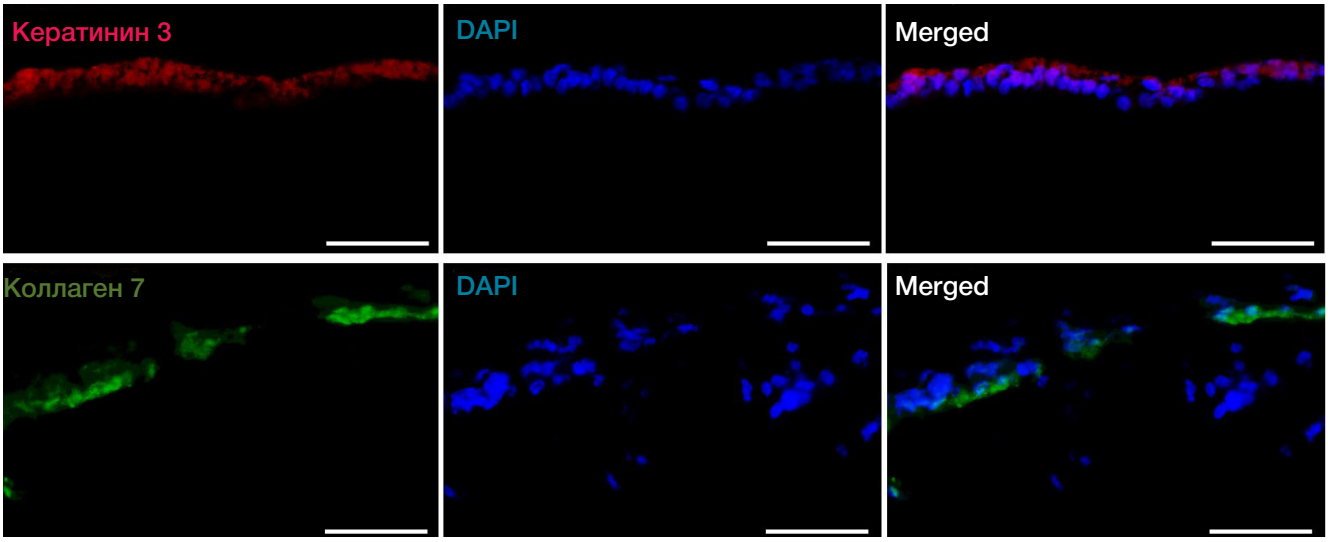

Fig. 3. Immunohistochemical staining of cryopresections of organoids on the 22nd day of differentiation. Reaction with antibodies to keratin 3 (red) and reaction with antibodies to type VII collagen (green). The cores were additionally colored with DAPI. The scale segment is 50 μm

by immunohistochemistry. In view of the corneal epithelium staining positive for the keratin markers K3, K7, and K19, we analyzed the expression levels of the corresponding corneal and conjunctival biomarkers [15].

The corneal epithelium is supported by a specialized basement membrane complex that anchors it to Bowman's layer. This attachment is mediated by a network of anchoring fibrils composed of type VII collagen and associated anchoring plaques that interact with the dense collagen matrix of Bowman's layer [16].

Figure 3 shows photographs of organoid sections that are a sphere with translucent contents bordered by cellular layers, with positive staining for markers of epithelial differentiation (K3) and basement membrane (type VII collagen). Cells positively stained for keratin 3 were observed on the periphery in the outer cell layer of the organoid. The expression of type VII collagen was seen in the underlying cell layers.

DISCUSSION

Corneal transplantation is one of the most successful organ transplantations in human beings. The orthotopic corneal allografts are used frequently (and successfully) in both humans and experimental animals because of the so-called immune privilege of the eye: any inflammatory processes is suppressed in order to preserve visual function. In case of corneal transplantation, this privilege is believed to be supported by three factors: 1) anatomical, cellular, and molecular barriers in the cornea; 2) tolerance associated with the presence of regulatory T cells and immune abnormalities in the anterior chamber of the eye; 3) immunosuppressive intraocular microenvironment [17].

We describe a protocol for differentiating functional corneal epithelium from human iPSCs in vitro. Under this protocol, some cells spontaneously form round colonies, spheroids, or embryoid bodies, which, during successive stages of the differentiation process that involves gradual change the culture medium and addition of growth factors and inhibitors of the WNT cascade, produce structures with the properties of the organoid of the eye. Our protocol was based on the methodology described in the Isla-Magrané article [18]. In this work, we also used a three-dimensional model of differentiation with forced aggregation of EB during cultivation and prevention of cell adhesion to the plastic surface. The advantages of this protocol include the possibility of co-cultivation of cells of various types, and the production of single three-dimensional models of controllable size [19]. The cells aggregate and form spheroids due to intercellular adhesion and the lack of adhesion to the culture plastic [20]. The differentiation protocol published in this paper can potentially be useful to researchers interested in eye development and/or regeneration of the eye surface, as well as specialists in the field of translational regenerative medicine.

The development of methods for the directed differentiation of iPSCs in RPE enabled some approaches allowing generation of the epithelium of the limb and cornea. Small molecules, inhibitors of the WNT/β-catenin cascade, or recombinant proteins and factors are used to produce RPE cells from iPSCs [22, 23].

When iPSC colonies differentiate under adhesive conditions, concentric SEAM (self-earned autonomous multi-zone) zones gradually form, in which cells begin to acquire different morphologies and a specific set of markers. This protocol has a distinct advantage. During cultivation, homogeneous cell types can be mechanically isolated and then sorted by flow cytometry to yield a population of progenitor cells analogous to the epithelial cells of the limb. Cells of this type can be multiplied and differentiated to form an epithelial layer expressing keratin markers 3 and 12, PAX6. The researchers confirmed the functionality of the obtained cells in an animal model of corneal epithelial dysfunction through surgical xenotransplantation. In 2024, the results of the first successful clinical trials on allogeneic transplantation of corneal epithelial cells differentiated from iPSCs using the SEAM protocol in four patients were published. Two patients suffered from limbal insufficiency syndrome, one from ocular mucosal pemphigoid, and one from toxic epidermal necrosis [24]. In all cases, after 52 weeks of follow-up, the researchers notes improved eye condition, better visual acuity, and a decrease in corneal opacity. Corneal epithelial defects, subjective symptoms, quality of life indicators, and corneal neovascularization have mostly improved or remained unchanged. Through the follow-up period, there were no serious adverse events observed, including tumor formation or clinical rejection.

Obtaining an eye organoid from iPSCs using the developed protocol allowed the detection of type VII collagen expression in the area adjacent to the basal membrane. The attachment fibrils formed by type VII collagen are an important structural element that supports the integrity of the tissue. Patients

affected by hereditary disorders involving defects in type VII collagen, such as recessive dystrophic epidermolysis bullosa, are classified as disabled from childhood due to chronic inflammation of the skin and epithelial linings of internal organs. Current clinical treatments for ophthalmological manifestations thereof, such as contact lenses, lubricants, and antibiotics, may reduce symptoms but fail to prevent corneal scarring [10].

In such cases, allogeneic transplants of the epithelium of the limb and cornea, differentiated from iPSCs of healthy donors, may be applicable for cell therapy. The creation of stem cell banks with homozygous human leukocyte antigen (HLA) cell lines can provide an effective and cost-effective resource for this type of therapy.

Autologous iPSC-based treatments can be prohibitively expensive and time-consuming. However, patient-specific iPSCs (RDEB-iPSCs) can be differentiated into eye organoids to create disease models for testing new drugs and evaluating gene therapy approaches.

CONCLUSIONS

This study describes a protocol for obtaining an organoid of an eye with a formed corneal epithelium by differentiating iPSCs. The development of various methods for the generation of corneal and limb epithelial tissues contributes to the development of regenerative medicine in ophthalmology. Although a single type of corneal transplant is unlikely to provide a universal solution, combining different approaches may help address the shortage of donor corneas, support the development of bioengineered matrices — implantable with or without cells — to stimulate tissue repair and regeneration, and enable new treatments for diseases that cannot be cured with currently available methods.

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