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Epithelial cells removed in advanced surface ablation (ASA) surgery can be used as a source of corneal samples to perform *in vitro* studies

Laura García-Posadas [a,*], Miguel J. Maldonado [b], Yolanda Diebold [a,c]

[a] Ocular Surface Group, Instituto Universitario de Oftalmobiología Aplicada (IOBA), Universidad de Valladolid, Valladolid, Spain
[b] Refractive Surgery and Vision Quality Group, Instituto Universitario de Oftalmobiología Aplicada (IOBA), Universidad de Valladolid, Valladolid, Spain
[c] Biomedical Research Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain

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ABSTRACT

Human corneal epithelial cells are needed to study corneal pathophysiology *in vitro*. Due to the limitations of cell lines, the use of primary cells is highly desirable, but the scarcity of human tissues, along with ethical issues, make it difficult to accomplish all required experiments. In advanced surface ablation (ASA), the central corneal epithelium is removed and discarded. We hypothesized that ASA samples could be used to perform *in vitro* assays. In this study, 29 samples from patients undergoing ASA were recovered in supplemented DMEM/F12 culture medium, RIPA buffer, or RLT lysis buffer. The first aim was to determine whether cells could be maintained in culture. Although with the explant technique, tissue pieces did not attach to the culture surface, after disaggregation, cells showed high viability (90.0 ± 6.0%), attached to plates, and remained viable for up to 14 days. The second aim was to elucidate if ASA samples could be used to study protein or gene expression. Cytokeratin-3, ZO-1, Ki67, and E-cadherin protein expression were confirmed by immunofluorescence. Total protein (485.8 ± 115.8 μg) was isolated from cells in RIPA buffer, and GAPDH was detected by Western blotting, indicating that samples are adequate for protein studies. RNA (9.0 ± 3.6 μg) was isolated from samples in RLT lysis buffer, and GAPDH gene expression was studied by PCR, confirming that samples were also suitable for gene expression studies. These results suggest that samples obtained from corneal surface ablation procedures may constitute a valuable source of human cells to accomplish *in vitro* studies.

The corneal epithelium is the outermost layer of the cornea. In humans, it is a stratified structure that has 2–3 layers of superficial cells, 2–3 layers of wing cells, and a monolayer of cuboidal, mitotically active basal cells (Sridhar, 2018). The corneal epithelium forms a tight barrier that is in contact with the tear film and the external environment, and it can be altered in numerous diseases affecting the ocular surface. Therefore, it is crucial to study it in the laboratory when trying to understand the pathophysiology of corneal diseases or when testing new drugs for them.

To perform those initial *in vitro* studies, corneal cells are needed. There are different sources of cells for *in vitro* experiments. Immortalized cell lines are an important and widely available tool that has allowed researchers to advance in the knowledge of many diseases and to perform initial drug screenings. There are several cell lines of human corneal epithelium, with HCE-T being the most widely used (Araki-Sasaki et al., 1995). However, immortalized cells present some differences from primary cells and a huge problem with misidentification and cross-contamination that have also affected human corneal epithelial cell lines (Di Girolamo et al., 2016). For those reasons, results obtained exclusively using cell lines should be taken with caution (Clark et al., 2013). Consequently, primary cells are more valuable but also more difficult to obtain. Due to the scarcity of human tissues for research, many experiments are done in primary cells from mice, rats, or other laboratory animals. But the use of animal cells has ethical concerns and the inconvenience of species extrapolation (Combes, 2004).

Removing the central cornea epithelium is the first step during corneal surface ablation procedures, such as advanced surface ablation (ASA), alcohol-assisted photorefractive keratectomy (PRK), or epi-off Laser-Assisted Epithelial Keratomileusis (LASEK). In these procedures, the corneal epithelium is removed before the laser remodels the corneal stroma to compensate for refractive errors. There are several methods to debride the epithelium in refractive surgeries, including mechanical debridement, transepithelial laser ablation, abrasion with a brush, and alcohol debridement (Sakimoto et al., 2006). Alcohol debridement with

* Corresponding author. IOBA, Paseo de Belén 17, 47011, Valladolid, Spain.
E-mail address: laura.garcia.posadas@uva.es (L. García-Posadas).

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diluted ethanol is one of the most widely used techniques. Ethanol disrupts hemidesmosome attachments between the basal layer of the corneal epithelium and the basement membrane, causing a smooth cleavage of the flap that allows delaminating the basement membrane before laser application (Browning et al., 2003).

Therefore, during ASA, a cell sheet from the central cornea with all epithelial cell layers is removed and considered surgical waste. In this study, we aimed to determine whether those cells are viable and useful to be cultivated *in vitro* and analyze protein and gene expression for biomedical research.

The following materials and methodologies were used in this study. This research followed the tenets of the declaration of Helsinki and was approved by the Institutional Review Board of the University of Valladolid. All patients enrolled in this study provided informed consent. Twenty-nine samples from 15 donors (eight females, seven males) undergoing ASA surgery at the Institute of Applied Ophthalmobiology (IOBA) from the University of Valladolid (Valladolid, Spain) were included in the study. Epithelial cells were obtained from the central cornea (9 mm diameter) after applying 20% ethanol for 30 s. Excess ethanol was absorbed with a sponge, and the corneal surface was rinsed with a topical cold balanced salt solution before removing the epithelium with a blunt spatula. Epithelial samples from 10 patients were immediately recovered in 1 mL of supplemented DMEM/F12 culture medium and used for cell culture, cytocentrifugation with a Shandon Cytospin (Southern Products, Cheshire, UK), or inclusion in Optimal Cutting Temperature (OCT, from Sakura, Tokyo, Japan) compound for further immunohistochemical analysis. The rest of the samples were collected in 700 μL of RLT lysis buffer for RNA isolation (n = 12) or in RIPA buffer (n = 7) for protein extraction and frozen until used.

Three samples collected in cell culture medium were included in the OCT compound, sectioned at 4 μm, and used for histological or immunohistochemical detection. Seven samples collected in cell medium were used for cell culture, using either explant or disaggregation techniques. For the explant technique, the cell sheet was placed on a plastic 4-well tissue plate (1.9 cm^2/well). For the disaggregation technique, 0.05% trypsin/EDTA (Invitrogen) was added to the tube, mixed with the pipette, and incubated for 15 min until the cell sheet was disaggregated and single cells were floating on the media. Then, cells were placed on a 4-well plate. In this last method, cells were counted using a hemocytometer, and cell viability was measured using the Trypan blue (Sigma-Aldrich, St. Louis, MO, USA) exclusion assay.

Primary cells were cultured with DMEM/F12 culture medium (Invitrogen-GIBCO, Inchinnan, UK) supplemented with 15% FBS (Invitrogen-GIBCO), 100 U/mL penicillin/0.1 mg/mL streptomycin (Invitrogen-GIBCO), 10 ng/mL EGF (Invitrogen, Eugene, OR, USA), 5 μg/mL insulin (Invitrogen), and 0.1 μg/mL cholera toxin (Gentaur, Brussels, Belgium). Cell cultures were maintained at standard conditions (37 °C and 5% CO_2) and observed daily under the inverted bright field microscope Nikon Eclipse TS 100 (Nikon, Tokio, Japan). Culture media was exchanged every other day to guarantee that cells received enough nutrients.

To determine whether the cell sheet maintained adequate structure and marker expression, immunofluorescence staining against cytokeratin (CK) 3 (MP Biomedicals 69143, 1:50 dilution), zonula occludens (ZO) 1 (Invitrogen 617300, 1:100 dilution), Ki67 (Dako M7240, 1:50 dilution), and E-cadherin (BD Bioscience 610151, 1:100 dilution) was performed. Preparations were observed under Leica DMI 6000B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Total soluble proteins were isolated from seven samples collected in RIPA buffer with a cocktail of proteases inhibitors (100 μl/mL phenylmethylsulfonyl fluoride, 6 μl/mL aprotinin, and 100 nM sodium orthovanadate, all from Sigma-Aldrich), and quantified with the bicinchoninic acid (BCA) protein quantification method (Pierce, Rockford, IL, USA). Then, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels and proteins transferred to nitrocellulose membranes. The expression of the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with an anti-GAPDH antibody (Santa Cruz Biotechnology) and visualize with the ChemiDoc® gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA) and Quantity One software.

Total RNA was isolated from 12 samples collected in RLT lysis buffer, using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantified with the Quant-iT™ RNA assay and reverse transcribed to cDNA with the SuperScript Vilo cDNA Kit (Invitrogen). Then, RT-PCR for GAPDH (primers from Sigma) was conducted using the Mastercycler Personal Thermocycler (Eppendorf, Hamburg, Germany) using 50 ng cDNA, 5 μL PCR buffer 10X, 1 μL dNTPs, 1 μL Taq polymerase, and 1 μL GAPDH primers (Sense: 5′-GAACGTGAAGGTCGGAGTCAAC-3'; antisense: 5′-CGTGAAGATGGTGATGGGATTTC-3; Size product: 250 base pairs (bp); from Sigma). Thermocycling conditions were 95 °C for 2 min, 39 cycles of 95 °C for 20 s, 60 °C for 30 s, 72 °C for 40 s, and a final cycle of 72 °C for 10 min. Products were resolved on 2% agarose gels, and images were captured with the ChemiDoc system.

Data from cell numbers, viability, and protein and RNA concentrations were presented as mean ± standard deviation.

The mean age of donors included in this study was 34.7 ± 7.1 years old. This is representative of the patients undergoing refractive surgery (Torricelli et al., 2014). All the corneal samples obtained during ASA were circled, homogeneous, and transparent (Fig. 1A). Epithelial cells had a polygonal shape and were tightly adhered to each other. Half samples were included in OCT, sectioned, and stained with hematoxylin/eosin to observe the general structure (Fig. 1B), formed by 5–6 cell layers. Several "holes" were observed in central layers, probably due to the ethanol or to the fixation and inclusion procedure.

The first purpose of this study was to determine whether it was possible to culture corneal epithelial cells discarded from ASA surgeries. To do so, explants from the corneal samples were placed in the culture immediately after surgery (n = 3). However, tissues did not attach (Fig. 1C), and no proliferation was observed. On the contrary, after disaggregation, 408,571.4 ± 169,551.2 cells with 90.0 ± 6.0% initial viability (n = 7) were recovered, and those epithelial cells attached and remained viable *in vitro* for up to 14 days (Fig. 1D). After 14 days in culture, these cells remained attached to plastic substrate, had adequate polygonal morphology, grew adhered to surrounding cells, showed no vacuoles in the cytoplasm and no nuclear aberrations. Although just a small percentage of cells proliferated *in vitro*, cultures from 2 donors reached confluence. Since the purpose of this study was to evaluate the suitability of ASA primary cells, those confluent cells were not passaged.

The second objective of this research was to evaluate the feasibility of accomplishing protein and gene analysis with the ASA corneal samples. Thus, immunofluorescent detection in corneal epithelium sections, protein detection after electrophoresis and Western blotting, and gene expression analysis with PCR were performed.

Immunofluorescent detection confirmed the expression of the corneal epithelial marker CK3 and the tight junction protein ZO-1 in all layers of the corneal epithelium (Fig. 2A). However, the proliferation marker Ki67 was exclusively detected in a small number of cells located in the basal layer of the corneal epithelium (Fig. 2A, arrows). This expected limited amount of Ki67 positive cells probably accounts for the low proliferation rates observed in the cell cultures. Finally, E-cadherin, the main protein that forms the adherens junction, was also observed in the cell membrane of epithelial cells that were fixed on slides by cytocentrifugation.

Total soluble proteins were extracted from samples collected in RIPA buffer. We obtained 485.8 ± 115.8 μg of protein (n = 7). This is enough quantity to perform tens of proteomic assays. For instance, for the extensively used Western blotting technique, 10–30 μg of protein are usually loaded, although less quantity can even provide better results (Murphy and Lamb, 2013). Other high-throughput techniques required much less concentration. Thus, these ASA samples provide adequate amounts of material for proteomic analysis. We tested protein analysis

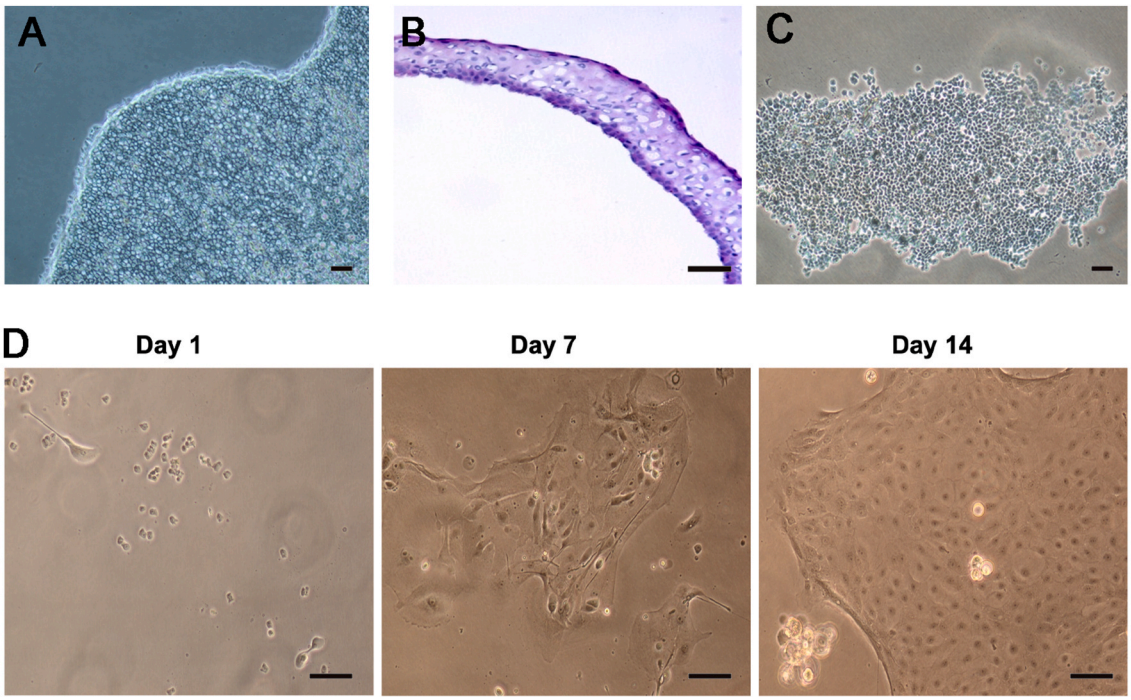

Fig. 1. Characteristics and culture of corneal epithelial samples. A) Light microscope photograph of the corneal epithelium sample obtained after PRK surgery, showing the edges of the epithelial sheet. Bar = 100 μm. B) Cell sheet included in OCT and stained with hematoxylin/eosin. Bar = 50 μm. C) Explant in culture after seven days. The explant did not attach and was floating on the medium. Bar = 100 μm. D) Disaggregated corneal epithelial cells in culture. On day 1 (left), just a few cells were attached to the plastic surface of the culture plate. At seven days (middle), more cells can be observed. Cells remained viable after 14 days in culture (right). Bar = 50 μm.

by electrophoresis and Western blotting loading 10 μg of the extracted protein in each line and observed a single band for the housekeeping protein GAPDH at 37 KDa, as described (Fig. 2B).

Similar to what was done with proteins, we isolated total RNA from samples collected in RLT lysis buffer. We obtained 9.0 ± 3.6 μg of RNA (n = 12), sufficient quantity to perform different gene expression assays that usually just need nanograms of sample in each technique. For instance, isolated RNA from ASA samples was reverse-transcribed to cDNA, and the GAPDH gene was amplified by PCR using 10 ng of cDNA sample, showing a single band in agarose gels between 200 and 300 bp, according to the expected size product of 250 bp (Fig. 2C).

In this study, we have shown that epithelial cells removed from ASA surgery are viable and useful to detect protein and gene expression. Moreover, we demonstrated that it is possible to maintain these cells in culture for up to two weeks. This increases the range of experimental procedures that can be performed in the laboratory with this human corneal material. General morphological alterations typical of senescence (enlarged and irregular size, vacuoles, or darker chromatin, among others) were not observed in our cultures. Nonetheless, evaluation of senescent cells by different techniques (such as detection of phosphorylated p53, determination of DNA synthesis rate by BrdU/EdU-incorporation assays, or others) may be needed for long term studies.

As mentioned before, there is a great need to obtain human primary cells. Primary cells are usually obtained from biopsies or from cadaveric donors. In the case of the cornea, biopsies from healthy donors are not an option for obvious ethical reasons, and scarcity of cornea cadaveric tissue is especially drastic since a great amount of the limited donations are used for corneal transplantation. In addition, when received, cadaveric tissues usually arrive in experimental laboratories 1–3 days after decease, and they are usually from aged donors. Although there is controversy regarding the effect of donor age on corneal graft survival (Mannis et al., 2013), the performance of cells in culture in other tissues has shown to be limited by age (Choudhery et al., 2014). Interestingly, in the case of the corneal epithelium, different reports using limbal tissue from corneo-scleral rings found no correlation between growth rate and donor age, but discrepancies were observed in the correlation of cell growth with the time from death to culture (Baylis et al., 2013; Kim et al., 2004). It is important to note that both Kim et al. and Baylis et al. investigations used limbal tissue containing limbal epithelial stem cells, whereas in the current study only central corneal epithelial cells were obtained. This may cause differences in the observed correlations. Nevertheless, even though it has been demonstrated that corneal cells can be cultured from old donors, it may be presumed that having younger donors may benefit cell culture outcomes. All these potential inconveniences of age and time from cell recovery to culture are overcome with the use of cells obtained from ASA surgery, apart from the obvious difference of collecting cells from alive donors. Refractive surgery patients are usually young, healthy, and cells can be retrieved and cultured immediately after the surgery. Thousands of patients have surface treatments each year, and ASA is a commonly used procedure. Regardless of the presence of postoperative pain and discomfort (Sobas et al., 2017), ASA has gained popularity (Moisseiev et al., 2013) due to the absence of flap complications that can occur in laser in situ keratomileusis (LASIK). Thus, it is widely performed in hospital and clinics and pre-clinical researchers may effortless find a center with which to collaborate in sample retrieval.

Despite the use of ethanol, the viability of the recovered cells is around 90%, and cells are healthy enough to perform protein expression analysis by immunofluorescence and western blotting and gene expression studies by PCR. Alcohol has been widely used in ophthalmology, and Dua et al. recently reviewed it (Dua et al., 2021). Several reports have shown that at low percentages of ethanol and short contact

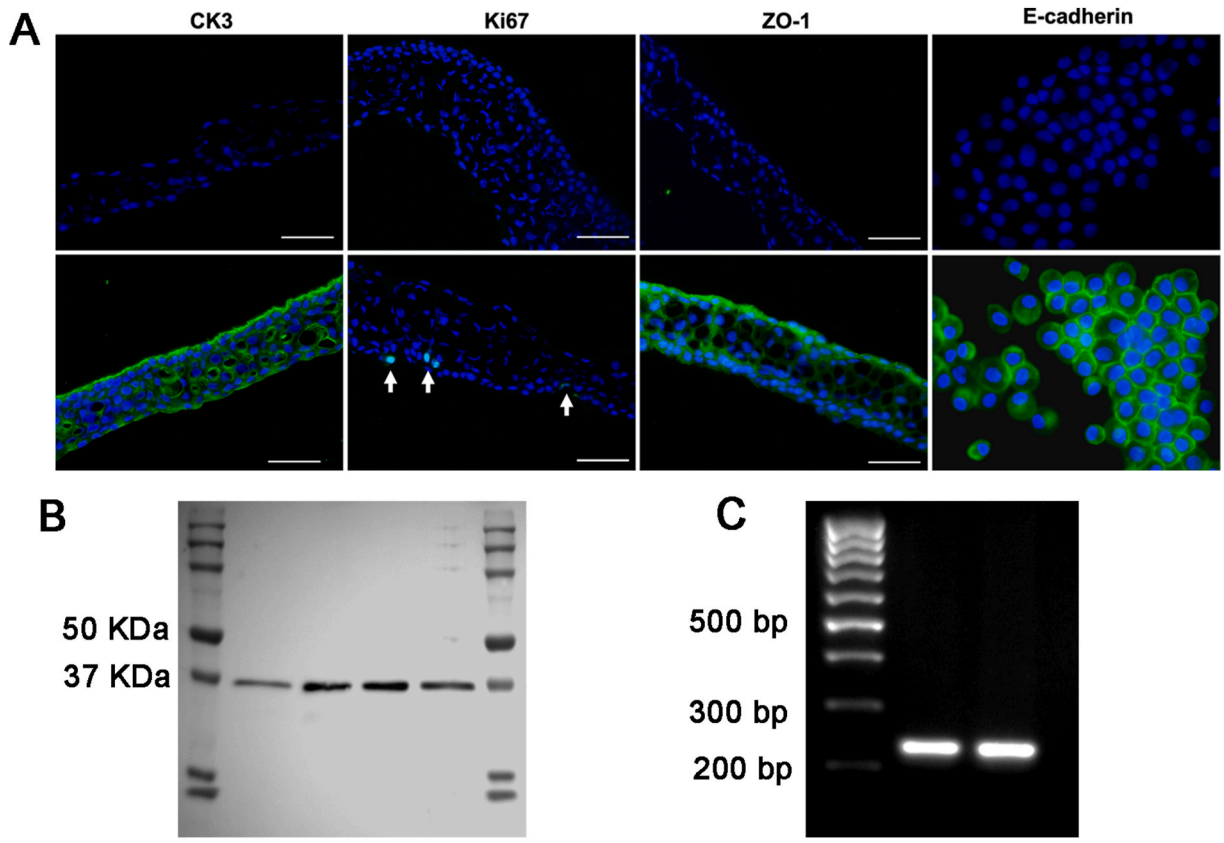

Fig. 2. Protein and gene analyses in PRK samples. A) Negative controls (top) and immunodetection (bottom) of CK3, Ki67, and ZO-1 in epithelial sheets, and E-cadherin in cytospin of corneal samples. Arrows indicate cells labeled with anti-Ki67. Bar = 50 μm. B) Immunoblot of corneal samples showing the presence of GAPDH at 37 KDa. C) Agarose gel showing a result of PCR for GAPDH gene at approximately 250 bp.

times, the corneal epithelial cells remained alive (Browning et al., 2003; Chen et al., 2002; Oh et al., 2013). In their review, Dua et al. summarizes that these results suggest "that the alcohol solution passes from the surface to the basal layer intercellularly rather than exclusively via an intracellular path.". Thus, our results are in accordance with these previous reports. Nevertheless, the alcohol may be affecting different signaling pathways in the exposed cells, and that should be considered when using these cells for specific signaling or gene expression purposes. Interestingly, Serrano et al., demonstrated that the toxic effect of 25% ethanol applied for 3 min to rabbit corneas did not affect to wing cells, and for the rest of cells the effect did not persist over time (Serrano et al., 2003).

We showed that ASA samples maintained the tight junction protein ZO-1 and the adherens junction protein E-cadherin. However, further research to analyze the interactions between cultured cells and the cell barrier function is needed to use these cells *in vitro* for specific functional experiments, such as permeation studies.

In summary, the ASA surgery samples allow to obtain enough protein for Western blot assay and enough RNA to perform gene expression studies. But most importantly, we have demonstrated that it is possible to culture these cells, widening the range of applications of these samples. Further research to stablish the specific conditions needed for each type of *in vitro* experiment is warranted. Bearing all this in mind, we can conclude that this study opens new avenues for the use of epithelial cells removed in corneal surface ablation procedures (such as ASA surgery, alcohol-assisted PRK, or epi-off LASEK) in the execution of different *in vitro* studies.

Author contributions

LGP: conceptualization, investigation, writing - original draft, writing - review & editing, funding acquisition. YD: conceptualization, writing - review & editing, funding acquisition. MJM: resources, writing - review & editing. All authors have read and approved the final version of the manuscript.

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Declaration of competing interest

The authors declare no conflicts of interest.

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