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Bacterial Cellulose Membranes Constitute Biocompatible Biomaterials for Mesenchymal and Induced Pluripotent Stem Cell Culture and Tissue Engineering

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Introduction

Tissue engineering refers to an "interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [1]. In order to develop such biological substitutes, non-immunogenic cells, signaling molecules and scaffolds are required in large scale.

In animal models of bone injury, for instance, up to 10^5-10^6 cells may be used in tissue engineering strategies [2]. Considering the translation of such techniques to human proportions, the number of cells required for tissue engineering may reach up to a hundred million cells. Currently, adult stem cells are being used for such situations, mostly in clinical trial settings, and results are encouraging. Unfortunately, though, many limitations still hinder adult stem cell therapy.

Induced Pluripotent Stem (iPS) Cell technology, which has recently crowned its inventor with a Noble Prize, holds great promise as a potential platform for generating cells for regenerative medicine purposes, able to attend the unmet need of tissue engineering, which requires large scale production of non immunogenic cells.

Likewise, Bacterial Cellulose (BC) is a unique and promising material for use as implants and scaffolds and has also attracted much attention in the tissue engineering field, due to its highly desirable characteristics of biocompatibility, mechanical strength, chemical and morphologic controllability [3-5]. Bacterial cellulose is a type of biological material, derived from the *Acetobacter xylinum*, which grows in the liquid sugar matrix, and it is a product of extracellular of bacterial cell wall without the structural components. Besides, it is characterized by easy purification manipulation, elastic modulus and high hydrophilicity. It is readily degraded into monosaccharide by acidoid, microorganism and cellulose in nature. Cellulose biodegradation resulting from enzymolysis generally occurs in nature rather than in the human body because of the absence of cellulose degrading enzymes. In order to achieve *in-vivo* degradation in human body for *in-vivo* tissue regeneration applications, some studies have been done concerning to the integration of cellulases into bacterial cellulose [6]. Taken together, these characteristics make BC popular and having a wide applicability for regeneration medicine [7].

Our aim in this work was to bring the advantages of iPS cells in terms of self-renewing, absence of ethical issues and the huge potential of differentiation in any lineage, together with the attractive physicochemical properties and biocompatibility of BC membrane for further application in tissue engineering. In order to achieve this goal, we first addressed the *in vitro* biocompatibility of BC membrane by colonization with hASC. Thereafter, we head to verify the capacity of BC membrane to harbor iPSC and to maintain their pluripotent state. Of our knowledge, this is the first time that this scaffold is studied for culturing pluripotent stem cells and regarding their promising features it constitutes a great tool for the field of tissue engineering.

Material and Methods

Mesenchymal stem cell isolation

Human adipose tissue was obtained with written informed consent from healthy patients who had undergone liposuction surgery for esthetic reasons in the "Plastic Surgery Center-Dr. Luiz Alberto Lamana" in Belo Horizonte, Minas Gerais, Brazil. The liposuction tissue was collected after obtaining informed consent from the patients according to procedures approved by Ethics Committee of Federal University of Minas Gerais. No diabetes, hepatitis, metabolic diseases, or other systemic complications were reported for these donors. The isolation and culture of human adipose tissue-derived stem cells (hASCs) was performed as described elsewhere [8]. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin (Basal medium). Medium was changed every two days. Cells were then cultured for 7 to 10 days until they reached confluence, when they were harvested by digestion with 0.05% trypsin-EDTA, centrifuged at 1400 rpm for 5 minutes, suspended in culture medium, and plated at a density of approximately 26×10^4 cells/cm^2. Cells were utilized at passage 4.

IPS cell culture

Induced Pluripotent Stem Cells from the FN052 lineage were generated and kindly provided by Dr. Stevens K. Rehen, from the LaNCE-The National Laboratory of Embryonic Stem Cells. Those cells were cultured on Matrigel-hESC qualified Matrix (BD Biosciences) and using mTeSR (Stem Cell Technologies) and subcultured every 5-7 days in a passaging ratio of 1:3, using Dispase (BD Biosciences, USA).

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Bacterial cellulose membrane production

Bacteria *A. xylinum* ATCC 23769, acquired from "Collection of Tropical Culture (CCT)" (André Tosello Foundation) was used for the cellulose production.

Porous, nanofibrous bacterial cellulose membranes were produced as previously described [9]. Shortly, *A. xylinum* inoculum was prepared by its cultivation under static conditions, at 30°C, in a 125 mL Erlenmeyer flasks, containing 25 mL of Hestrin & Schramm medium [10]. Cellulose production was carried out by the cultivation of the bacterium for 3 days, under static conditions at 30°C, in 24 well plates containing 450 µL of modified Hestrin & Schramm medium, pH 6.6, containing (per liter): 5.0 g peptone, 5.0 g yeast extract, 1.15 g citric acid, 2.27 g Na_2HPO_4 and 20 g glucose plus 50 µL of bacteria inoculum.

After the cultivation period, the formed gel at the liquid/air surface was removed, transferred to a flask containing 0.1 M NaOH solution and incubated there for 24 h at 50°C to remove bacterial impurities and eventual cell debris. The membrane was then washed 3 times in deionized water until neutral pH and then autoclaved for 20 min at 121°C.

In vitro bacterial cellulose biocompatibility analysis

In order to assess the BC's biocompatibility, MTT and alkaline phosphatase (AP) assays were initially performed with human adipose tissue-derived mesenchymal stem cells.

Cellular viability and proliferation: hASCs were seeded on bacterial cellulose or on standard culture plate and their viability and proliferation were determined by the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay, which is based on the reduction of tetrazolium salt to formazan crystals by the dehydrogenase present in living cells mitochondria [11]. After 24 h, 7, 14 and 21 days of cell seeding, the medium was removed and 210 µl of fresh culture media and 170 µl of MTT solution (5 mg/mL in PBS) were added to each well, followed by incubation for 2 h at 37°C in a 5% CO2 atmosphere. The resulting formazan salts were solubilized with 210 µl of SDS-10% HCl (sodium dodecyl sulfate – hydrochloric acid) for 18 h at 37°C in a 5% CO_2 atmosphere, and the optical density of the solution was evaluated with a microplate spectrophotometer at 595 nm. Cells cultured without the scaffold were employed as the control for the proliferation experiment, which evaluated the proliferation of the hASCs or iPS cultured with the basal medium and mTeSR, respectively.

Alkaline phosphatase activity: The AP activity was evaluated with the BCIP-NBT Kit assay as described by the manufacturer (Cat. 00-2209, Invitrogen). hASCs were seeded on bacterial cellulose and on standard culture plate and cultured with basal medium at 37°C, 5% CO_2 and humidified atmosphere. After 24 h, 7, 14 and 21 days, the supernatant of each well was removed and washed twice with PBS. Then, 200 µl of BCIP-NBT solution was added to each well and incubated. Two hours later, the insoluble purple precipitants were dissolved with SDS-10% HCl. After 18 hours, 100 µl of solution was transferred to a 96-well plate and the OD was measured at 595 nm. Cells cultured without the scaffold were subjected to the same procedure, as a control.

Colonization of BC membrane with iPS cells: After confirming *in vitro* biocompatibility of BC membrane with hASC colonization, we head to look whether BC membrane is also suitable to harbor iPS cells and to maintain their pluripotent state. For this purpose, iPS cells were seeded on BC membrane and cultured in mTeSR media. After 120 h, viability and proliferation were determined by MTT and AP assays, as previously described.

iPS Cells adhesion and proliferation in BC membrane: To evaluate cell attachment and proliferation rates in BC membrane in comparison to standard culture conditions, iPSC were seeded onto the substrates (1×10^4 cells/cm^2) and cultured in the mTeSR medium. The cells were trypsinized the next day (day 1) or at day 3 and counted with the hemacytometer.

SEM sample preparation for cell adhesion on scaffolds and morphological analysis: iPS cell adhesion, distribution and morphology of iPS cells seeded on BC membranes were observed by SEM. The samples were washed in PBS and fixed with 2.5% glutaraldehyde in PBS for at least 2 hours. Afterward, the samples were washed 3 times with PBS for 10 minutes and incubated with 1% osmium tetroxide (Electron Microscopy Sciences) in PBS at 4°C in the dark for 1 hour. The constructs were again washed with PBS and incubated in 1% tannic acid in water for 20 minutes. The constructs were subsequently washed with PSB and incubated in 1% osmium tetroxide in water at 4°C in the dark for 1 hour. Then, the constructs were washed with PBS and dehydrated through stepwise incubation in a series of graded ethyl alcohols: 30%, 50%, 70%, 90% and 100% ethanol; each construct was incubated twice in each alcohol solution for 10 minutes. Finally, the samples were coated with gold and analyzed in the Microscopy Center of Biological Sciences Institute, Federal University of Minas Gerais, Brazil by SEM (DSM950- Zeiss).

Immunocytochemistry analysis: To further confirm the maintenance of pluripotent state of iPS cultured on BC membrane, iPS cells were cultured for one week on BC membranes and then submitted to immunocytochemistry analysis to evaluate the expression of Oct-4 and SSEA-4, all pluripotency markers. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Samples were then blocked with 0.1% Triton 3% BSA in PBS for 10 min at RT. After blocking, the cells were incubated overnight at 4°C with 1:100 dilution of rabbit anti-human Oct-4 (also called POU-5F1) (Santa Cruz) or mouse anti-human SSEA-4 (ab16287, Abcam) primary antibodies. Alexa Fluor dye conjugated secondary antibodies (Invitrogen, USA) were used for detection of mouse or rabbit primary antibodies through 1 h incubation at RT. The nuclei were counterstained with 0.2 mg/mL Hoechst in PBS for 20 min. The slides were, then, mounted with Hydramount Aqueous media (Cat. HS-106, National diagnostics) and analyzed with a Zeiss LSM 510-Meta confocal microscope.

Results

Isolation and characterization of human adipose tissue derived stem cells

Stem cells from the stromal fractions of adipose tissue were isolated by enzymatic dissociation following centrifugation. The isolated cells displayed a fibroblastic morphology and were adherent to plastic. Also, the cells were able to differentiate in adipocytic, chondrocytic and osteocytic lineages, confirming their multipotency (data not shown).

Bacterial cellulose showed *in vitro* biocompatibility after hASC colonization

A metabolic activity-based assay (MTT) was performed after determined time points to evaluate the hASC viability and proliferation when cultured on BC membrane or in tissue culture plate. MTT results are directly proportional to the number of living cells. The results indicated that hASCs proliferated in both surface and at the day 14,

hASC cultured on BC membrane or tissue culture plate presented equivalent cell viability levels (Figure 1A).

The alkaline phosphatase activity results obtained from the NBT-BCIP assay showed a pattern of AP activity from hASCs cultured in BC membrane that was similar or even high than the AP activity of the hASC cultured in standard conditions during evaluated times. Again, at day 14, alkaline phosphatase activity of hASC on BC membrane achieved the same levels of hASC activity on standard substrate condition (Figure 1B).

These results showed that BC membrane is a suitable scaffold to mesenchymal stem cell colonization because it could promote cell proliferation with no cytotoxic effect and maintained a typical pattern of alkaline phosphatase activity.

Bacterial cellulose as a substrate for culturing iPS cells

Cell viability and alkaline phosphatase activity essay: Based on the promising results with hASC culture on BC membrane, we aimed to evaluate the ability of this scaffold to support pluripotent cell culture as well. In order to do so, iPS cells were seeded on BC membranes and their cell viability and proliferation, as well their alkaline phosphatase activity, were assessed after 120 h. It can be observed that iPS cells were kept viable and producing alkaline phosphatase as desirable (Figures 2A and 2B).

Cell adhesion and proliferation assay

To compare the efficiency of cell attachment and proliferation onto BC membranes and tissue culture plate, the iPSCs were seeded at the same density (1×104 cells/cm^2) and cultured in mTeSR medium. Quantitative analyses of cell densities showed that iPSCs adhered to both substrates 1 day after seeding. However, the adherent cell percentage on the BC membranes (~25.5%) was lower than that on the Matrigel tissue culture plate (~69.6%), suggesting less efficient cell adherence onto BC membrane. However, the cells proliferated more rapidly ~5 fold to ~125.8% on the BC membrane at day 3 when compared with Matrigel culture surface to ~193.8% (~2.8 fold proliferation).

Morphology analysis of BC membrane and growing iPS cells by SEM

In the humid state, the biofilm formed on the surface of the culture medium of the bacterium *A. xylinum* is a homogenous transparent, moldable and handle-resistant gel. Its macroscopic morphology (top surface) of BC membrane can be visualized at figure 4A. SEM image showed a structure composed of randomly distributed fibers with diameters ranging from 200 nm to 1.3 µm with mean of 750 nm (Figure 4B). This structure is similar to that of a natural ECM.

The morphology of the iPS seeded in BC membrane was also examined by SEM, which indicated that the cells adhered to the surface and formed colony-like structure with defined boundaries similar to iPS culture in standard conditions (Figure 4C).

Expression of pluripotency markers by immunocytochemistry

In order to further confirm the pluripotent state of iPS culture in BC membrane, cells were seeded on the scaffold or in Matrigel and after one week, an immunofluorescence essay was performed for labeling Oct-4 and SSEA-4. As can be observed in figure 5, both makers were labeled in cells cultured in BC membrane as well as in tissue culture plate. Oct-4 and SSEA-4 are proteins expressed in the nuclei and in the membrane of pluripotent cells, respectively, and the images for cell

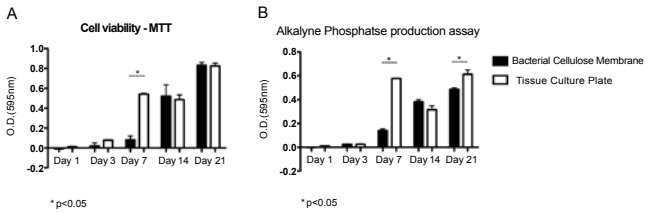

Figure 1: Evaluation of *in vitro* BC's membrane biocompatibility with human adipose tissue-derived mesenchymal stem cells. During the period evaluated, hASC cultured on BC membrane could achieve the same levels of MTT methabolization capacity-at day 14 and day 21: (A) as well as alkaline phosphatase production-day 14: (B) compared to standard culture conditions.

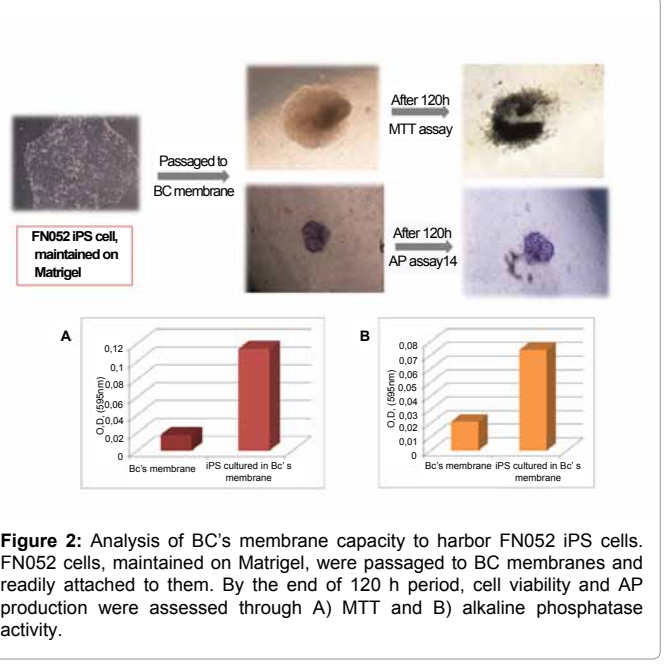

Figure 2: Analysis of BC's membrane capacity to harbor FN052 iPS cells. FN052 cells, maintained on Matrigel, were passaged to BC membranes and readily attached to them. By the end of 120 h period, cell viability and AP production were assessed through A) MTT and B) alkaline phosphatase activity.

cultured in Matrigel shows the expected labeling pattern. For the cells cultured in BC membrane, accurate labeling could not be obtained; due to the three-dimensional structure of BC membrane does not allow to get images in the focus.

Discussion

In this study, we attempted to utilize bacterial cellulose membranes in combination with hiPSCs for further tissue engineering applications. The iPSCs hold great promise for cell therapies and tissue engineering as they can be derived from somatic cells making possible patient-specific cell therapies, which bypasses immune rejection issue and ethical concerns of deriving and using embryonic stem cells (ESCs). The unlimited expansion potential of iPSCs also makes them a valuable cell source for tissue engineering [12]. The BC membrane used as a cell-supporting matrix scaffold presented proper features like being analogous to native extracellular matrix (ECM) in terms of both chemical composition and physical structure. Besides, BC meets others requirements of a suitable tissue-engineering scaffold that should have good biocompatibility without causing inflammation or distortion response *in vivo* and porosity of scaffolds and more specific surface area that is suitable for cell growth and differentiation [13]. BC biocompatibility was confirmed by colonization with hASC, which

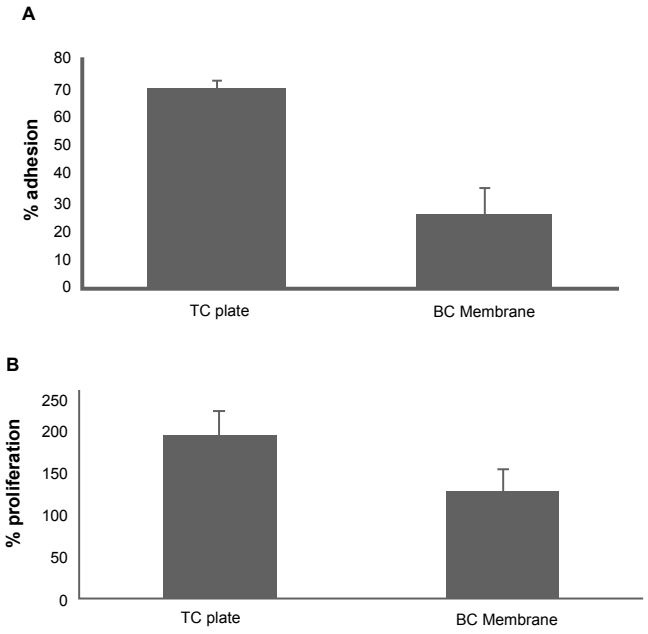

Figure 3: BC membrane enables iPSCs attachment and proliferation. The cells were seeded at the same density (1×10^4 cells/cm^2) onto the BC membrane or standard TC plate and cultured in the mTeSR medium. Percentage of cell adhesion (A) and percentage of cell proliferation (B) were evaluated after 1 and 3 days of seeding, respectively.

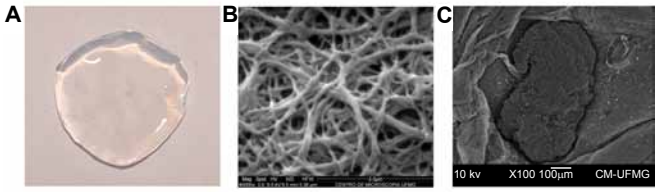

Figure 4: Morphology analysis of BC membrane and growing iPS cells. Digital photograph of the A) CB membrane surface; B) SEM micrography of the superficial structure of the BC membrane illustrating the disposition of fibers; C) SEM micrography illustrating the morphology of iPS cultured in BC membrane with colony-like structures with defined boundaries.

presented standard behavior as proliferation and cell viability levels, as well alkaline phosphatase activity, parameters that could achieve the same magnitude in BC membrane comparing with tissue culture plates (Figure 1). Additionally, MEV analysis of BC membrane supports its similar structure to extracellular matrix containing their dispersed nano-fibers in its surface (Figure 3B).

Despite of all their attractive features, the culture of pluripotent cells, though, still constitutes an extremely expensive and difficult technique, which rely on limited options of culture media and growing surfaces. Therefore, the look for alternative substitutes for growing pluripotent cells is required. Recent work in developing alternative systems represents key progress in simplifying hESC/hiPSC production, but these methods still require components that are difficult to synthesize. A variety of substrates are used in these methods: synthetic polymers [14], peptide-modified surfaces [15], embryonic ECM laminin isoforms [16], fibronectin from ECM with a small molecule mixture [17], and various vitronectin proteins [18]. Some of these culture methods use animal products, which makes potential transplantation applications problematic [19]. Bacterial cellulose, otherwise, can be produced and purified by a simple protocol and it was never tested for culture pluripotent cells, at least of our knowledge. At this point, we had to evaluate the capacity of BC membrane to harbor iPS cells and maintain their pluripotent state. When seeded to BC membrane, iPS cells readily attached to it and remained viable as can be confirmed by MTT essay (Figure 2). Alkaline phosphatase is one of the markers of the indifferentiation state of embryonic stem cells [20] and its activity was observed in iPS cultured in BC membrane (Figure 2), as expected for pluripotent cells.

When assessing the efficiency of adhesion and proliferation, iPSCs seeded in BC membrane showed impaired adherence compared to tissue culture plate (Figure 3). Nevertheless, iPSCs proliferation seems to occur in faster rate in BC membrane than in TC plate, which suggests that after several days, the yield of iPSC in BC membrane can be as satisfactory as standard conditions. More experiments need to be performed to confirm this hypothesis.

Subsequently, MEV analysis showed that iPS cells displayed structure-like colonies with defined boundaries when cultured in the scaffold (Figure 4). Unlike typical and spread colonies cultured in Matrigel, iPS colonies in BC membrane seems to present a "stacked" morphology, which is probably due to the 3D character of BC membranes that impairs cells to spread. To circumvent this issue, we are addressing to produce BC membrane as a thinner film covering standard tissue culture plates to verify whether colonies can display a similar behavior of cells cultured in Matrigel.

Finally, in order to further confirm pluripotency of iPS cultured in BC membrane, we assessed expression of Oct-4 and SSEA-4 by immunofluorescence. As can be observed in figure 5, iPS cells cultured in Matrigel as well in BC membrane were labeled for both markers. The cells in Matrigel presented the accurate pattern of staining in which Oct-4 is located at nuclei and SSEA-4 at membrane surface. When

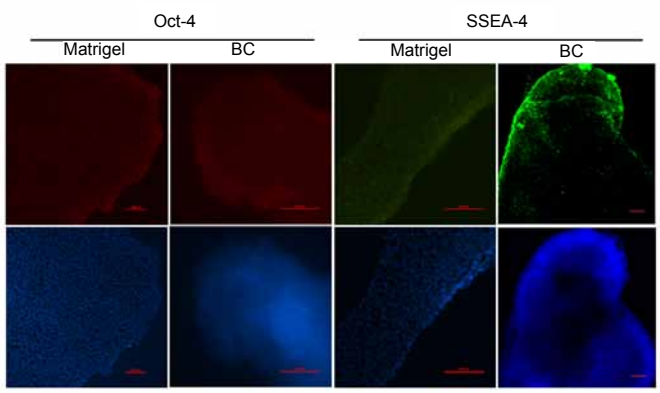

Figure 5: FN052 cultured on Matrigel and BC membranes maintained Oct-4 and SSEA-4 pluripotent markers expression, as observed in imunofluorescence assays. The nuclei are stained in blue, Oct-4 in red and SSEA-4 antigen in green. Scale bars: 100 µm.

related to cells in BC membrane, the labeling was not accurate due to the three dimensional morphology of the membrane, which limits to get images at the main focal plan. Besides, SSEA-4 labeling was not homogenous in the colony, but this could be due the fact that colony possibly has more than one layer, which prevents the access of antibody to all cells in the colony. On the other hand, concerning to Oct-4, the labeling in BC membrane was present at the whole colony, and, as it is known, when cells start their differentiation process, Oct-4 in one of the first markers that has its expression interrupted [21], what suggests that cells are still in their pluripotent state after seeded in BC membrane.

In conclusion, the resultant scaffold, BC membrane was shown to have suitable features to harbor both hASC and iPS cells, where cells are able to maintain their primary characteristics of adherence, viability and proliferation, enzymatic activity and expression of specific markers. The association of BC membrane with iPS cells holds, therefore, a promising tool and it requires continuing studies to explore their vast potential of application in different areas of the tissue engineering.

Acknowledgments

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