Original Article

Comparative Histological Study on the Effect of Early and Late Administration of Adipose Derived Stem Cells on Corneal Alkali Burn in Adult Male Albino Rats

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ABSTRACT

Introduction: Corneal alkali burns cause corneal infection, ulceration, perforation, neo-vascularization (NV) and opacification resulting in permanent visual impairment.

Aim of the work: This study was aimed at comparing the outcome of early and late administration of adipose derived stem cells on corneal alkali burn.

Materials and methods: Twenty eight adult male albino rats (12 weeks old), 180-200 grams body weight, in addition to ten rats used as a source for ADSCs. Rats were divided into 4 groups. Group I (control); group II (corneal alkali burn): subjected to corneal alkali burn injury using NaOH. Group III (early administration of stem cells): the injury was performed as in group II then the rats were received an injection of adipose derived stem cells (ADSCs) one hour after injury. Group IV (late administration of stem cells): rats were subjected to corneal alkali burn then were given ADSCs 3 weeks after injury. Rats of group III and IV were sacrificed 3 and 6 weeks post injury respectively. Corneal sections were stained with hematoxylin & eosin, masson's trichrome and immunohistochemichal stains for anti-vascular endothelial growth factor (VEGF) and anti-Caspase 3. Morphometric and statistical studies were done.

Results: In alkali burn group, abnormal architecture of the corneal tissue with complete loss of part of the epithelium, widely separated collagenous bundles and congested blood vessels were observed. However, ADSCs therapy resulted in regeneration of corneal epithelium and reduction of apoptotic cells. In addition, less separation of collagen bundles and disappearance of neovascularization were more pronounced in early ADSCs treatment. These results were confirmed morphometrically, in comparison to alkali burn group.

Conclusion: Both early and late treatment with ADSCs had effective role in corneal repair. However, early treatment with ADSCs had better effect than late treatment on corneal wound healing.

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Key Words: ADSCs; alkali burn; caspase3; corneal injury; VEGF.

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INTRODUCTION

Corneal damage can result from many clinical disorders, including a chemical, mechanical or thermal injury. In severe injury, it may cause loss of vision. The prognosis of corneal injury in many cases is determined by the degree of lack of limbal epithelial stem cells (LSCs)[1]. Therefore, stem cell transplantation is a new therapeutic way for corneal tissue regeneration that relies on their multipotency. In terms of stem cell therapy, it was demonstrated that both intra venous (I.V) and intraperitoneal (I.P) administration of human mesenchymal stem cells (hMSCs) significantly decrease inflammation in the cornea. Also, it was found that the anti-inflammatory and anti-fibrotic, tumor necrosis factor-α stimulated gene (TSG-6) was considered as therapeutic factor that hMSCs secrete in response to corneal epithelial cells injury[2].

Subcutaneous adipose derived stem cells has many advantages over other stem cell sources, such as easy accessibility and easy isolation[3]. Additionally, many researchers suggested that the adipose derived stem cells are less immunogenic and immunosuppressive[4].

Therefore, this work aimed at comparing the outcome of early and late administration of adipose derived stem cells on corneal wound healing following chemical injury in male albino rats.

MATERIALS AND METHODS

Animals

Twenty eight adult male albino rats (12 weeks old), and 180-200 grams body weight were used in this experiment, in addition to ten rats used as a source for ADSCs. The

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rats were bred at the animal house of Faculty of Medicine, Cairo University. They were housed in metal cages with mesh and wood chips for bedding, fed with standard laboratory chow and had free access to tap water. This study was conducted according to the guidelines for the care and use of laboratory animals and was approved by the ethics committee in faculty of medicine, Cairo University.

Experimental Design

The rats were divided into four groups

Control Group (Group I): eight rats were deeply anesthetized by an intraperitoneal (i.p.) injection of 50 mg/kg tiletamine plus zolazepam (Zoletil; Virbac, Carros, France) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer, Leuverkeusen, Germany)[5].

Control Group was subdivided into two subgroups, four rats each

Group Ia: rats' corneas were covered by round filter paper, which had been rinsed by saline then covering the rat corneal surface for 45 seconds (Corresponding to Group II).

Group Ib: included rats that were injected with 1 ml Phosphate Buffered Saline (PBS) solution via the rat tail vein (corresponding to Group III & Group IV).

Control animals were sacrificed with the corresponding experimental groups.

Alkali Burn Group (Group II): included eight rats that were deeply anesthetized as control group. Rats' corneas were covered by round filter paper which had been rinsed by (1N) NaOH then covering the rat corneal surface for 45 seconds. Then the wound surface was washed with 0.9% physiological saline. Filter paper covered 100% of the corneal area (7 mm in diameter). NaOH (1N): one mole (= 4 g) of purified NaOH dissolved in one liter of water. It was used to induce chemical corneal injury (alkali burn)[5].

Alkali Burn Group was subdivided into two subgroups, four rats each

Group IIa: rats that were sacrificed 3 weeks post injury.

Group IIb: rats that were sacrificed 6 weeks post injury.

Early Treatment with Stem Cells Group (Group III)six rats were injured as in group II then they received an injection of one million adipose derived stem cells (ADSCs) suspended in 1 ml PBS via the rat tail vein one hour after injury. Four rats were sacrificed 3weeks post injury[6]. (Ye *et al.*, 2006)

Late Treatment with Stem Cells Group (GIV): six rats were injured as in group II then they received an injection of one million ADSCs in 1 ml PBS via the rat tail vein 3weeks after injury. Four rats were sacrificed 3 weeks post injury.

Two rats from each group (Group III & GIV) were sacrificed 3 days after injection with ADSCs to detect homing of the cells.

I- Preparation of adipose-derived stem cells from rats

a) Isolation and culture of ADSCs from rats

Subcutaneous white adipose tissue was excised from the inguinal pad of fat in rat under complete aseptic condition. The adipose tissue was resected and placed into a labeled sterile tube containing 15 ml of a phosphate buffered solution (PBS; Gibco/ Invitrogen, Grand Island, New York, USA). Enzymatic digestion was performed using 0.075% collagenase II (Serva Electrophoresis GmbH, Mannheim) in Hank's Balanced Salt Solution for 60 minutes at 37^{o}c with gentle shaking. Digested tissue was filtered and centrifuged, and erythrocytes were removed by treatment with erythrocyte lysis buffer. The cells were transferred to tissue culture flasks with Dulbecco Modified Eagle Medium (DMEM, Gibco/ BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco/BRL) and, after an attachment period of 24 hours, non-adherent cells were removed by a PBS wash. Attached cells were cultured in DMEM media supplemented with 10% fetal bovine serum FBS, 1% penicillin-streptomycin (Gibco/ BRL), and 1.25 mg/L amphotericin B (Gibco/ BRL), and expanded in vitro. At 80-90% confluence, cultures were washed twice with PBS and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (Gibco/ BRL) for 5 min at 37^{o}c. After centrifugation, cells were resuspended with serum- supplemented medium and incubated in 50 cm^2 culture flask (Falcon). The resulting cultures were referred to as first-passage cultures and expanded in vitro until passage four[7].

b) Characterization of the cultured cells

ADSCs in culture were identified by their adhesiveness, fusiform shape and by surface marker for ADSCs characterization like CD45& CD29 (eBioscience, San Diego, CA, USA) that was detected by Flow cytometry analyses of the cultured cells on flow cytometer (BD Biosciences).

c) Labeling of ADSCs with PKH26 dye

ADSCs were harvested during the 4th passage and were labeled with PKH26 fluorescent linker dye.

PKH26 is a red fluorochrome. It has excitation (551 nm) and emission (567 nm). The linkers are physiologically stable and show little to no toxic side-effects on cell systems. Labeled cells retain both biological and proliferating activity, and are ideal for in vitro cell labeling, in vitro proliferation studies and long term, in vivo cell tracking.

The dye itself is stable and will divide equally when the cells divide. After staining with PKH dyes, it is possible to observe as many as 8 divisions depending on how brightly

the cells were stained initially and the amount of surface area on the cells. Most commonly 4-6 divisions can be visualized[8].

ADSCs were labeled with PKH26 according to the manufacturer's recommendations (Sigma, Saint Louis, Missouri, USA). Cells were injected via rat tail vein. Rat's corneas were examined with a fluorescence microscope to detect ADSCs stained with PKH26

d) Administration of ADSCs

One million units of PKH26 labeled ADSCs were loaded in a 1 ml volume sterile syringe and were injected via the tail vein of rat[6] .

e) Detection of injected ADSCs in rat corneal tissue

Unstained sections were examined 3 days after injection with ADSCs by a fluorescent microscope to detect the cells stained with PKH26 dye to ensure homing and to trace the injected cells in the corneal tissue. The fluorescent microscope model is Olympus B x 50 F4 Optical Co – Ltd, made in Japan (No. 7M03285).

II- Histological Study

All animals of the four groups were sacrificed according to their scheduled time in the animal house of faculty of medicine, Cairo University being anesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg tiletamine plus zolazepam and 15 mg/kg xylazine hydrochloride. The eye ball of both eyes were fixed in (10%) buffered formalin, then specimens were dehydrated in ascending grades of alcohol, cleared in xylene then embedded in paraffin. Paraffin blocks were cut at 5-6 µm thickness. Serial sections from the central part of the cornea were subjected to the following techniques:

a. Hematoxylin and eosin stain for histological evaluation.

b. Masson's trichrome stain for connective tissue staining.

c. Immunohistochemical staining for:

    1. Vascular endothelial growth factor (VEGF) (marker for neovascularization), using the avidin–biotin peroxidase complex technique.

    2. Caspase-3 (marker for cell apoptosis).

Negative sections were processed in the previously mentioned sequence but without adding the primary antibody and instead, PBS was added in this step (Figure A).

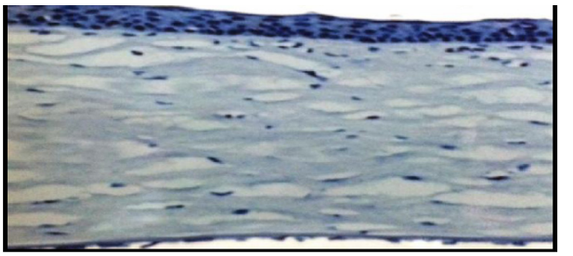

Fig. A: A photomicrograph of control section of an adult male albino rat treated with non-immune serum showing –ve immunostaining (x200).

III- Morpthometric study

Data were obtained using "Leica Qwin 500 C" image analyzer computer system Lt, in Histology Department, Faculty of Medicine, Cairo University. The following parameters were measured:

a. Mean area % of collagen in Masson's trichrome stained sections.

b. Mean number of VEGF immunoreactive cells.

c. Mean number of Caspase 3 immunoreactive cells.

All measures were taken in 10 non overlapping fields from central part of cornea in each section of all groups with magnification x400.

IV- Statistical Analysis

Quantitative morphometric studies using one-way analysis-of-variance (ANOVA). *P-values*<0.05 was considered statistically significant. Post-hoc test was used for pair wise comparisons. Calculations were made on SPSS software version 9 to compare between different groups.

RESULTS

ADSCs culture, identification & homing

Isolated and cultured undifferentiated ADSCs reached 70-80% confluence at 15 days (Figure1 A, B&C).

In addition, ADSCs were identified by surface marker CD29 (+ve), and CD105 (+ve) detected by flow cytometry (Figure2 A&B).

Three days post administration of ADSCs corneal tissue was examined with the fluorescent microscope. ADSCs labeled with PKH26 fluorescent dye were detected in the corneal tissues confirming that these cells homed into the corneal tissue (Figure 3).

Hematoxylin &Eosin - stained sections

Corneal sections from the central part of the cornea of control subgroups (group Ia & group Ib) revealed the normal histological picture of the cornea with non-keratinized stratified squamous epithelium, straight basement membrane and Bowman's layer. Substantia propria formed the main bulk of the cornea with its regular arrangement of collagenous bundles. Descemet's membrane and endothelium were seen lining the cornea from inside (Figures 4A and 5A). Alkali burn group (group IIa) showed complete loss of part of the epithelium and most of the remaining epithelial cells showed dark, shrunken nuclei and vacuolated cytoplasm. The collagen bundles lost their regular parallel arrangement and were widely separated (Figures 4B and 5B). In group IIb, abnormal architecture of the corneal tissue with reduced corneal thickness was noticed. This group also revealed complete loss of part of epithelium in some areas. Additionally, epithelial vacuolations were seen and some epithelial cells exhibited dark nuclei. The substantia propria appeared with thin collagen bundles in the upper half and compacted bundles in lower half. Extravasation of RBCs, congested blood vessels and inflammatory cellular infilteration could also be detected in the corneal stroma. The Descemet's membrane was separated from the corneal stroma and the endothelium. The endothelial layer showed vacuolated cytoplasm (Figures 4C and 5C). Group III revealed restoration of the corneal epithelium with straight basement membrane but some cells were still having vacuolated cytoplasm with darkly stained nuclei. Additionally, regular arrangement of collagen bundles was regained although in some areas it was still separated. Descemet's membrane and endothelium were intact (Figures 4D and 5D). Corneal sections of Group IV also showed restoration of the corneal epithelium with straight basement membrane in most parts of the cornea. However, many cells with dark nuclei and vacuolated cytoplasm were still seen. Separation of collagen bundles also was still detected. Additionally, this group didn't show inflammatory cellular infiltration. Descemet's membrane and endothelium were intact in most corneal parts (Figures 4E and 5E).

Fluorescence detection of PKH26 labeled adipose-derived stem cells (ADSCs) by fluorescent

microscope in unstained corneal sections:

PKH26 labeled ADSCs appeared as bright fluorescent dots that was heavily distributed in the corneal tissue 3 days post injection (Figure 6A &C). However, few PKH26 labeled ADSCs were seen in the corneal tissue 3 weeks and 6 weeks post injection in groups III and IV (Figure 6B &D).

Masson's trichrome – stained sections

Sections from the central part of the cornea of Group I showed corneal stroma formed of regular collagen bundles and continuous Bowman's layer and Descemet's membrane (Figure 7A). On the other hand, separation of faintly stained collagen bundles and Descemet's membrane were illustrated in group IIa (Figure 7B). Additionally, group IIb showed more advanced affection in the form of loss of collagen bundles from the upper half of substantia propria, irregular Bowman's layer and continuous Descemet's membrane (Figure 7C). However, corneal sections of Group III and Group IV revealed stroma with regular collagen bundles and continuous Bowman's layer (Figures 7 D&E).

Immunohistochemical results

Examination of VEGF immuno-stained sections from the central part of the cornea of Group I showed negative immunoreactivity within corneal tissues (Figure 8A). However, in group IIa, positive cytoplasmic reaction within epithelial cells, endothelial cells lining the blood vessels, keratocytes and corneal endothelial cells was detected (Figure 8B). group IIb revealed positive cytoplasmic reaction within keratocytes (Figure 8C). Corneal sections of Group III showed immunoreactivity in few keratocytes and corneal endothelial cells (Figure 8D). As regards, Group IV positive immunoreactivity within the cytoplasm of epithelial cells, endothelial cells of few blood vessels and keratocytes was detected (Figure 8E).

Caspase-3 immuno-stained sections from the central part of the cornea exhibited negative immunoreactivity within corneal tissues in Group I (Figure 9A). On the other hand, group IIa recorded positive immunoreactivity within the cytoplasm of many epithelial cells, keratocytes and corneal endothelial cells (Figsure 9B). However, positive immunoreactivity were detected within the cytoplasm of many epithelial cells and keratocytes in group IIb (Figure 9C). Group III showed immunoreactivity within the cytoplasm of few keratocytes (Figure 9D). Regarding Group IV, positive immunoreactivity for Caspase-3 immuno-stained sections was recorded within the cytoplasm of few epithelial cells, keratocyes and corneal endothelial cells (Figure 9E).

Morphometric & Statistical results

The mean area % of collagen in Masson's trichrome stained sections of GIII & GIV showed significant increase when compared to gIIa & gIIb for each of the corresponding durations. There was no significant difference in the mean area % of collagen in Masson's trichrome stained sections of groups III and IV (Table 1).

The mean number of VEGF and Caspase-3 immunoreactive cells in epithelium and corneal stroma of GIII & GIV was significantly decreased when compared to gIIa & gIIb for each of the corresponding durations. There was significant decrease in the mean number of VEGF immunoreactive cells in GIII versus GIV (Table 1).

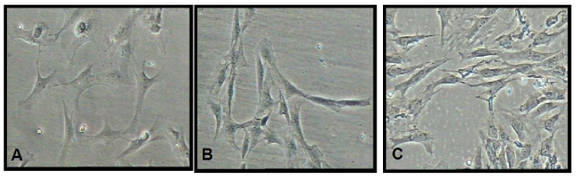

Fig. 1: Morphology of adipose-derived stem cell (ADSC). The phenotype of adipose-derived stem cell (ADSC) were observed, A (ADSC At the 3rd day of the culture), B (ADSC At the 10th day of the culture) cells showed heterogeneous morphology with various shapes; flat, star, sperm, triangular and elongated with long cytoplasmic processes and C (ADSC At the 15th day of the culture) cells became relatively homogeneous showing a similar morphology with abundant cytoplasm and had large nuclei with multiple nucleoli. The fibroblast-like appearance was clearly seen that photographed using phase-contrast microscopy (x100).

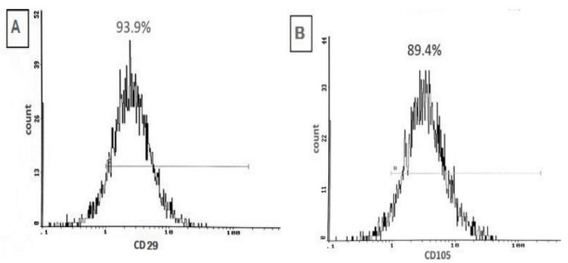

Fig. 2: Identification of surface markers on adipose-derived stem cells (ADSCs).The surface markers including CD29 (A), and CD105 (B), were analyzed by flow cytometry .The expression levels of CD29+ve (A) & CD105 + ve (B) of ADSCs are presented as a histogram. The percentage of expression of the indicated markers was defined in the figure.

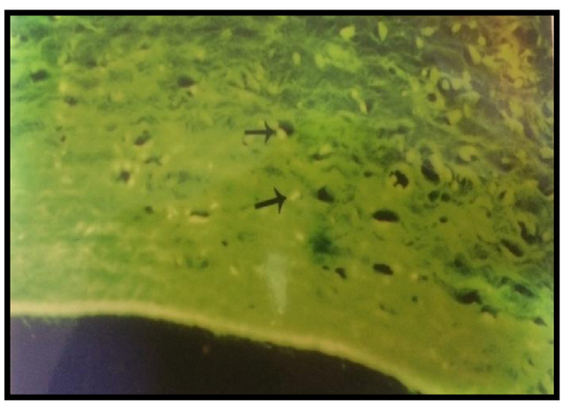

Fig. 3: Homing of injected ADSCs in rat corneal tissue stained with PKH26 dye (arrows) by a fluorescent microscope (Three days post injection of ADSCs GIII X 200).

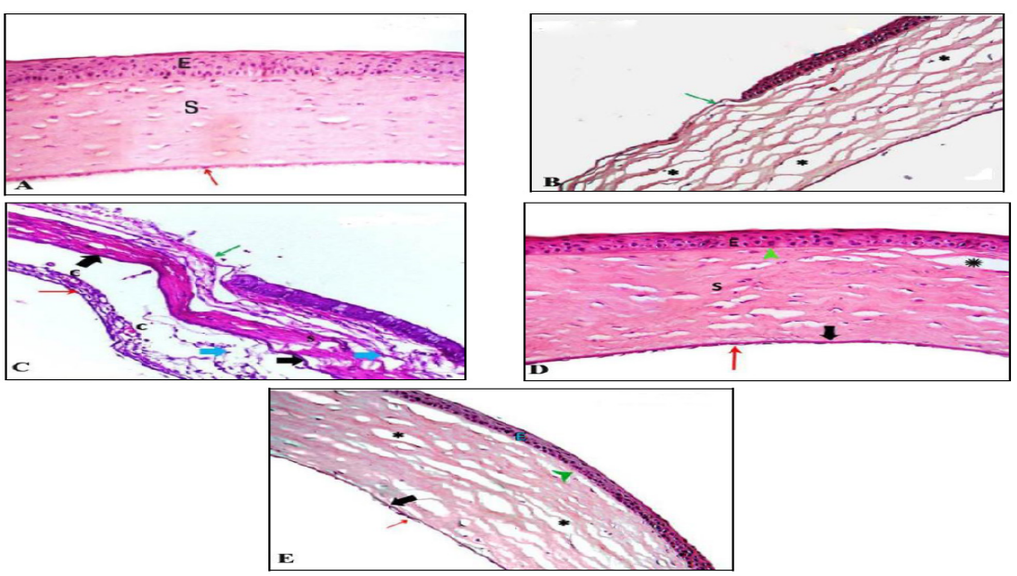

Fig. 4: Photomicrograph of sections from the central part of the cornea of (A): Group I showing normal histological structure of cornea, epithelium (E), substantia propria (S) that forms the main bulk of the cornea and the endothelium (red arrow). (B): group IIa showing complete loss of part of epithelium (green arrow). The widely separated collagen bundles (astrix) can be noted. (C): group IIb showing reduced corneal thickness with abnormal architecture. There is complete loss of part of epithelium (green arrow). Note the appearance of congested blood vessels (C) and inflammatory cells (blue arrows) in the stroma. Descemet's membrane (black arrows) appears separated from the stroma (S) and endothelium. The cells of endothelial layer are vacuolated (red arrow). (D): Group III showing restoration of the epithelium (E) resting on a straight basement membrane (arrow head). Collagen bundles of substantia propria (S) appear regular although some are still separated (astrix). Descemet's membrane (black arrow) and endothelium (red arrow) are intact. (E): Group IV showing restoration of the epithelium (E) with straight basement membrane (arrow head) in most parts of the cornea. The substantia propria still shows separation of collagen bundles (astrix). Note the absence of inflammatory cellular infiltration. Descemet's membrane (black arrow) and endothelial cells are intact in most corneal parts (red arrow). H&E (x200)

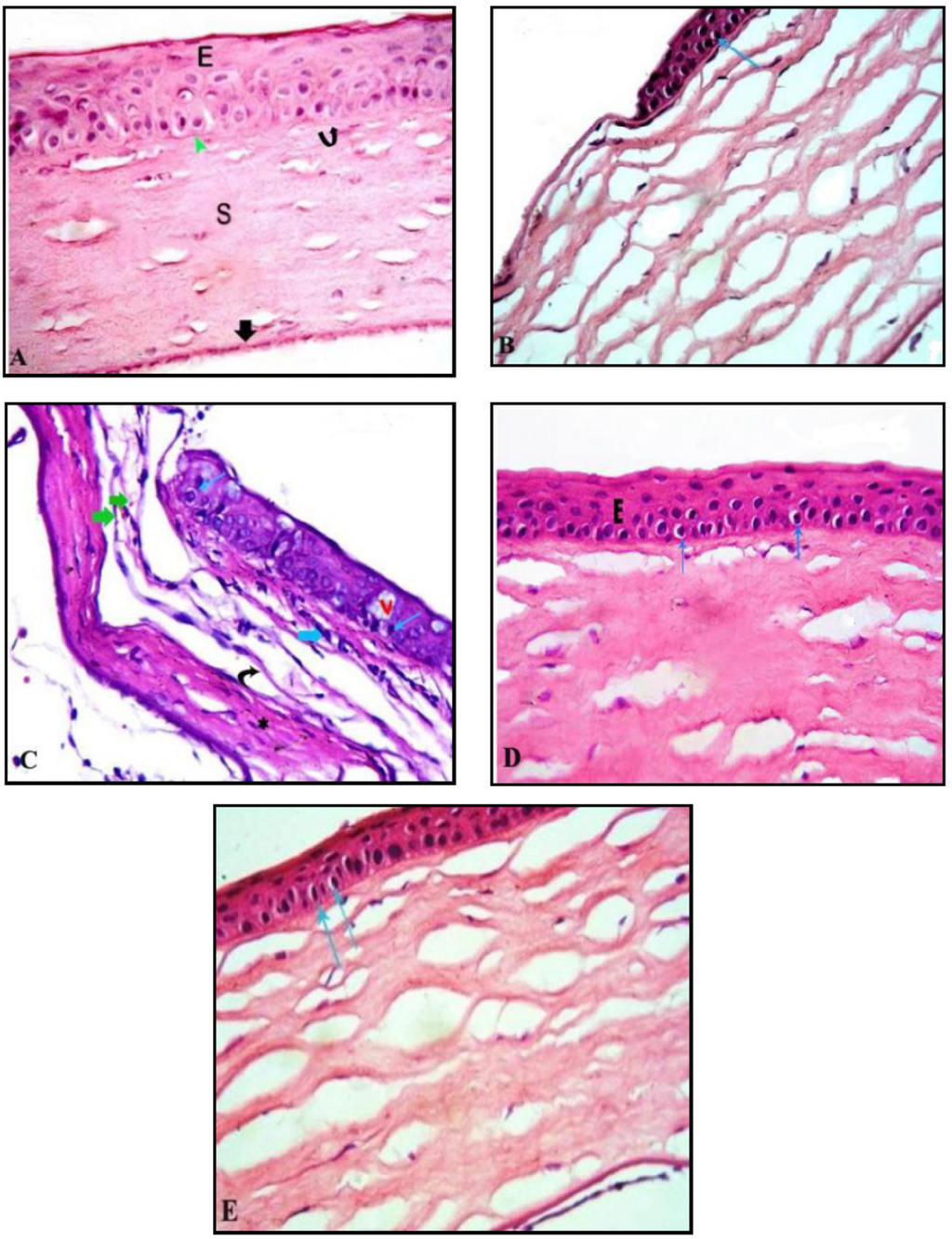

Fig. 5: Higher magnification from the previous sections, (A): Group I showing non keratinized stratified squamous epithelium (E), straight basement membrane (arrow head) and Bowman's layer (curved arrow). Substantia propria (S) with its regular arrangement of collagenous bundles and the Descemet's membrane (black arrow) can be seen. (B): group IIa showing multiple epithelial cells with dark, shrunken nuclei and vacuolated cytoplasm (blue arrow). (C): group IIb showing epithelial vaculations (V). Some nuclei of nuclei epithelial cells appear dark (thin blue arrows). Leucocytic infiltration (thick blue arrow) and extravasation of RBCs into the stroma can be observed (green arrows). Curved arrow points to the thinned out collagen bundles and compacted fibers (astrix) in lower half. (D): Group III showing many epithelial cells with dark nuclei and vacuolated cytoplasm (blue arrows). (E): Group IV showing nuclei of some epithelial cells with dark nuclei (blue arrows). H&E (x400)

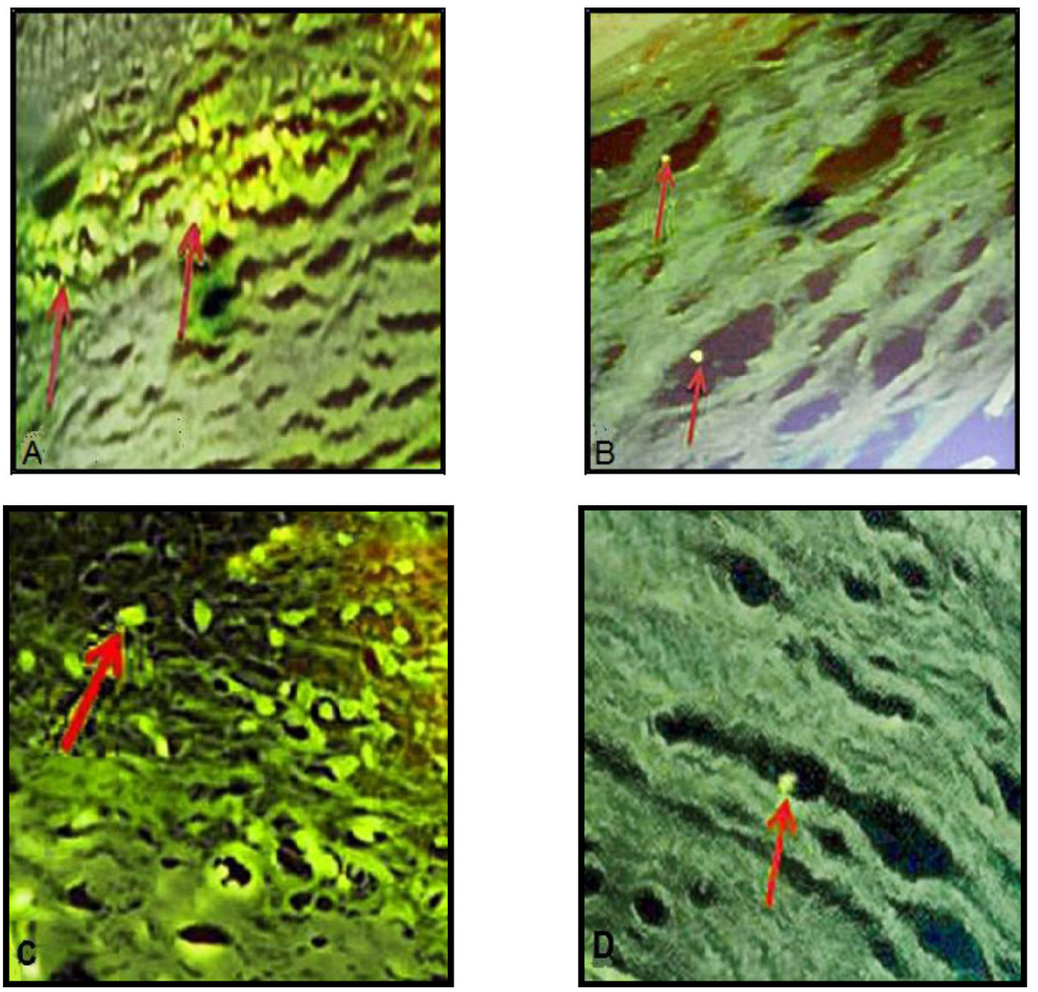

Fig. 6: ADSCs group (A): corneal sections GIII (3 days after injection with ADSCs one hour after alkali-burn) demonstrates bright fluorescence of ADSCs labeled with PKH26 (red arrows) that were heavily distributed in the stroma. (B): GIII (sacrificed 3 weeks after injection with ADSCs one hour after alkali-burn) shows few ADSCs labeled with PKH26 in the stroma (red arrows). (C): Corneal sections GIV (3 days after injection with ADSCs 3 weeks after injury) demonstrates bright fluorescence of ADSCs labeled with PKH26 (red arrows) that are widely distributed in the stroma. (D): GIV (sacrificed 3 weeks after injection with ADSCs (6 weeks after injury) illustrates few ADSCs labeled with PKH26 in the stroma (red arrow). PKH26 labeled ADSCs immunofluorescence (x200)

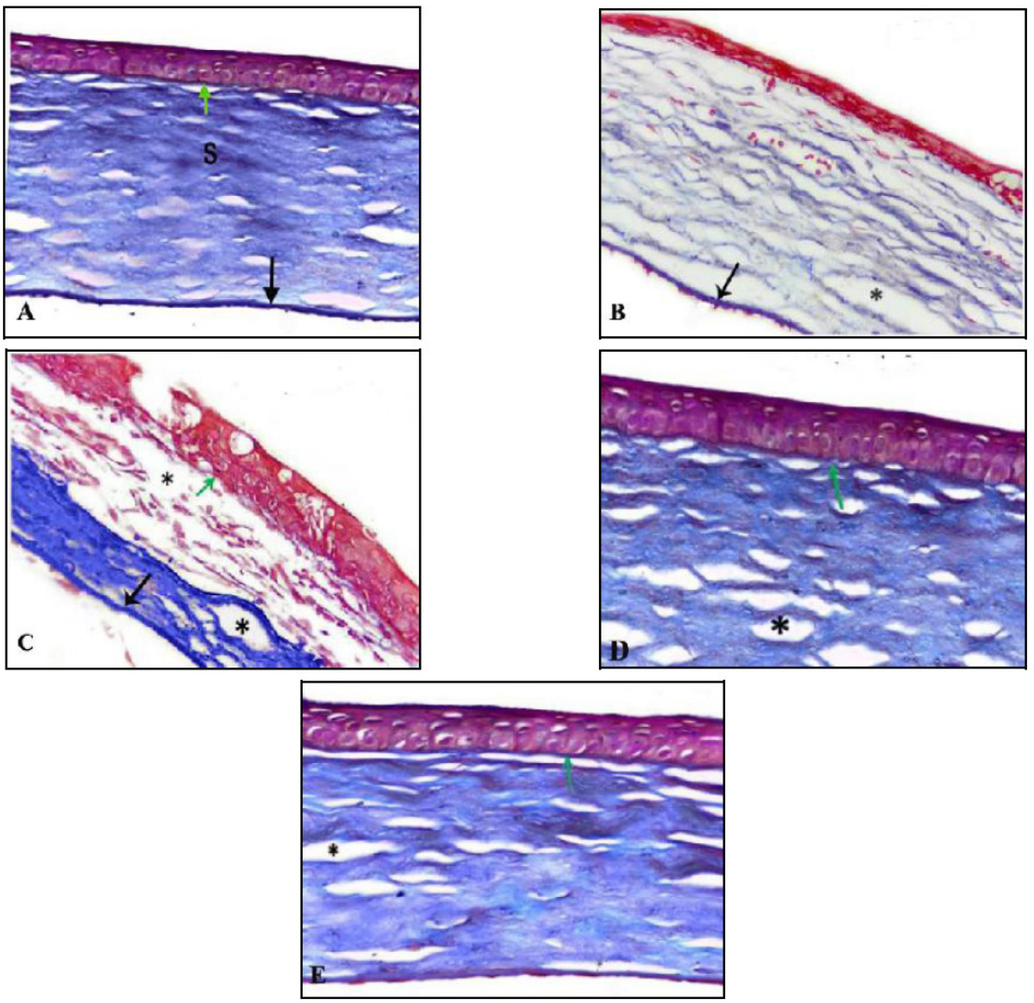

Fig. 7: Photomicrographs of sections from the central part of the cornea from (A): Group I showing corneal stroma (S) consists of regular collagen bundles, regular continuous Bowman's layer (green arrow) and Descemet's membrane (black arrow). (B): group IIa showing separation of faintly stained collagen bundles (astrix). Descemet's membrane (black arrow) appears separated from the stroma. (C): group IIb illustrating loss of collagen bundles from the upper half of substantia propria (astrix), irregular Bowman's layer (green arrow) and continuous Descemet's membrane (black arrow). (D): Group III demonstrating less separation of collagen fibers (astrix) and continuous Bowman's layer (green arrow). (E): Group IV showing the stroma with regular collagen bundles (astrix) and continuous Bowman's layer (green arrow). Masson's trichrome (x400)

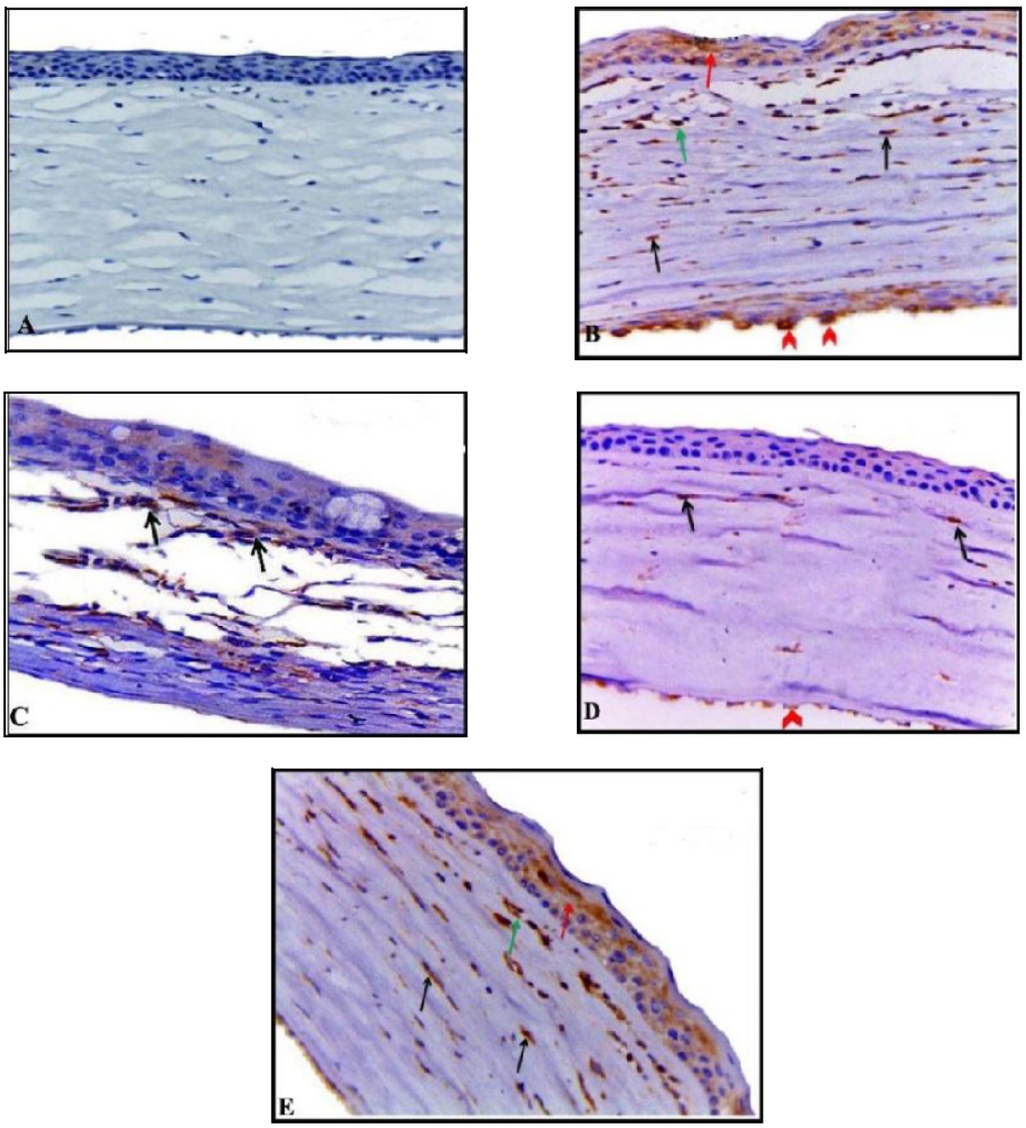

Fig. 8: Photomicrograph of sections from the central part of the cornea from (A): GI showing negative immunoreactivity within corneal tissue. (B): gIIa showing positive cytoplasmic reaction within epithelial cells (red arrow), endothelial cells lining the blood vessels (green arrow), corneal corpuscles (black arrows) and corneal endothelial cells (arrow heads). (C): gIIb illustrating positive cytoplasmic reaction within corneal corpuscles (black arrows). (D): GIII showing positive cytoplasmic reaction within few corneal corpuscles (black arrows) and corneal endothelial cells (arrow head). (E): GIV showing positive immunoreactivity within the cytoplasm of epithelial cells (red arrow), endothelial cells of few blood vessels (green arrow) and corneal corpuscles (black arrows). Immunohistochemical stain for VEGF (x400)

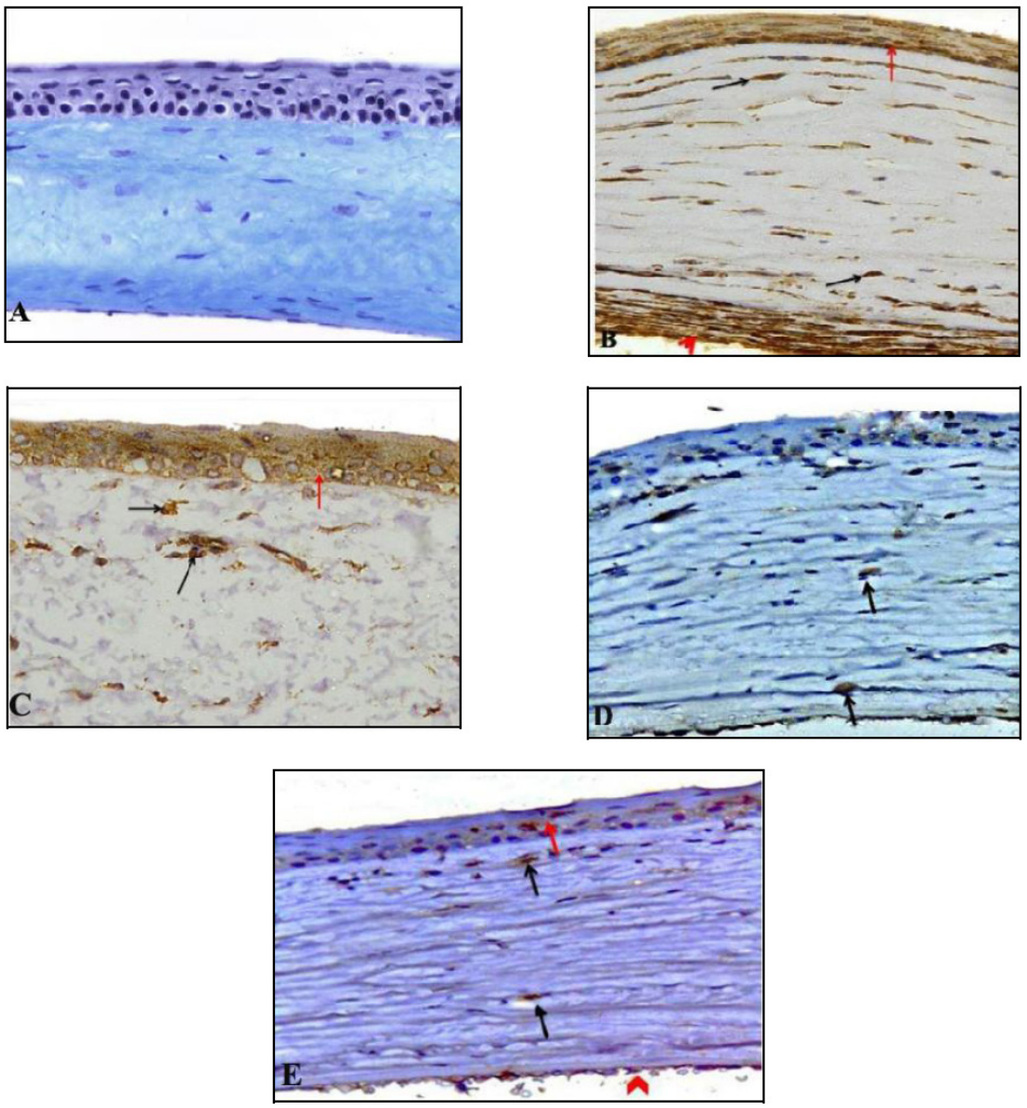

Fig. 9: Photomicrograph of sections from the central part of the cornea from (A): GI showing negative immunoreactivity within the corneal tissue. (B): gIIa showing positive immunoreactivity within the cytoplasm of many epithelial cells (red arrow), keratocytes (black arrows) and corneal endothelial cells (arrow head). (C): gIIb showing positive immunoreactivity within the cytoplasm of many epithelial cells (red arrow) and keratocytes (black arrows). (D): GIII showing positive immunoreactivity within the cytoplasm of few keratocytes (black arrows). (E): GIV showing positive immunoreactivity within the cytoplasm of few epithelial cells (red arrow), keratocytes (black arrows) and corneal endothelial cells (arrow head). Immunohistochemical stain for caspase3 (x400)

Table 1: Morphometric parameters of all studied groups

Group	area % Mean ± SD of collagen in Masson's trichrome stained sections	Mean number ± SD of VEGF immunopositive cells		Mean number ± SD of Caspase-3 immunopositive cells	
		Epithelium	Stroma	Epithelium	stroma
GI	10.5± 0.7	0	0	2 ± 0.8	0
gIIa	4.6 ± 0.4^{abc}	27.7 ± 1.25^{abc}	39.9 ± 0.99^{abc}	44.5 ± 1.18^{abc}	39.8 ± 1.4^{abc}
gIIb	2.2 ± 0.2^{abc}	18.4 ± 1.17^{abc}	30 ± 1.4^{abc}	35.2 ± 0.79^{abc}	19.5 ± 1.18^{abc}
GIII	8.3 ± 0.2^{a}	3.3 ± 1.15^{a}	6.8 ± 1.22^{a}	6.3 ± 1.16^{a}	7.1 ± 0.99^{a}
GIV	8.9 ±1.5^{a}	11 ± 0.8^{ab}	24.7 ± 1.25^{ab}	6.1 ± 1.2^{a}	7.5 ± 1.18^{a}

a $P < 0.05$ as compared to GI. b $P < 0.05$ as compared to GIII. c $P < 0.05$ as compared to GIV.

DISCUSSION

The present study was performed to compare the outcome of early and late administration of stem cells on chemically induced corneal injury. Many studies have evaluated the toxic effects of NaOH on the corneal surface where it was used to induce chemical corneal injury (alkali burn).

The results obtained from the present work in group IIa (alkali burn group) demonstrated complete loss of part of the epithelium. Epithelial cells appeared having dark, shrunken nuclei and vacuolated cytoplasm. Our results agree well with those of other researchers who reported that apoptosis in the corneal epithelial cells and the stromal keratocytes occurred after alkali burn exposure[9].

Sections of group IIa also showed that collagen bundles lost their parallel arrangement and were widely separated. This was in agreement with previous study which reported that the distortion of the stroma and separation of collagen bundles were attributed to increased corneal hydration (edema) that might affect corneal transparency[10].

To detect the progress of healing process that normally occurred without management, corneal sections were examined 6 weeks after injury. Sections of group IIb showed abnormal architecture of the corneal tissue and complete loss of part of epithelium. Some epithelial cells showed vacuolations and their nuclei appeared dark and pyknotic. These findings coincided with those of former researchers who examined the effect of corneal alkali injury two, four and ten weeks after injury and stated that all injured eyes showed persistent corneal opacity with obvious neovascularization[11].

Leucocytic infiltration in the stroma was also observed. Thin collagen bundles in the upper half of substantia propria and compacted bundles in the lower half were also seen in group IIb. These findings were in agreement with those of previous authors who noticed the association between the distribution of inflammatory cells and the amount of collagen fibers; as fewer filaments were seen in the region of dense inflammation whereas high concentration was noticed more commonly with minimal inflammation[12].

Extravasation of RBCs with congested blood vessels and inflammatory cells were also detected in the corneal stroma in group IIb. These results were in line with previous researchers who noticed that corneal neovascularization was a sight-threatening condition usually related to inflammatory or infectious conditions of the ocular surface[13]. Corneal chemical burn usually caused abundant corneal neovascularization in which severe inflammation and corneal lysis were involved[14].

In the present work, rats received ADSCs after alkali burn and comparative study of the outcome of early and late administration of stem cells was done. Examination of corneal sections by fluorescent microscope showed heavily distributed PKH26 labeled ADSCs 3 days post injection in Group III and Group IV. This finding indicated homing of ADSCs in the injured corneal tissue after intravenous injection. Going with these results ,researchers also found ADSCs in high number in sites of trauma and ischemia after several days[15]. However, few PKH26 labeled ADSCs were detected in the corneal stroma 3 and 6 weeks post injection in Group III and Group IV. This might be explained by the ability of ADSCs to differentiate into various cell types to promote tissue regenerations.

Regarding Group III, examination of sections revealed restoration of the corneal epithelium with straight basement membrane. This might be the result of ADSCs capability to differentiate and regenerate the damaged cornea. Some cells with vacuolated cytoplasm and dark nuclei were still seen. This was explained previously and stated that ADSCs has the ability to secrete different types of molecules with anti-apoptotic, immunomodulatory, and anti-scarring properties providing an opportunity of using them in regenerative medicine[16]. These findings denoted that adipose-derived adult stem cells could be a source for corneal regeneration and repopulation in corneal diseases[17]. This group also showed regular collagen bundles but some still appear separated. Descemet's membrane and endothelium were intact. No vascularization or inflammatory cells in the central parts of the cornea were seen. These findings indicated that ADSCs had anti-inflammatory and anti-angiogenic properties which had profound effects on accelerating the healing process in corneal injury and avoiding the complications. This was in agreement with whom stated that stem cells treatment suppressed the infiltration of inflammatory cells and CD68+ macrophages which were closely related to the degree of inflammation[18&19].

Corneal sections of Group IV were examined where restoration of the corneal epithelium in most parts of the cornea with a straight basement membrane was detected. Many epithelial cells with dark nuclei and vacuolated cytoplasm were still seen. It was supposed that injection of ADSCs in a late stage was not highly effective in all cases where many apoptotic cells were still present.

The functions of ADSCs in corneal wound repair could be achieved through two mechanisms: Trans-differentiation and paracrine action through the cytokines which probably mediate an anti-inflammatory and anti-angiogenic effect[20].

Moreover, group IV showed separation of the collagen bundles. This might indicate delayed healing due to late treatment where collagen separation was persistent. No inflammatory cellular infiltration was detected as compared to the injury group. This finding was matching with that of Oh *et al.* who declared that ADSCs increased level of IL-10, IL-6 and TSP-1 (thrombospondin), which reduced infiltration of inflammatory cells and reduced the expression of the inflammatory markers IL-2 and MMP-2 (matrix metalloproteinase-2) in the chemically burned cornea[20].

Descemet's membrane and endothelium were intact in most parts of the cornea. These restoration of corneal

structure might be supported by the characteristics of ADSCs that included secretion of anti-apoptotic, anti-angiogenic and hematopoietic factors (cytokines and growth factors), such as macrophage colony-stimulating factor, granulocyte macrophage colony-stimulating factor, insulin-like growth factor IGF and hepatocyte growth factor (HGF)[21].

The changes in the stroma in the current study were verified by Masson's trichrome stained sections. The corneal stroma of control group was formed of regular collagen bundles. Bowman's layer and Descemet's membrane were regular and continuous. Corneal sections of alkali burn subgroup group IIa revealed separation of faintly stained collagen bundles while collagen bundles were lost from the upper half of the stroma in group IIb. Similar results were also obtained by previous authors, who suggested that the damage to the corneal stroma was most likely mediated by proteolytic enzymes released by the polymorphonuclear leukocytes (PMNs), epithelial and stromal cells[22]. They also found accumulation of variable-sized electron-dense particles, in association with collagen fibrils that might result from breakdown products of collagen and/or proteoglycans. Furthermore, they attributed the dispersion of the collagen bundles to the edema caused by inflammation.

On the other hand, Group III showed stroma with less separation of collagen fibers and continuous Bowman's layer. Sections of Group IV showed corneal stroma formed of regular collagen bundles and continuous Bowman's layer. These findings might be explained that human ADSCs can be procured easily and transplanted into the corneal stroma of rabbits and differentiate into functional keratocytes when injected into corneal stroma (creating a niche), as assessed by the expression of the cornea-specific proteoglycan, keratocan, and aldehyde dehydrogenase (ALDH) and formation of newly formed tissue with successful collagen remodeling and less stromal scarring[17].

Neovascularization is an important complication that clearly affects the acuity of vision, also essential for repair, remodeling and regeneration of damaged tissue during corneal wound healing[23]. On examination of VEGF immuno-stained sections of control group negative immunoreactivity was recorded. While positive cytoplasmic reaction within epithelium, endothelial cells lining the blood vessels, keratocytes and corneal endothelial cells was seen in group IIa, On the other hand, Positive cytoplasmic reaction within keratocytes was seen in group IIb. It was observed that corneal epithelial and endothelial cells, vascular endothelial cells of limbal vessels, fibroblasts and macrophages in diseased tissue all secrete VEGF, mainly in inflamed and vascularized corneas. The receptors of VEGF (VEGFR1 and VEGFR2) were also originated in a new proliferating vascular endothelial cells in inflamed cornea[24]. Sections of Group III showed positive cytoplasmic reaction within few keratocytes and corneal endothelial cells. On the other hand, positive immunoreactivity within cytoplasm of epithelial cells, endothelial cells lining few blood vessels and keratocytes was seen in Group IV. These findings indicated the anti-angiogenic effect of ADSCs during healing of the cornea. This was in accordance with some authors who suggested an anti-angiogenic action of ADSCs in the chemically burned corneas through paracrine pathways involving soluble factors such as IL-10, TGF-β1, IL-6 and TSP-1[16]. In contrast to its anti-angiogenic activity, ADSCs stimulate VEGF in an ischemia or tumor model[25].

Caspase-3 (apoptotic marker) immuno-stained sections showed positive immunoreactivity in the cytoplasm of many epithelial cells, keratocytes, as well as cytoplasm of the corneal endothelial cells in group IIa and only cytoplasm of many epithelial cells, keratocytes in group IIb. The above-mentioned immunohistochemical findings were discussed clearly by some researchers who found that corneal exposure to applied stresses (as alkali burn application) led to activation of Fas receptor of plasma membrane. These resulted in the formation of a death-inducing signal complex and led to the activation of caspase 8. The activated caspase 8 then propagated the apoptotic signal by activating downstream proteins through proteolytic cleavage. Among the proteins activated by this cascade were caspase 3 and a bcl-2 interacting protein. This bcl-2 protein was a pro-apoptotic one that triggers mitochondrial release of cytochrome C, which in turn activated caspases 9 and 3[26]. On the other hand, Group III showed positive cytoplasmic reaction in few keratocytes. Group IV recorded positive immunoreactivity for Caspase-3 immuno-stained sections in the cytoplasm of few epithelial cells, keratocytes, and cytoplasm of corneal endothelial cells. This finding was in accordance with former investigators who noticed that stem cells were found to promote corneal epithelial cells survival, and inhibit apoptosis of these cells by secretion of anti-apoptotic factors[27]. To verify these findings, the mean area % of collagen in Masson's trichrome stained sections, mean number of VEGF and Caspase-3 immunopositive cells were assessed by morphometric measurements using the image analysis system. Morphometric analysis proved significant increase in the mean area % of collagen in Masson's trichrome stained sections of Group III and Group IV when compared to group IIa and group IIb for each corresponding duration. These results indicated a significant effective regenerative role of ADSCs. This might be one of its mechanisms of action in accelerating corneal wound healing. A significant decrease in mean number of VEGF and Caspase-3 immunopositive cells of Group III and Group IV was detected in epithelium and stroma when compared to group IIa, group IIb for each corresponding duration. There was significant decrease in the mean number of VEGF in Group III versus Group IV. It was supposed that injection of ADSCs in a late stage was not highly effective in all cases where neovascularization were still increased.

In the present study, the use of ADSCs as a new cell therapy resulted in regeneration of corneal epithelial cells. In addition, no epithelial erosions, less separation of

collagen fibers and diminished corneal neovascularization and cellular infiltration were found. The regression of neovascularization and apoptotic nuclei as well as increase area of collagen fibers were confirmed morphometrically, in comparison to alkali burn group. It was concluded that early treatment with ADSCs had superior role than late treatment as noticed by the incomplete healing and persistent complications with late treatment.

CONFLICTS OF INTEREST

There are no Conflict of Interest

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الملخص العربى

دراسة هستولوجية لمقارنه تأثير الأعطاء المبكر والمتأخر للخلايا الجذعية المستخرجة من الدهون علي حروق القرنيه القلويه فى ذكور الجرذان البالغه البيضاء

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المقدمه: يسبب الحرق القلوي للقرنية إلتهابات وقرحة وثقب بالقرنية وتكوين الأوعية الدموية والعتامة والتي غالباً ما ينتج عنها ضرر بالغ. إن تدمير الخلايا الجذعية الطرفية بواسطة هذه الحالة المرضية غالباً ما يتسبب في مضاعفات.
الهدف من الدراسه: تم تصميم هذه الدراسة لمعرفة تأثير الأعطاء المبكر والمتأخر للخلايا الجذعية المستخرجة من الدهون بعد إحداث حروق القرنيه القلويه في الجرذان البيضاء.
المواد وطرق البحث: وقد أجريت هذه الدراسة على ثمانيه وعشرون من ذكور الجرذان البيضاءالبالغه. وتم تقسيمها إلى اربعه مجموعات0المجموعة الأولى(المجموعة الضابطة.(المجموعة الثانية:المجموعة المصابة بالحرق القلوي:خضعت هذه المجموعة لحرق قلوي في أعينهم باستخدام هيدروكسيد الصوديوم.المجموعة الثالثة: (مجموعة العلاج المبكر بالخلايا الجذعية) :وقد تم تعرضهم لحرق قلوي كالمجموعة الثانية ثم تم حقنهم عن طريق الوريد الذيلي بمليون خلية جذعية مستخرجة من الدهون]في 1 مل محلول الفوسفات الملحى الموازن [(PBS) بعد ساعة من الإصابةوتم تشريح الجرذان بعد ثلاثة أسابيع من الحقن.المجموعه الرابعه: (مجموعه العلاج المتأخر بالخلايا الجذعية) وقد تم تعرضهم لحرق قلوي كالمجموعة الثانية ثم تم حقنهم عن طريق الوريد الذيلي بمليون خلية جذعية مستخرجة من الدهون]في 1 مل محلول الفوسفات الملحى الموازن [(PBS) بعد ثلاثة أسابيع من الإصابة وتم تشريح الجرذان بعد ثلاثة أسابيع من الحقن.وقد تضمنت خطة هذا البحث: دراسة التغيرات الشكلية باستخدام:صبغة الهيماتوكسيلين والإيوسين ,صبغة الماسون ثلاثى الألوان,الصبغة النسيجية الكيميائية المناعية لتحديد: عامل نمو بطانة الأوعية الدمويةو كاسباس 3.وايضا تم إستخدام الفلورسنت ميكروسكوب لإيجاد الخلايا الجذعية المستخرجة من الدهون الموسومة ب PKH26, وكدلك الدراسات القياسية المتريةوالتحليل الإحصائي0
النتائج: أظهر فحص قطاعات القرنية لمجموعة الحرق القلوى تركيب غير طبيعي لنسيج القرنية وفقدان كامل لجزء من النسيج الطلائى.و أن الإصابة أدت إلي تغيرات كبيرة في النسيج الطلائي، ما بين تقشره في بعض الأجزاء إلي سقوط كامل في أماكن أخري. كما لوحظ العديد من الخلايا الطلائية ذات الأنويه الداكنة وفراغات بالسائل الخلوي. وترتبت حزم الكولاجين في سدى القرنية ترتيباً غير منتظم. مع وجود إرتشاح كبير للخلايا الالتهابية وإتساع الأوعية الدموية في بعض الاماكن بسدى القرنية. وأيضا لوحظ في بعض الاماكن إنفصال غشاء ديسميت والطبقة البطانية عن سدى القرنية. على الرغم من ذلك، بالنسبة للمجموعة المعالجة مبكرا بالخلايا الجذعية المستخرجة من الدهون، ظهر النسيج الطلائي للقرنية متصل وإستقر على غشاء قاعدي منتظم.اما بالنسبة للمجموعة المعالجة متأخراً بالخلايا الجذعية المستخرجة

من الدهون أظهرت قطاعات القرنية إستعادة النسيج الطلائى فى معظم أجزاء القرنية. كما ظهر الغشاء القاعدى مستقيم. وكان إستخدام الفلورسنت ميكروسكوب لإيجاد الخلايا الجذعية المستخرجة من الدهون الموسومة ب PKH26 ونتائج الماسون ثلاثي الألوان و النتائج النسيجية الكيميائية المناعية والنتائج المترية مؤكده0

الإستنتاج: إن إصابة القرنية بالحرق القلوي حالة خطيرة و ان العلاج بالخلايا الجذعية المستخرجة من الدهون له تأثير مفيد في سرعة الشفاء من حروق القرنية ومنع مضاعفاتة. العلاج المبكر بالخلايا الجذعية المستخرجة من الدهون له دور أعلى من العلاج المتأخر كما لوحظ شفاء غير مكتمل و إستمرار المضاعفات في العلاج المتأخر0