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Original Article

Clinical and Histopathological Study of the Effect of Adipose-Derived Mesenchymal Stem Cells on Corneal Neovascularization following Alkali Burn in a Rabbit Model

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Abstract

The cornea, the transparent part of the eye, performs a significant function in eyesight by refracting the light to focus a visual image. Since the cornea is indispensable for vision, corneal inflammation may induce visual disturbance and blindness. Several investigations have reported that various corneal inflammatory diseases cause visual impairment and chronic inflammation of the cornea, which can lead to blindness. The present study aimed to assess the effect of adipose-derived mesenchymal stem cells (ADMSCs) on corneal healing after alkali injuries. Corneal alkali injuries were induced in the eyes of 20 rabbits. The MSC group (n=10) was treated with subconjunctival injections, while the control group (n=10) was left without any treatment. Rabbits underwent slit-lamp examination and photography and were evaluated for corneal neovascularization. Based on the histological evaluation, the eyes treated with MSCs showed better recovery. Furthermore, the MSC and control groups were significantly different in the degree of corneal neovascularization and re-epithelialization, as well as the elevation of the neovascular tissue at two and four weeks post-surgery.

Keywords: AD-MSCs, Alkali burn, Corneal neovascularization, Rabbit model

1. Introduction

The cornea, the transparent part of the eye, performs a significant function in eyesight by refracting the light to focus a visual image. Since the cornea is indispensable for vision, corneal inflammation may induce visual disturbance and blindness. Several investigations have reported that various corneal inflammatory diseases cause visual impairment and chronic inflammation of the cornea, which can lead to blindness (1). As an avascular tissue, the cornea is an immunologically privileged structure (2) and owes its clarity to its unique arrangement of stromal collagen fibrils and lack of vascularization, at least centrally. Corneal transparency can be maintained through a dynamic balance between pro-and antiangiogenic mechanisms (3). Corneal neovascularization (NV) constitutes a defense mechanism against harmful agents (4).

Ocular alkali burn is a common trauma worldwide, particularly in developing countries. Caustic agents often cause extensive corneal damage, resulting in permanent visual impairment (5). Clinically, except for recurrent epithelial erosions, corneal ulceration, and severe stromal inflammation, secondary corneal neovascularization (NV) is one of the most sight-threatening conditions (6). The cornea is an avascular and transparent tissue that acts as the refractive surface of the eye (7). After alkali burn, a large number of leukocytes and macrophages infiltrate the cornea (8). Moreover, the levels of numerous cytokines, such as the inflammatory factors IL-1β, IL-6, and vascular

endothelial growth factor A (VEGF-A), are dramatically elevated in the alkali-burned cornea (9). The disruption of the balance between proangiogenic and antiangiogenic molecules will lead to **corneal** neovascularization (10).

Mesenchymal stem cells (MSCs) are a type of multipotent cell originally isolated from bone marrow that has subsequently been isolated from other tissues, such as adipose tissue (11), heart tissue (12), cord blood (13) and oral tissue. Recently, an increasing body of evidence has indicated that MSCs possess multifunctional properties from tissue repair/regeneration to immunomodulatory/ anti-inflammatory functions (14). More recently, MSCs have been studied for the treatment of corneal chemical burns with encouraging results (15). In light of the aforementioned issues, the present study aimed to investigate the effects of subconjunctival administration of MSCs on the corneal healing acute phase in a rabbit after alkali burn.

2. Materials and Methods

2.1. Isolation and Control of Adipose Tissue-Derived Mesenchymal Cells

The MSCs were isolated from rabbit adipose tissue as previously described (16). In brief, animals were anesthetized by intramuscular administration of Ketamine/ Xylazine, a solution with intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) (17). The inguinal fat pad was obtained and washed with Phosphate-buffered saline (PBS). Thereafter, it was minced and digested with collagenase type-1 at 37°C for 1 h with constant shaking. An equal volume of PBS was added and the mixture was incubated for 15 min at room temperature until the formation of three distinct layers. The middle layer containing MSCs was aspirated and centrifuged for 10 min at 600×g. The cellular pellet was re-suspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (penicillin (100 IU/ml), and streptomycin (100 µg/ml). It was then cultured at 37°C in a humidified incubator containing 5% CO_2.

2.2. Animal Model

A total of 20 New Zealand rabbits were included in the study (n=10 eyes). Each rabbit was approximately 2:5 kg in weight and 10 months old. The rabbits were kept in a room with a standard 12-hour light-dark cycle. The study was approved by the local Ethics Committee of the Baghdad University and the Committee of the Department of Veterinary Medicine of Baghdad. The rabbits were randomly assigned to two groups. Group 1 was the MSC group (n=10), and group 2 was the control group (n=10). All the rabbits in each group were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5mg/kg), while proparacaine hydrochloride 0.5% was applied topically. Povidone-iodine 5% was instilled into the conjunctival sac. Alkali injuries were created with the application of a 3-mm diameter Whatman Filter Paper, soaked in 1 N NaOH, on the upper cornea for 30 s, followed by a rinse with a balanced salt solution for 1 min. Subsequently, all animals received a solution of dexamethasone 0.1%, and tobramycin 0.3% was instilled three times daily for the first week. Immediately after the creation of the alkali injury and the application of eye drops, 2×10^6 MSCs in 0.5 ml PBS were injected into the subconjunctival in the injured eyes. In the control group, the subjects were left without any treatment.

2.3. Clinical Evaluation

The eyes of the animals were evaluated for neovascularization and opacification. Each rabbit underwent slit-lamp examination. Photos were taken in a follow-up, starting after the creation of the alkali injury, as well as at postoperative weeks 2 and 4.

2.4. Histology

Eye tissue samples were fixed in 10% formalin and embedded in paraffin. Eye sections (2.5 µm) were stained with hematoxylin and eosin (H&E) for morphological evaluation and connective tissue staining. All samples were evaluated by a pathologist.

2.5. Statistical Analysis

Data were analyzed in SAS software (version 9.1). Two-way ANOVA and the least significant difference (LSD) post hoc test were performed to assess significant differences among means. A *P*-value≤0.05 was considered statically significant.

3. Results

3.1. Effect of ADMSCs on Corneal Neovascularization

Representative images demonstrated that the area of NV markedly increased over time in the control group, while less NV was observed in the ADMSC group. Neovascularization occurred in the corneas of both control and ADMSC groups. Despite this, the corneal surface was covered with neovascular tissue in the ADMSC and control groups, as evidenced by photographs taken two weeks after corneal alkali burn for the control and ADMSC- group in both groups (acute and chronic). This demonstrated that new vessels appeared fine, dense, and emerged from the adjacent limbus. The majority of vessels were grown around the limbus toward the injured corneal area at this time (Figures 1 and 2, taken at four weeks). After corneal alkali burn for control and ADMSC groups in both groups (acute and chronic), the neovascularization was increased in both control groups, and neovascular tissue disappeared in the ADMSC group, compared to the control group (Figures 3 and 4).

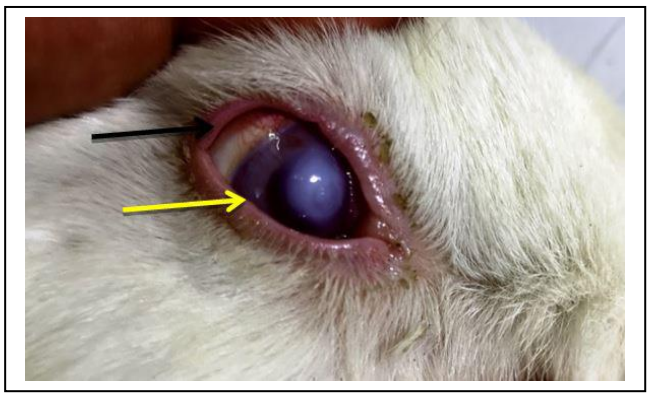

Figure 1. Morphological appearance of the acutely injured cornea at two weeks post alkali burn, demonstrating conjunctival and perilimbal neovascularization (black arrow) with grade 2 moderate opacity (yellow arrow)

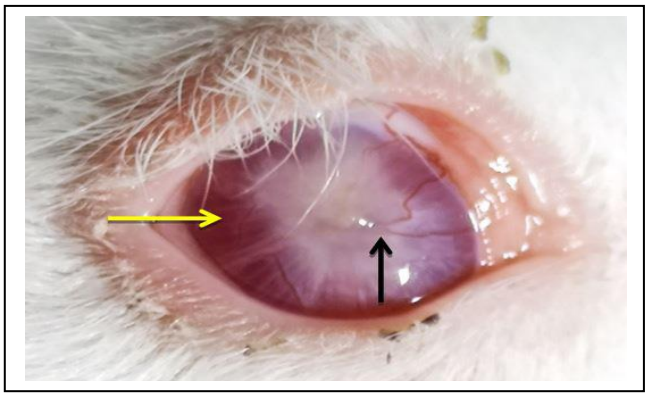

Figure 3. Morphological appearance of the injured cornea at four weeks post-Alkali, demonstrating moderate corneal neovascularization(black arrow) with grade 2 moderate opacity (yellow arrow)

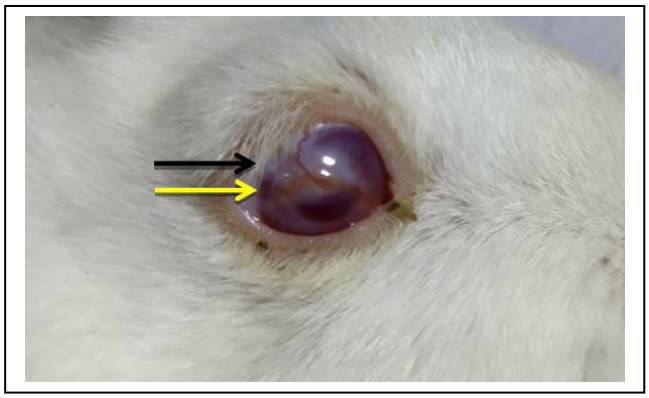

Figure 2. Morphological appearance of the acutely injured cornea at two weeks post-treatment, illustrating severe corneal neovascularization(black arrow) with grade 2 moderate opacity (yellow arrow)

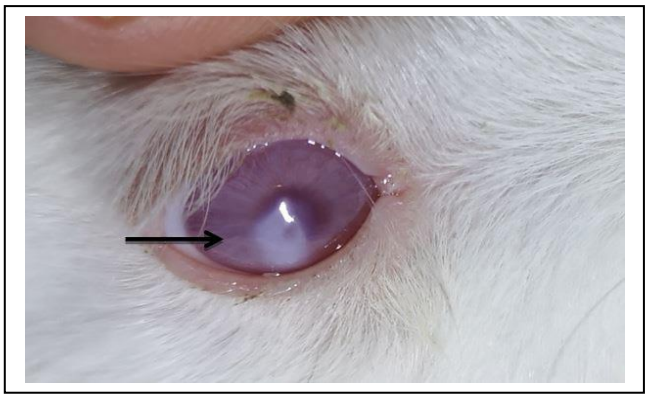

Figure 4. Morphological appearance of the acutely injured cornea at four weeks post-treatment, illustrating the mild corneal opacity grade 1 (arrow)

3.2. Histopathologically

Figure 5 depicts the distinct manifestation characterized by marked irregular thinning of corneal epithelium with long fingerlike projection, accompanied by a focal increase of basal epithelium, a cellular fibrotic deposition, and severe disruption of corneal stromal structure mainly in the interior portion with evidence of cysts formation and corneal edema. As illustrated in figure 6, the corneal epithelium appeared wrinkled and thin with goblet cells. Formation (bubbles) can be observed, as well as complete loss of basal epithelium and bowman layer. Marked stromal edema with areas of necrotic stromal is recognized anteriorly.

Based on figures 7 and 8, corneal observation illustrated mild irregular epithelialization with a lack of basal epithelial and absence of bowman layer sitting on highly vascular collagenous stroma with mixed cellular infiltration and sparsely distributed keratocyte. Figure 5 depicted the corneal surface linning with a multilayer of epithelium and prominence basal epithelium, as well as a bowman layer (similar to normal) and regular anterior stromal containing numerous keratocytes with minimal vessels.

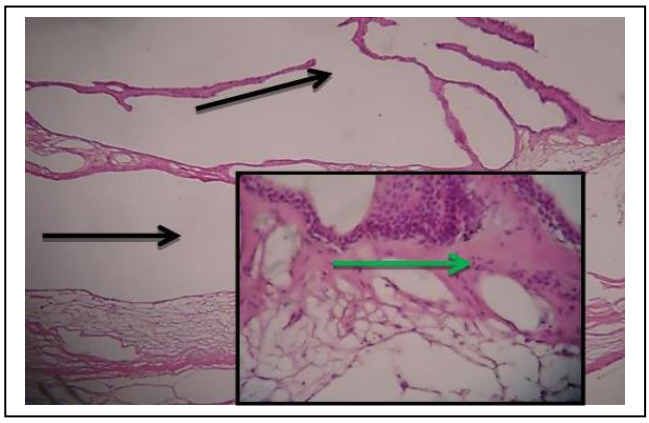

Figure 5. Histological section of rabbit cornea at two weeks post-injury (acute control group), displaying a marked disruption of both corneal epithelium and stroma with moderate stromal edema (black arrow), focal epithelial hyperplasia, and subepithelium fibrosis deposition (green arrow) (H and E X10, 40)

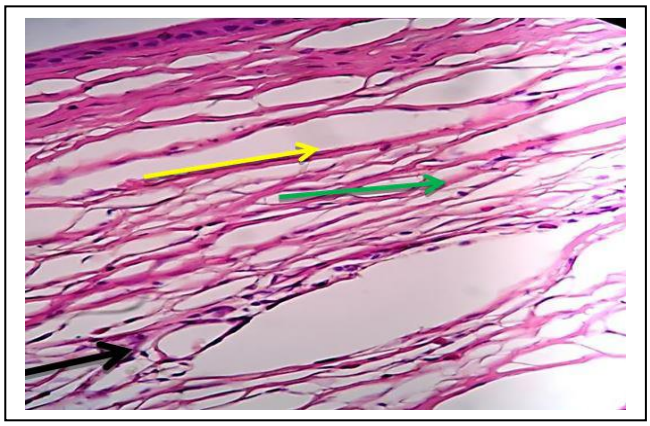

Figure 6. Histological section of rabbit cornea at two weeks post-injury (acute control group), demonstrating a mild stromal vascularization (black arrow), mild inflammatory infiltration (yellow arrow), and the irregularity of collagen bundles (green arrow) (H and E X40)

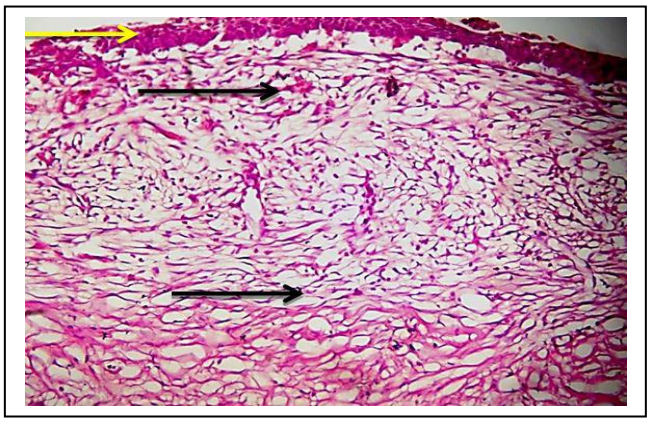

Figure 7. Histological section of rabbit cornea at two weeks post-MSCs implantation(acute treated group), illustrating mild irregular epithelialization (yellow arrow) and vascular collagen stromal with mixed cellular infiltration (plasma cells and lymphocyte) (black arrow) (H and E X10)

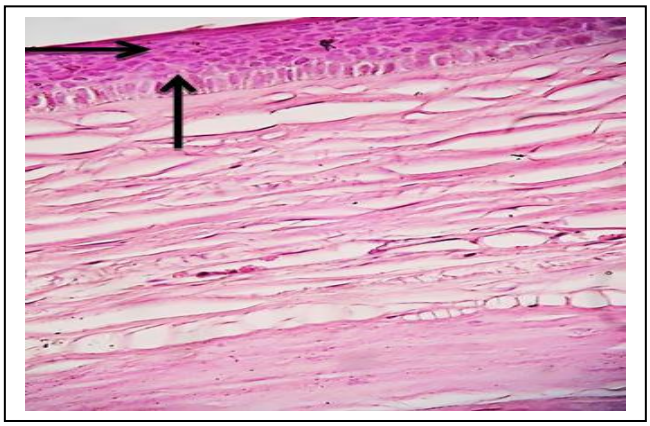

Figure 8. Histological section of rabbit cornea at four weeks post-MSCs implantation (acute treated group), illustrating corneal surface lining with multilayer epithelium with a prominence of basal epithelium and bowman layers (black arrow) (H and E X40)

4. Discussion

The MSCs have a lot of pros, making them a promising therapeutic tool for corneal reconstruction. Firstly, they are multipotent progenitor cells that can be found in many tissues, such as bone marrow (18), adipose tissue, peripheral blood (19), as well as the limbal stroma of the human eye (20), and they can be easily harvested (21). It has been reported that MSCs can be self-renewed as undifferentiated cells and then differentiate into different types of mesenchymal tissues (22). Most importantly, MSCs can escape the monitoring of the immune system and infuse into an allogeneic host without being rejected (23).

The results of the present study indicated that allogeneic MSCs led to a more significant reduction in corneal NV when applied in a rabbit model of corneal injury. This finding is in agreement with a previous study conducted by Pirounides, Komnenou (24), who demonstrated that MSC applied by three different routes effectively inhibited the formation of new vessels when administered immediately after incision in the rabbit. Different routes of administration have been used for MSC transplantation, such as intrastromal injection (25), subconjunctival administration (26), and systemically (6), with nanofiber scaffolds (27).

In accordance with the results of the current study, Ghazaryan, Zhang (28) suggested that subconjunctival injection overcomes the hurdles of amniotic membrane-associated MSC transplantation and reduces VEGF levels, resulting in better outcomes. We evaluated the therapeutic efficacy of MSCs by sub-conjunctival administration of $2x10^6$ MSCs in 0.5ml PBS, an image that was taken after two and four weeks. Post-injury, the neo-vascularized area was lower in the MSC than that in the control group. The MSC group displayed a better recovery in their corneas, as compared to the control group in a follow-up of 28 postoperative days. Mesenchymal stem/stromal cells secrete soluble molecules that inhibit angiogenesis and reduce the levels of VEGF (15), via a paracrine signaling process, with a concomitant upregulation of TNF-stimulated gene-6 (TSG-6) (29). Several studies investigating vascular and arterial diseases have pointed out that MSC paracrine activity may be mediated through exosome secretion (30).

Authors' Contribution

Study concept and design: H. H. A.
Analysis and interpretation of data: H. H. A.
Drafting of the manuscript: A. H. F. A.
Critical revision of the manuscript for important intellectual content: A. H. F. A.
Statistical analysis: A. H. F. A.
Administrative, technical, and material support: A. H. F. A.

Ethics

All the procedures in this study were evaluated by the Ethics committee at the College of Veterinary Medicine, University of Baghdad, Iraq. During the experiment, the inspector of the committee checked the procedure.

Conflict of Interest

The authors declare that they have no conflict of interest.

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