Original Research Article

COMPARATIVE STUDY OF THE EFFICACY OF STEM CELLS IN CORNEAL REGENERATION IN A CHEMICAL BURN IN RABBITS

Abstract

Purpose: This study will compare the efficacy of stem cell transplantation in corneal regeneration and restoration of the limbic deficit in an experimental chemical burn in rabbits.

Methods: We performed the biopsy of the limbus and the chemical burns for all rabbits, and we collected the amniotic membranes from a pregnant female rabbit. We kept a control group without transplantation, to study spontaneous and natural healing, and we transplanted the stem cells produced in vitro under the corneal epithelium burned. To compare the result, we tested a group for amniotic stem cell transplantation, a group for limbal stem cell graft, and another group for combined transplantation of both types of stem cells.

Results: Transplanted rabbits develop permanent unilateral blindness due to a severe limbic deficit. The group receiving only amniotic stem cells shows temporary anatomical improvement without functional recovery. The two groups receiving limbal stem cells alone or combined with amniotic stem cells showed anatomical and functional satisfaction with quick recovery time for the combined transplantation.

Conclusions: A simple chemical burn can establish permanent blindness. When the limbic deficit is important, spontaneous healing is not available. Transplantation of stem cell transplant is the only way to repair this deficit and regenerate the cornea. Only limbic stem cells can be sufficient. Amniotic stem cells can support and speed up the healing time when it combined to limbal stem cells graft.

***Keywords*:** stem cell; chemical burn; limbic deficit; transplantation; corneal regeneration.

Introduction

The ocular surface contains three adjacent epithelia: conjunctiva, limb, and cornea. Cornea shows many roles in protecting internal structures from germs and particles and in protecting against ultraviolet rays.[1,2,3,4,5,6] It is the main lens of the eye, its location on the front surface of the eyeball often exposes it to accidental injuries. All damage can lead to infectious keratitis, chronic ulcer, limbal deficiency, or even permanent blindness.

Corneal blindness is the 4th leading cause of blindness in the world (according to the World Health Organization), responsible for 5.1% cases.[7] It was necessary to protect it or even regenerate it if possible. Various studies confirm the therapeutic success of amniotic membrane grafting associated or not with limbal stem cells. This success is limited in time and often linked to later complications. We don't know which of the transplanted cells are the most effective, and we haunt for long-term therapy. In this study, we explored two types of stem cells used for corneal repair in an experimental limbal deficit in rabbits; autologous cells from the limbus and amniotic cells amplified in vitro and then administered as a single or combined transplant. We hope to discriminate between their efficacy.

Methods

We declare that sex does not affect the outcome and that we have followed the guidelines of the animal department of the faculty (ARRIVE guidelines, and EU Directive 2010/63/EU for animal experiments), during all these trials without the need to have any ethical approval from the responsible authority.

We declare that we conducted this study using the Dutch rabbit as the experimental model, with a cohort of 17 individuals, including a pregnant female for amniotic membrane collection and 16 rabbits, all males, for screening, which we divided into four groups:

- The group without transplantation as a control (A),
- The group that underwent amniotic cell transplantation only (B),
- The limbic cell transplant group only (C),
- The group with the combined transplant (D).

We keep all rabbits on an empty stomach for four hours. We perform sedation with Midazolam (0.5 mg/kg intramuscularly), and anesthesia with Propofol (5 mg/kg intravenously) injected slowly to avoid the risk of apnea. We placed an eyelid retractor, and we performed the biopsy in the limbic region of the left eye over an arc of about 70° to 80°, 1 mm deep and 2 mm towards the cornea (Fig. 1).

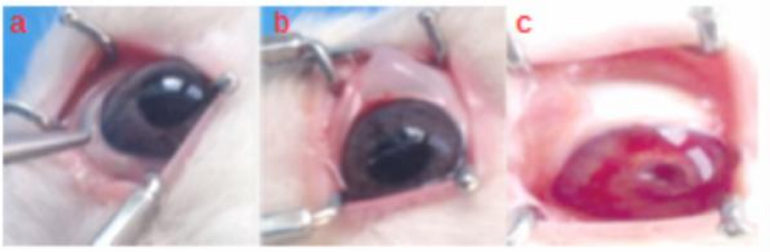

Fig. 1. limbal biopsy process.

Before biopsy (a), Immediately after biopsy (b) et 1 hour after biopsy (c)

We applied a cotton swab immersed in 4% hydrogen chloride, at the center of the cornea for 2 seconds, and we washed immediately with a saline solution to limit diffusion of caustic to the rest of the ocular surface (Fig. 2).

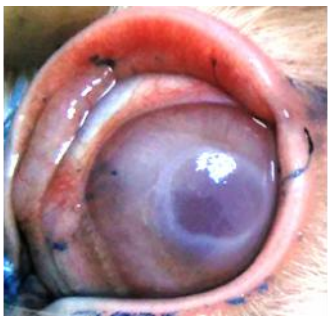

Fig. 2. Corneal opacity

We transported each biopsy in a sterile vial containing a culture medium. In the laboratory, we transferred them to an identified Nunc dish for microscopic observation (Fig. 3: a), then to a conical tube containing 1 ml of trypsin solution at 0.25%. After 5 minutes of contact, we washed each tube with 3 ml of PBS to inhibit the enzymatic action. We centrifuge all tubes at 500 G for 5 minutes, and we incubated the recovered pellets in identified Petri dishes at 5% CO2 and 37°C.

We extracted amniotic membranes from a pregnant rabbit and transported it to the laboratory in a sterile vial containing culture medium. We performed a microscopic examination by emptying the vial into an identified Nunc box (Fig. 3: b). We transferred the contents to a large Petri dish for cutting and cleaning from the conjunctive tissue. We perform washing in a conical liquid containing a PBS solution. We centrifuged the tube twice in PBS at 500 G for 5 minutes, and we cultivated the pellet in a Petri dish under the same conditions as mentioned above.

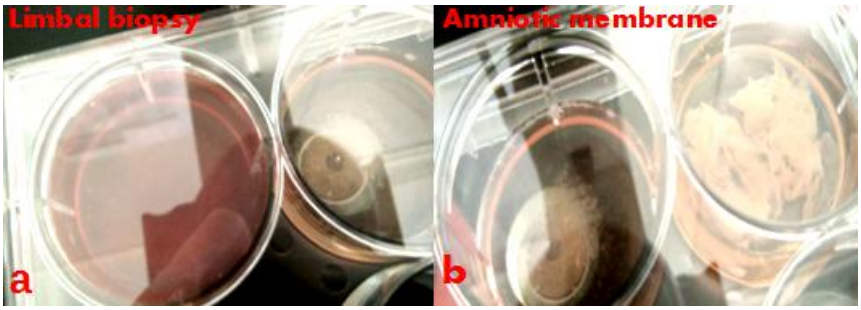

Fig. 3. Sample processing

We worked under optimal conditions of sterility (Fig. 4). The culture medium used contains a base of DMEM/F12 with fetal calf serum 10 %, glutamine 2 mM, non-essential amino acids0.1 M and antibiotic 100x (penicillin 10,000 u/ml, streptomycin 10,000 µg/ml) and antifungal agent (Nystatin) 0.1 %.

Fig. 4. Workstation (Microbiological Safety Station)

During the proliferation process, we changed the culture medium every two days and performed trypsinization and passage at the level of 70% cell confluence. We stopped the proliferation after two passages. We divided the proliferated stem cells into two tubes per individual, each containing a volume of 500 ml (10^6 cells/ml); to the first tube intended for transplantation we added 100 µl of sodium hyaluronate, and to the second tube intended for cryopreservation we added an equal volume of a freezing solution (20% DMSO in FBS). We left the tubes at - 80 °C for one night before transferring them into liquid nitrogen.

We kept the rabbit candidates for transplantation on a fast stomach for four hours before the anesthesia. After applying the eyelid retractor, we made a small incision in the cornea through which we passed a curved knife to separate the damaged epithelium from the stroma. Under this epithelium, we introduced the cell content for grafting using a syringe equipped with a fine, flexible catheter.

We treated the four test groups with local treatment (antibiotic and anti-inflammatory) for 15 days, and we extended the postoperative monitoring for up to 2 months. To explore the return of vision, we adopted two functional tests: the light reaction test and the labyrinth test in search of food after covering the right eye.

Results

When we examined the samples under the microscope, we found the amniotic membranes in a perfect state, and we found limbic cells in all the biopsies. The caustic induced an immediate opacity of the cornea, visible with Trypan blue (Fig. 2). The in vitro culture provided sufficient cells for grafting and freezing in only two passages. The rabbits showed very good clinical performance after biopsies and transplantation, with no postoperative complications, no signs of infection, no neovascularization of the cornea, and no graft rejection.

In only 10 days, rabbits that received amniotic stem cells alone developed temporary anatomical improvement without functional recovery. Rabbits that received a combined graft of limbal and amniotic stem cells showed a clear anatomical and functional improvement compared to those that received limbal stem cells alone, but both regained their visual abilities. The control group (rabbit without grafting) showed unilateral blindness without anatomical improvement during the two months of follow-up (Fig.5).

Fig.5. Anatomical evolution of the ocular surface for the four study groups

Discussion:

All groups tested developed a corneal ulcer (Fig. 2). Without stem cell transplantation, the ulcer was complicated by a severe limbic deficit and unilateral blindness (Group A) [14]. There was a temporary anatomical improvement, but no improvement in visual acuity for the group that received an amniotic cell transplant (Group B). The anatomical and functional recovery observed for groups C (limbic stem cells) and D (mixed limbic and amniotic stem cells) was so rapid and significant for group D.

Various cellular and molecular processes started; first, the caustic agent destroys the epithelial cell membranes around the cornea and limbus and the extracellular matrix composed of structural proteins (collagen, laminin, and fibronectin) and signaling proteins (integrin and metalloproteine). [15-17] Two systems are activated, the metalloproteinases that break down proteins in the extracellular matrix and the system that converts plasminogen to plasmin. Plasmin intervenes in the cleavage of extracellular matrix proteins and activates the TGF-b pathway and pro-collagenases.[15,16,18-20] This hyperactivity leads to the fusion of the stroma.[21] Secretions from the limbic blood vessels, the tear film, or the aqueous humor, inhibit the expansion of the lesion into the underlying tissues. [1,2,5]

Underlying cells that escape caustic action modify their cytoskeleton and increase their metabolism to produce the various proteins of the cytoskeleton (vinculin, actin, talin, and integrin). [22,23] Fibronectin, fibrinogen, and fibrin reach the site of damage by limbic blood vessels and participate in the reconstitution of a temporary extracellular matrix consisting of tenascin, lumican, and laminin, which facilitates the migration of epithelial cells.[24,25] Laminin reduces gene expression in integrin subunits by altering the level of sp1 and sp3 transcription

factors, which reduces integrin production and facilitates the detachment of intact epithelial cells from the basement membrane. These cells modify their differentiation and proliferation properties to regenerate a neo-epithelium [26,27]. This process is known as vertical renewal and requires continuous multiplication and migration of stem cells from the limbus to satisfy the need, which becomes impossible if the niche is damaged.

The amniotic cells contain epithelial stem cells (ESC) and stromal or mesenchymal stem cells (SSC, MSC) [28], while the limbic cells contains epithelial stem cells (ESC) and mesenchymal stem cells (MSC) [3,29,30]. After transplantation, only the epithelial cells migrate to the recently synthesized fibronectin matrix, [31,32] and forme intercellular and matrix contacts as a protective barrier. [10,11,20,21,33] Reassembly of the hemidesmosomes at the basal pole of the limbic epithelial cells facilitates their adhesion to the basement membrane to form a temporary corneal epithelium. [21,33]

The limbal graft cell joins the limbus and divides asymmetrically into a small cell that remains in the niche (pool renewal) and a large differentiated cell called the transient amplifier cell (TAC). This TAC proliferates and migrates from the limbus to the centre of the cornea.[4,11-13] This explains the permanent renewal provided by limbal stem cell transplantation.

Studies on laboratory animals describe the beneficial effect of MSCs on corneal healing after the application of their conditioned medium or after their implantation in injured tissue. [5,9] These cells produce growth factors (KGF; HGF; EGF; TGF and bFGF) and cytokines that facilitate corneal reepithelialization,[20,31,34,35] prevent apoptosis of epithelial cells,[24,36-38] promote their differentiation and migration,[12,39] and enhance their adhesion.[11,40] They also have anti-adhesive, antibacterial, and antifungal properties that inhibit microbial colonization; [37] and anti-angiogenic properties that reduce neo-vascularization and the invasion of conjunctival tissue (Ptérygion).[8,24,37,38,41] Amniotic epithelial cells produce anti-inflammatory cytokines such as IL-1Ra and IL-10 that block the inflammatory cascade and inhibit metalloproteinases.[36,38,41,42] Finally, these cells have the advantage of not expressing histocompatibility antigens and therefore do not cause a rejection reaction.[38,42] Amniotic epithelial and mesenchymal cells can synthesize thrombospondin-1, endostatin, and metalloproteinase tissue inhibitors (TIMPs).[15-18,43] Simultaneous amniotic cell transplantation has shown that it can be an important complement to auto or limbal allograft techniques.[31,32,44]

We discovered a temporary therapeutic result with the amniotic stem cell transplantation compared to a synergistic, rapid and permanent result obtained with the combined amniotic and limbal stem cell transplant, thanks to the supportive effect of amniotic stem cells and the regenerative effect of limbal stem cells.

This therapeutic alternative has successfully repaired the caustic corneal burn and restored the limbic deficit. In humans, it will replace and surpass the various therapies used in this sense, as it is technically fast, inexpensive, and with a short time of recovery.

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