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RESEARCH ARTICLE

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

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ABSTRACT

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

Methods: The biopsy of limbus and the chemical burn were performed in all rabbits, and the amniotic membranes were obtained from a pregnant rabbit. A control group without transplantation, to study spontaneous and natural healing, and transplanted the stem cells produced *in vitro* under the corneal epithelium burned. To compare the result, a group was tested 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: Chemical burn, corneal regeneration, limbic deficit, stem cell, transplantation.

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INTRODUCTION

The ocular surface contains three adjacent epithelia: conjunctiva, limbus, and cornea. Cornea shows many roles in protecting internal structures from germs and particles and in protecting against ultraviolet rays^{1,2}. It is the transparent part 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 fourth leading cause of blindness in the globe according to the

World Health Organization (WHO), responsible for 5.1% cases³. Various studies confirm the therapeutic success of amniotic membrane grafting associated or not with limbal stem cells⁴. This success is limited in time and often linked to later complications. Current study search about most effective transplanted cells. In current study, two types of stem cells were 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.

Figure 1: Limbal biopsy process.

(a): Before biopsy (b): Immediately after biopsy (c): 1 hour after biopsy.

MATERIALS AND METHODS

Drugs and chemicals

The following drugs were used in the studies: Midazolam (0.5 mg/kg), Propofol (5 mg/kg); 4% hydrogen chloride, antibiotic (penicillin 10,000 µg /ml, streptomycin 10,000 µg/ml) and antifungal agent 0.1 % Nystatin. was purchased from Ibn Sina pharmacy at Rabat-Morocco^{5,6}.

Animals

The study was performed on adult male rabbits (1.50–2.50 kg), bred at the laboratory of Pharmacology, Faculty of Medicine and Pharmacy of Rabat. All animals were kept in a room maintained under environmentally controlled conditions of 23°C and 12h light–12h dark cycle. The food was withdrawn on the day before the experiment, the animals were handled according to the prescribed ethical guidelines for laboratory animals^{7,8}. This study was conducted using the 17 Dutch rabbits as the experimental model, and one a pregnant female for amniotic membrane collection, four groups were prepared as below:

- 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 (amniotic cell÷ limbic cell) (D),

All rabbits were kept on an empty stomach for four hours. The sedation was performed with Midazolam (0.5 mg/kg intramuscularly), and anesthesia with Propofol (5 mg/kg intravenously) injected slowly to avoid the risk of apnea⁹.

Figure 2: Corneal opacity.

An eyelid retractor was placed, and the biopsy was performed in the limbic region of the left eye over an arc of about 70° to 80°C, 1 mm deep and 2 mm towards the cornea (Figure 1). A cotton swab immersed in 4% hydrogen chloride was applied, at the center of the cornea for 2 seconds, and washed immediately with

a saline solution to limit diffusion of caustic to the rest of the ocular surface (Figure 2). Each biopsy was transported in a sterile vial containing a culture medium (DMEM), after that transferred to an identified Nunc dish for the observation by electronic microscopic (Figure 3: a), then to a conical tube containing 1ml of trypsin solution at 0.25%. After 5 minutes of contact, each tube was washed with 3 ml of PBS to inhibit the enzymatic action. All tubes were centrifuged at 500 G for 5 minutes, and incubated the recovered pellets in identified Petri dishes at 5% CO₂ and 37°C⁸. Amniotic membranes were extracted from a pregnant rabbit and transported in a sterile vial containing culture medium (DMEM). A microscopic examination was performed by emptying the vial into an identified Nunc box (Figure 3b). The contents were transferred to a large Petri dish for cutting and cleaning from the conjunctive tissue. Washing was performed in a conical tube containing a PBS solution. The tube was centrifuged twice in PBS to clean cells at 500 G for 5 minutes, and the pellet was cultivated in a petri dish under the same conditions as mentioned above⁹. The culture medium used contains a base of DMEM/F12 with fetal calf serum 10 %, glutamine 2mM, non-essential amino acids 0.1M and antibiotic (penicillin 10,000 u/ml, streptomycin 10,000 µg/ml) and antifungal agent 0.1 % Nystatin¹⁰. During the proliferation process, the culture medium was changed every two days and performed trypsinization and passage at the level of 70% cell confluence. The proliferation was stopped after two passages. the proliferated stem cells were filled into two tubes per individual, each containing a volume of 500 ml (10⁶ cells/ml), the first tube intended for transplantation and the second tube intended for cryopreservation, in which an equal volume of a freezing solution (80% DMSO + 20% FBS) was added. The tubes were left at - 80°C for one night before transferring them into liquid nitrogen¹². The rabbit candidates were kept for transplantation on a fast stomach for four hours before the anesthesia. After applying the eyelid retractor, a small incision was made in the cornea through which passed a curved knife to separate the damaged epithelium from the stroma. Under this epithelium, the cell content was introduced for grafting using a syringe equipped with a fine, flexible catheter. The four test groups were treated with local treatment (antibiotic and anti-inflammatory) for 15 days, and the postoperative monitoring was extended for up to 2 months. To explore the return of vision, two functional tests were adopted: the light reaction test and the labyrinth test in search of food after covering the right eye.

Figure 3: a-b . a microscopic examination for limbal and amniotic biopsy.

RESULTS

Microscopic examination of the samples showed that the amniotic membranes were in perfect condition and that limbic cells were present in all biopsies. The caustic induced an immediate opacity of the cornea, visible with Trypan blue (Figure 2). 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 (Group B). 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 (Group D), 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 (Group A) (Figure 4).

Figure 4: Anatomical evolution of the ocular surface for the four study groups.

DISCUSSION

All groups tested developed a corneal ulcer (Figure 2). Without stem cell transplantation, the ulcer was complicated by a severe limbic deficit and unilateral blindness (Group A)⁵. 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 metalloproteinase)^{6,7,8}. 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- β pathway and pro-collagenases^{6,7,8-10}. This hyperactivity leads to the fusion of the stroma²¹. 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}. 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 contain 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 consist the 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 colonization³⁷; and anti-angiogenic properties that reduce neovascularization and the invasion of conjunctival tissue (Pterygion)^{8,24,37,38}. Amniotic epithelial cells produce anti-inflammatory cytokines such as IL-1Ra and IL-10 that block the inflammatory cascade and inhibit metalloproteinases³⁶⁻³⁹. Finally, these cells have the advantage of not expressing histocompatibility antigens and therefore do not cause a rejection reaction³⁸. Amniotic epithelial and mesenchymal cells can synthesize thrombospondin-1, endostatin, and metalloproteinase tissue inhibitors (TIMPs)^{15-18,39}. Simultaneous amniotic cell transplantation has shown that it can be an important complement to auto or limbal allograft techniques^{31,32,40}. It was discovered that 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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AUTHOR'S CONTRIBUTION

The manuscript was carried out, written, and approved in collaboration with all authors.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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