

The use of bone marrow derived mesenchymal stem cell for cornea regeneration in rabbit model

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Abstract

Aim or purpose: To evaluate the use of autologous bone marrow derived mesenchymal stem cells (BM-MSCs) to treat cornea stromal defect in a rabbit model.

Methods: A non-randomized interventional controlled animal study involving twenty-one adult New Zealand white rabbits. Corneal deep lamellar dissections were created in three groups of rabbits and treated accordingly. Group I: Autologous bone marrow derived MSCs with autologous fibrin and human amniotic membrane. Group II: Autologous fibrin with human amniotic membrane without MSCs. Group III: No treatment. Clinical outcome was evaluated by corneal re-epithelization, corneal opacity, corneal thickness and histology.

Results: BM-MSCs were successfully isolated from bone marrow of seven rabbits based on the adherence property of the cells to the plastic of the cell culture plate. At day 60 corneal thickness was significantly thicker in Group I. The localization of PKH26-labeled BM-MSCs showed an increase in cell density at the transplanted site, proving its role in cornea stromal regeneration. Although the cornea clarity was not achieved in this study, we believe that cornea stromal remodeling requires many months to years to regain its original optical quality.

Conclusion: Locally transplanted BM-MSCs may be a useful source for cornea stromal regeneration. The use of autologous BM-MSCs offers a promising option for treating corneal disorder without the risk of immune-rejection and calcification.

Keywords: BMSCs, corneal stroma, rabbit, tissue engineering, transplantation

Introduction

The cornea is damaged in various diseases, such as trauma and injuries. Corneal melt is a debilitating disease resulting in severe loss of vision. Such cases usually result in scarred and vascularized corneas or evisceration. At present, the only treatments available are inorganic tissue glue or corneal transplantation. However, the main risk of corneal transplantation in an inflamed eye is tissue rejection. Furthermore, in many countries access to corneal tissue is severely hampered by the lack of organ donors. The use of inorganic tissue glue is also hampered by the fact that

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they are very toxic to the underlying cell. This may sometimes result in a larger corneal hole than before. Using a person's own cells in corneal regeneration may address such problems.

Autologous corneal tissue engineering is a promising therapeutic approach to overcome the problems of primary immune rejection and the limitation of corneal donors. Norzana *et al.*⁹ reported that corneal epithelium can be serially expanded in serum-free and feeder layer-free culture system, and be used for corneal tissue engineering. In clinical cases, the epithelial cells can be obtained from donors or from the healthy eye of the same patient. However, the former may cause tissue rejection, whilst the latter may not be suitable in cases where both eyes are damaged.

A recent report suggested that transplantation of human mesenchymal stem cells could reconstruct the damaged cornea.^{8,12,14} Ye *et al.*¹⁴ showed that systemically transplanted MSCs can engraft to an injured cornea and promote wound healing by differentiation, proliferation and synergizing with hematopoietic stem cells. The therapeutic effect of the transplantation may be associated with the inhibition of inflammation and angiogenesis. However, these studies are mainly looking at cornea epithelial regeneration as opposed to stromal regeneration. The latest study by Gu *et al.*⁵ showed MSCs could differentiate into corneal epithelial like cells *in vivo* and *ex vivo*. However, most corneal diseases involve the corneal stroma, which accounts for 90% of corneal thickness. Diseases that can cause cornea melt include autoimmune keratitis, infection and ectatic diseases. Arnalich-Montiel *et al.*¹ did a study to look at stroma regeneration. Their results showed that adipose-derived stem cells could be a source for cellular therapy for the corneal stroma.

Barbosa and co-workers² suggested that corneal myofibroblasts, which make up the corneal stromal layers, can be derived from MSCs in a chimeric mice study. MSCs also have the capacity to differentiate to corneal stromal cells when the cells were intravenously introduced, as reported by Harada.⁶ A recent study by Park *et al.*¹¹ reported that MSCs are able to be differentiated to keratocyte-like cells *in vitro* by using keratocyte conditioned-medium (KCM). The MSCs differentiated keratocyte-like cells expressed both keratocan and ALDH1A1 and consistently upregulated lumican. This study showed that conditioned medium promotes differentiation of human MSCs to corneal keratocyte-like cells *in vitro*.

The ability to bioengineer a person's own cornea using autologous MSCs in improving visual outcome will represent a significant improvement in corneal tissue engineering. This may delay or reduce the need for corneal transplantation and improves the patient's quality of life. It may also prevent the risk of corneal rejection, which is the main cause of graft failure. It also avoids the controversy surrounding the use of embryonic stem cell. The primary aim of a bioengineered cornea is to overcome the undesirable outcome of corneal melt following insult and the secondary aim is to restore the corneal thickness and clarity.

The objective of this study was to evaluate the use of autologous bone marrow derived mesenchymal stem cells (BM-MSCs) to treat cornea stromal defect in a rabbit model.

Materials and methods

Animals experiment approval

This study involved 21 adult male New Zealand white rabbits weighing 1.8-2.3 kg, obtained from Medical Faculty UKM Animal Unit after the approval of Universiti Kebangsaan Malaysia Animal Ethics Committee. They were treated according to the *Guide for The Care and Use of Laboratory Animals* (National Academy of Science 1996). The animals were randomly divided into three groups of seven rabbits each. Group I: Autologous BM-MSCs with autologous fibrin glue and human amniotic membrane (HAM); Group II: Autologous fibrin glue with human amniotic membrane without BM-MSCs; Group III: No treatment. Bone marrow was harvested from the left iliac crest bone in all the rabbits in group I. Four ml of blood were collected to produce autologous fibrin from all the rabbits in group I and II.

Isolation, expansion and labeling of BM-MSCS

Autologous BM-MSCs aspiration from the Group I was harvested and expanded in the laboratory using widely accepted protocol and commercially available tissue culture media.¹³ The animals were being anaesthetized by intramuscular injection of a mixture of zoletil 50 mg/kg (Virbac), ketamine hydrochloride 50 mg/kg (Bioketan) and 10 mg/kg of xylazine (Troy Lab) (0.2 ml/kg body weight). Once they were sedated, the animals were then placed in a wooden box restrainer. Intravenous animal anesthetic drugs regime (0.1 ml/kg body weight of the mixture) was given through the lateral marginal ear vein to ensure the animals were deeply sedated. Left iliac crest region of the rabbit was shaved, painted and draped. One centimeter skin incision was made over the iliac crest. Four ml of rabbit bone marrow was harvested using a percutaneous 18-G needle aspiration from the iliac crest. The bone marrow was kept in vacutainers containing 0.1 ml of heparin (3000 U/ml-B/ Braun) at room temperature and transferred to the cell culture facility.

Isolation and expansion of BM-MSCs were performed as previously described.¹³ They were isolated solely based on their plastic adherent property. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Fresh medium was added on the third day. Medium was changed upon substantial cell attachment and later, twice a week in a 75 cm² flask for the second passage and in a 175 cm² flask for the third passage. Haematopoietic cells and non-adherent cells were removed along with the media. Upon 80%-90% cell confluency, cells were detached by the addition of 0.05% trypsin-EDTA (Gibco USA) and viable cells counted using trypan blue dye-exclusion-method. Cells were subsequently sub-cultured at a standard density of 10,000 cells/cm². Adherent cells after the third passage referred to as third-generation MSCs, were used for this experiment.

Preparation of autologous fibrin

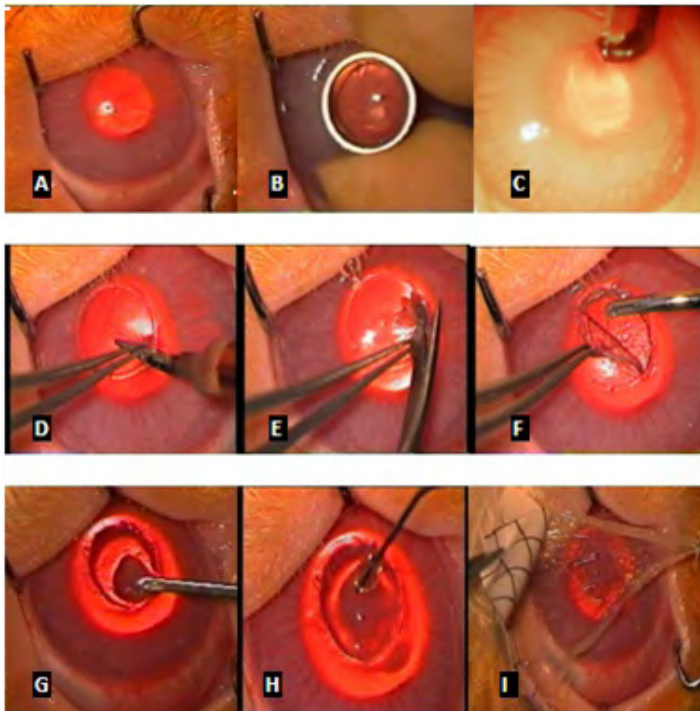
An amount of four ml of fresh rabbit blood was collected through the lateral marginal artery from the rabbit's ear. Blood was collected in sodium citrate tubes followed by rapid inversion of the tube to deter blood from clotting and kept at room temperature. It was then centrifuged at 3000 rpm for five minutes. The plasma

layer (top yellowish layer) was gently transferred to a new tube with a pipette without disturbing the bottom layer (red layer). This process was repeated three times to ensure complete removal of residual cellular components. The plasma was sterile-filtered using a 0.2 μ syringe filter (Sartorius, USA) to remove the cell debris that may cause spontaneous clotting of the plasma. Plasma was stored at -20 °C until the day of transplantation.

Animal model

The rabbits were anesthetized and the quality of ocular surface was evaluated by the degree of smoothness using slit lamp microscopy (SL-DC 1, Topcon, Japan) with a Topcon anterior segment camera. The cornea thickness was measured by anterior segment optical coherence tomography (OCT-Visante Carl Zeiss). The left eye was cleaned and draped and was kept open by a speculum. Left cornea defect was created by deep lamellar dissection (Fig. 1A-F). A five-mm trephine was used to standardize the size of the defect. A guarded diamond knife was calibrated to a desired corneal thickness. An incision of 80% to 90% thickness of the rabbit's cornea was created. The cornea was then dissected using Melles' corneal lamellar dissector and the edge was cut by corneal scissors. The thickness of lamellar dissection was then evaluated by anterior segment OCT and slit lamp to assure the desired corneal thickness was removed.

Fig. 1.



In-vivo transplantation

Regarding the rabbits in Group I, BM-MSCs were transplanted with an autologous fibrin. On the day of transplantation, monolayer cells were trypsinized and centrifuged at 3000 rpm for five minutes to obtain a cell pellet of approximately ten million cells. Autologous fibrin derived from plasma was used as BM-MSC cell carrier. Stored plasma was thawed on the day of surgery. Calcium chloride (0.5 mmol/l) was added to one ml of plasma to initiate the polymerization process. Cell fibrin mixtures were then filled into the cornea defect. A fresh frozen human amniotic membrane (HAM) was sutured over the site using 10/0 nylon sutures to be held in place for seven days (Fig. 1G-I).

In Group II, corneal defects were treated with the autologous fibrin without cells. As in group I, the treated eyes were covered with HAM. The HAM was removed on day seven post transplantation. Group III consisted of cornea defects without treatment and were left to heal on their own. All rabbits were returned to their cages after the procedure and were allowed to move freely. Topical dexamethasone 0.1% (Alcon®) and moxifloxacin 0.5% (Alcon®) were used four times daily for two weeks. Only dexamethasone was continued twice daily for another month to all corneas.

Follow-up and clinical evaluation

Each eye of the cornea defect underwent slit lamp examination, fluorescein staining and viewing under blue filter, and anterior segment OCT at postoperative day 0, 7, 14, 30, and 60. The corneal epithelial integrity was quantified by the ratio of the epithelial defect area to the total cornea using simple Imaging Measurement Software (Topcon, US). The corneal opacity was assessed using published corneal opacity grading technique by visualizing the ocular tissue beneath the cornea.⁴ The corneal clarity was graded as: grade 0, totally clear with no opacity seen by any method of slit lamp microscopic examination; grade 1, haze of minimal density seen with difficulty with direct and diffuse illumination; grade 2, mild haze easily visible with direct focal slit illumination; grade 3, moderately dense opacity that partially obscured the iris details; grade 4, severely dense opacity that completely obscured the details of intraocular structures. Corneal thickness was measured with anterior segment OCT at preoperatively and post operatively day 30 and 60. Six readings were taken of the center corneal thickness and mean thickness was used for the data.

Statistical analysis

Epithelial defect size and cornea thickness were compared with repeated measure ANOVA. To compare statistical association of cornea opacity among group, we used the Fisher exact test.

Histology examination and localization of MSC cells in the cornea

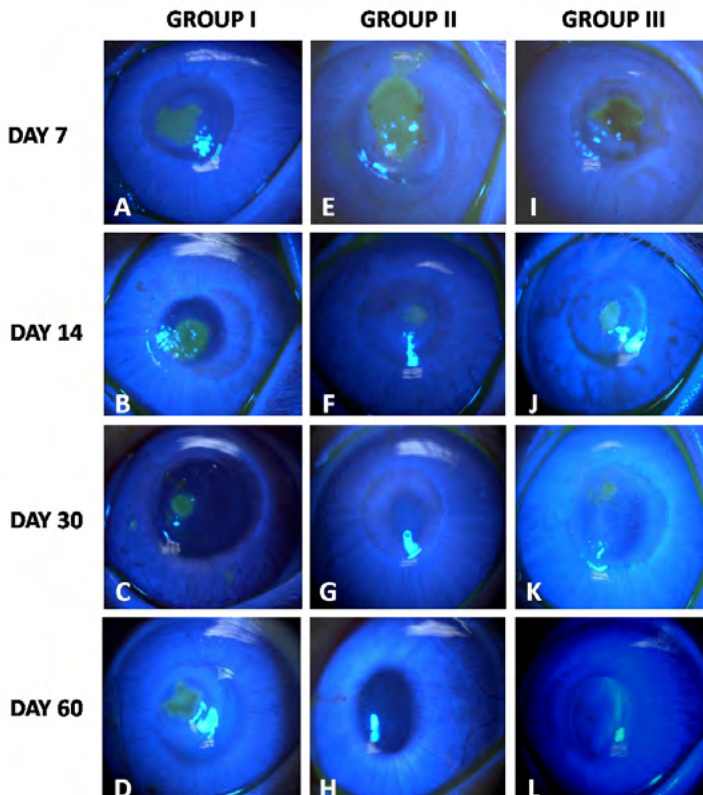
For localization of the BM-MSC cell experiment, one rabbit in group I was euthanized after two weeks and one after four weeks and the remaining were euthanized 60 days after cell transplantation. Several sections of each cornea were stained with hematoxylin and eosin and von Kossa staining for light microscopy examination

(Olympus BX51; Olympus Co. Ltd., Tokyo, Japan). DAPI stain was used to label all cells nuclei in the corneas in the biosafety experiment. Localization of PKH26 labeled BM-MSC cells using Red Fluorescent Cell Linker Kit (Sigma-Aldrich, Inc. Saint Louis, Missouri, USA) was achieved using confocal microscope (LSM 510 Meta, Zeiss). This technique was performed according to the manufacturer's instruction. Cell tracking was observed at week 2 and 4, following transplantation.

Result

The treatment groups were successfully performed, *i.e.*, Group 1: autologous BM-MSCs with autologous fibrin glue and human amniotic membrane (HAM); Group II: autologous fibrin glue with human amniotic membrane without BM-MSCs; Group III: no treatment. The mean area of baseline epithelial defect ranged from 19.04 mm² to 19.51 mm². The statistical analysis revealed there was no significant difference in baseline epithelial defect area among the three groups. This was not surprising as all the defects are created in a well-defined area using a trephine (Fig. 2). All the rabbits showed significant improvement in corneal re-epithelization by day 60 among their group.

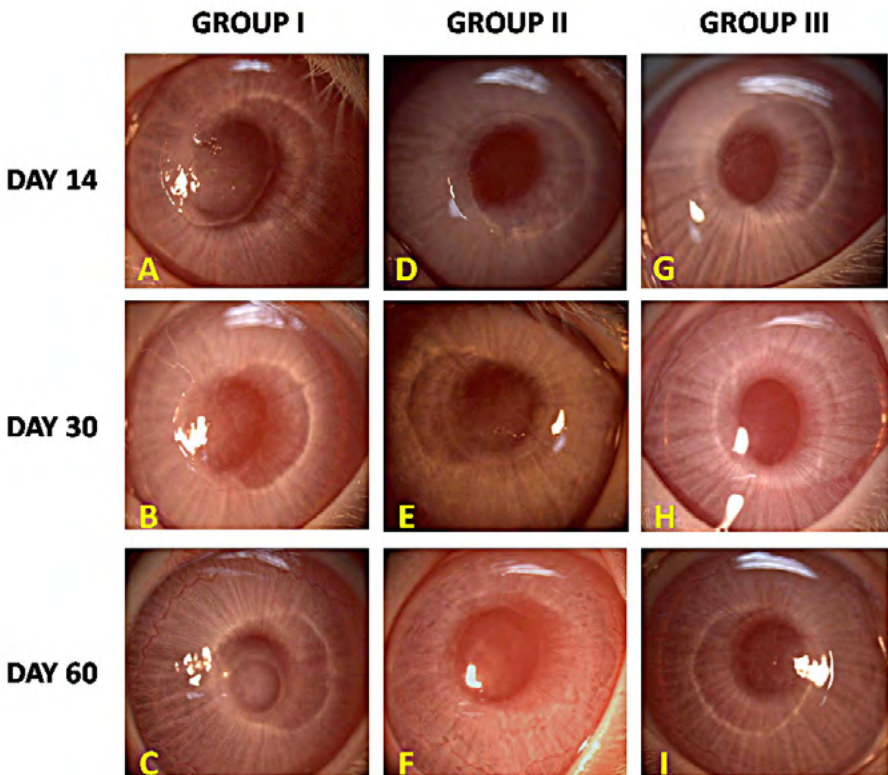
Fig. 2.



However, overall cornea re-epithelization in the three groups did not show any statistical significance (Table 1). The results showed that BM-MSCs transplantation did not improve the rate of epithelial healing.

Corneal opacity was observed in all three groups during follow-up. Fig. 3 shows a photography of one of the rabbits in each group on day 14, day 30 and day 60 of the follow-up. In group III, the corneas were much clearer and only one rabbit developed moderately dense opacity at day 30. In addition, the opacities observed in group I tended to be denser at the edge of the dissected cornea, forming a ring appearance. However, at day 30 the corneal opacity in the three groups did not show any statistical significance (Table 2).

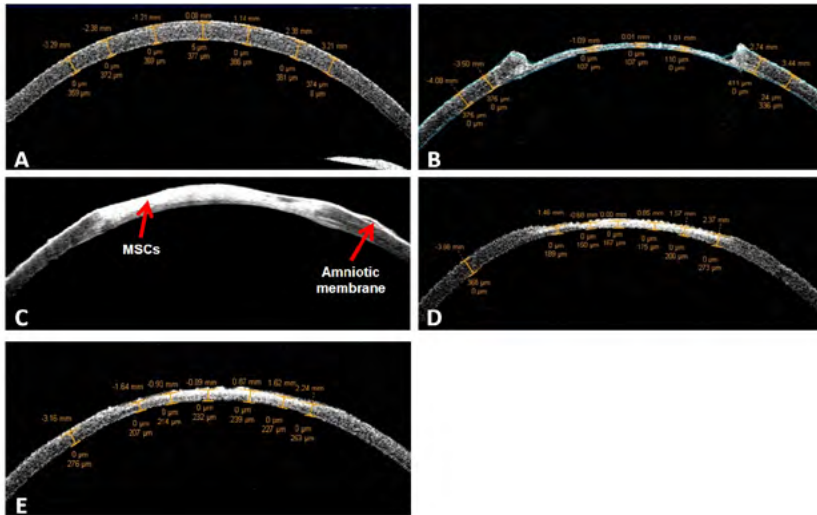
Fig. 3.



The normal corneal thickness in all rabbits ranges from 405 μm to 342 μm with mean thickness of 369 μm . The mean corneal thickness preoperatively in group I: 351 μm ; group II: 372 μm and group III: 374 μm . We removed almost 80% of corneal thickness during deep lamellar dissection. There appeared to be a significant increase in the corneal thickness in all three groups at day 30 and day 60. At day 60, the final corneal thickness was also significantly higher in group I (258.40 μm)

compared to group II (215.33 μm) and III (225.60 μm) (Table 3). With the advantage of high resolution anterior segment OCT, we managed to clearly visualize the depth of the corneal defect and the transplanted BM-MSCs covered with HAM (Fig. 4).

Fig. 4.



At day 30, a confocal microscope examination of the transplanted cornea which received a PKH26 labelled MSCs showed localization of PKH26 MSCs at the area of defect and edge of the wound (Fig. 5A). Histology staining with Hematoxylin and Eosin in all three groups (Fig. 5E-G) showed that the rabbit's epithelium had completely covered the damage cornea surface with variable epithelium layer. The epithelium in the peripheral corneal areas was normal in appearance with a total thickness of five to seven layers. It was noted that there was an increase in epithelial thickness in group II and III. Stromal regeneration was observed in the group I at the edge of the wound with increase in cellular density compared to group II and III which was lesser toward the centre.

Fig. 5.

Discussion

Our study was based on clinical observation as an objective measurement. The size of epithelial defect, corneal opacity and corneal thickness were used as parameters for stroma regeneration. In our study, corneal neovascularization was not observed in all three groups. It seemed that neither the MSCs nor fibrin scaffold are capable of angiogenesis.

Epithelial healing rates were the same in all three groups. It seemed that transplantation of BM-MSCs or fibrin alone did not affect the healing rate on epithelium.

This result differs from previous study in other groups.^{10,14} A study by Ma *et al.*⁸ also showed significant improvement in re-epithelization in MSCs-treated group. The re-epithelization was quantified by less than one fourth of cornea fluorescein staining. It differs from our study method in that we use autologous fibrin as a scaffold and then mixed with autologous MSCs pellets before transplantation. Their method uses amniotic membrane as a cultivating sheet for the MSCs before being transplanted. This may be due to the preservation of stromal layer in their cornea rabbit model whereas in our study, we removed almost 80% of the stroma layer. We postulated that cellular migration in our study might be inhibited by the corneal construct. Instead of enabling the epithelial cells to grow over the stroma as in group III, the cells in group I and II have to grow over the cell-fibrin and fibrin matrix, respectively.

Stroma remodeling is a major factor in contributing to the transparency of the cornea. Based on our result, we observed that the MSCs group developed more corneal opacity. This is consistent with other study by Gu *et al.*⁵ in that they also showed that groups treated with MSCs developed cornea opacity. In contrast, another study by Ye *et al.*¹⁴ showed significantly clear corneal in the treated group with MSCs compared to those without MSCs. These differences may be due to different methods of transplantation.

In the present study, we observed that MSCs tend to distribute more at the edge of the defect. The cellular distribution was not even due to the curvature of the stromal defect created. It also explained why the scarring tends to be annular in shape in the treated group. It developed opacity, but from the increase in corneal thickness, a new stromal matrix must have been produced.

The optical coherence tomography (OCT) can visualize the transplanted MSCs within the lamellar dissected cornea and amniotic membrane overlying it. We found that the normal corneal thickness in experimental rabbits range from 408 μm to 342 μm , which is similar to the findings in other studies.^{3,7}

We found that the corneal thickness had significantly increased from day 30 to day 60 and at day 60 in group I, the newly regenerated cornea is marked by the presence of a thick stroma layer with an increase in cellular density in the region close to the corneal defect that was created. It showed that MSCs-treated group has participated in stroma regeneration. The difference of corneal thickness in BM-MSCs treated group had significantly increased at day 60 compared to day 30, indicating that stromal regeneration continues throughout the time frame of this experiment. In groups II and III, corneal thickness also increased at day 30 but had not increased significantly at day 60. Further, epithelial hyperplasia noted histologically in group II and III. This study also found that stroma took a long time to regenerate as the thickness from postoperatively to day 30 was not significant between all groups.

The present study had the following limitations: transplantation method of MSCs results in excessive accumulation of cells at the edge of the wound and less remaining in the central region. We did not investigate whether the transplanted MSCs differentiated into functional keratocytes or if they exert a paracrine effect in promoting wound healing. Despite the limitations, we showed that transplanted

BM-MSCs are capable of regenerating corneal stroma. However, this beneficial effect comes at the expense of increased corneal haze.

Conflict of interest

The authors have no conflict of interest to declare in the conduct of the study.

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