***Comparison between the effect of hyaluronic acid and mesenchymal stem cell therapy on cornea of adult albino rats after exposure to alkali burn (light and electron microscopic study)***

**Abstract**

Introduction: Corneal epithelial injuries are common in both physician and veterinary ophthalmology that often causes extensive damage and results in permanent visual impairment.

**Aim of work:** to evaluate the beneficial effect of hyaluronic acid and MSCs in treatment of experimental Alkali corneal burn.

**Subjects and methods:** thirty adult albino rats of both sexes. Ten rats were used to harvest BM-MSCs while the others were divided into four groups. Group I was the control group. Group II with unilateral alkali-burnt cornea these rats were sacrificed after 1 day and the other sacrificed after 2 weeks. Group III were rats with unilateral hyaluronic acid-treated alkali-burnt corneas for 2 weeks. Group IV were rats with MSCs treated alkali-burnt corneas. Immune histo-chemical staining for CD44 and vimentin was performed. The corneas were examined under light microscopic, transmission electron microscopic and morphometric studies.

**Results:** Corneal alkali burn resulted in desquamation of corneal epithelium in group II. The epithelial cell layers had vacuolated cytoplasm with pyknotic nuclei. The stroma contained irregularly arranged collagen fibers with wide spacing and congested blood vessels with cellular infilteration. Groups III and IV showed improvement of the histological and electron microscopic changes described in group II.

**Conclusions:** The use of MSCs in the acute stage of corneal chemical trauma was associated with faster recovery of the wounded cornea when compared with the effect of Hyaluronic acid sodium salt

**Keywords**: cornea, alkali burn, MSCs, hyaluronic acid

**Abbreviations:** HA: Hyaluronic acid; BM-MSCs: Bone Marrow derived Mesenchymal Stem Cells; PBS: Phosphate Buffer Saline; H&E: Haematoxylin and Eosin; SD: Standard Deviation; ANOVA: One-way Analysis of Variance; EM: Electron Microscope.

***Introduction:***

Cornea is a transparent avascular tissue that. It provides a clear vision by refracting light onto the lens. Cornea acts as a barrier of the eye against infections, abrasions, and structural damage(1).

Alkali burn corneal wounds cause reduced transparency of the cornea and may cause permanent visual impairment or even blindness. Moreover, they do not heal properly spontaneously and there is a lack of satisfactory therapy(1).

 HA is a viscoelastic glycosaminoglycan composed of alternating β-1,4-glucuronic acid and β-1,3-N-acetylglucosamine. HA interacts with several cell surface receptors and provides anti-inflammatory and antiapoptotic signals to corneal cells exposed to Ultraviolent radiation.(2)

MSC are promising approaches in regenerative therapies. MSC have been used in a recent technique for wound repair, regeneration, and tissue engineering procedures because the isolated cells expand rapidly and differentiate into different cell types(3).

The aim of this study was to compare the therapeutic effects of HA and MSCs in the treatment of alkali-induced epithelial corneal defects.

***Material and methods:***

This study was conducted on 30 albino rats of both sexes weighing between 220 and 250 mg, taken from and housed at the laboratory animal house unit of Kasr Al-Ainy Faculty of Medicine, Cairo University (from July 2018 to November 2018). Strict maintenance and cleaning measures were applied to keep the animals in healthy state. All ethical rules for animal treatment were followed.

**Experimental Design**

Bone marrow was harvested by flushing the tibiae and femurs of ten rats for BM-derived MSCs culture.

The other animals were divided into: Group I (4 rats); the control group was sham operated. Group II (8 rats); corneal alkali burn was created. These rats were anesthetized with intramuscular injection of 0.5 mg/kg ketamine. The right eyes of each rat were gently opened wide. Filter paper soaked in 1% NaOH then placed on the cornea for 40 seconds. The cornea was then rinsed with 60 ml of saline for 1 minute(2). Four of these rats (subgroup IIa) were sacrificed at 1 day after alkali burn; other four rats (subgroup IIb) were sacrificed after two weeks.

Group III (4 rats); corneal alkali burn was done as in group II then HA sodium salt (polyfresh artificial tear) administered topically as eye drops, which was given every 2 h, six times a day, daily starting 1 day after corneal alkali burn. These rats were sacrificed after 2 weeks.

Group IV (4 rats); underwent corneal alkali burn as in group II then treated with intravenous injection of PBS containing 2 × 106 MSCs; sacrificed at 2 weeks.

**Isolation of BM-derived MSCs:** Bone marrow was harvested with Dulbecco’s modified Eagle’s medium (DMEM,GIBCO/ BRL) supplemented with 10% fetal bovine medium(GIBCO/BRL). Cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin- streptomycin (GIBCO/BRL). Cells were incubated at 37oC’ in 5% humidified Co2 for 12∼14 days as primary culture or upon formation of large colonies. The cultures were washed and cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL). After centrifugation, cells were resuspended and incubated in flask Falcon. The resulting cultures were referred to as first-passage cultures(4). The MSCs were recognized in culture by inverted microscope as spindle shaped cells (fig. 1a).

**Labeling of stem cells with PKH26 dye**

MSCs were labeled with the PKH26 fluorescent dye. PKH26 was purchased from Sigma Company (Saint Louis, Missouri, USA)(5). Cornea was examined using fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) at Kasr Al-Ainy Faculty of Medicine, Cairo University. Several MSCs labelled with PKH26 fluorescent dye were detected housed in the cornea by their strong red fluorescence (fig. 1b).

**Histological study:** Specimens were fixed immediately in 10% buffered formal saline for:

* **Light microscopic study:** 5 μm sections were cut and stained with hematoxylin and eosin (H&E) for histological study(6).
* **Transmission electron microscopic study:** the corneas were collected and ultrathin sections were performed(7). It was done and photographed in Tanta EM Unit, Faculty of Medicine, Tanta University using JOEL (JEM-100 SX, Akishima, Tokyo, Japan).

**Immunohistochemical study:**

Immunohistochemistry of paraffin-embedded corneal sections was performed with CD44 and vimentin antibodies by using the avidin–biotin peroxidase complex technique(8). The slides were studied by light microscopy in Anatomy Department, Benha faculty Of Medicine, Benha University.

**Morphometric study**: H&E slides were assessed to measure the corneal epithelial thickness (μm), stromal thickness (μm) and Descemet’s membrane thickness (μm) at a magnification of × 400. Data were expressed as group mean value ± SD. ANOVA with post hoc Scheffe's test was used to contrast differences between the groups. A value of P < 0.05 was accepted as statistically significant.

***Results:***

**H&E results**

Group I: the rat cornea consists of nonkeratinized stratified squamous epithelium which is formed of columnar basal cells with oval nuclei, intermediate layers of polygonal cells with rounded nuclei and superficial flattened squamous cells with flattened nuclei. Bowman's layer, where the columnar cells are resting on, is ill defined seen. The corneal stroma is the thickest layer of the cornea. It appears avascular. It is formed of regularly organized collagen fibers with spindle-shaped stromal cells (Keratocytes) in between. Descemet’s membrane is seen as a homogenous acidophilic noncellular layer interposed between the stroma and the underlying endothelial cells with their flat nuclei **(Fig.2a)**

Group II: Corneal sections for subgroup IIa show desquamation of surface and intermediate cells exposing the basal epithelial cells. Corneal stroma had disturbed collagen fibers with spaces began to appear in between fibers **(Fig.2b).**

Corneal sections for subgroup IIb show variability in the thickness of the corneal epithelium and disfigurement. Some of epithelial cells have vacuolated cytoplasm. Corneal stroma contained collagen fibers with wide spaces. Blood vessels are surrounded with inflammatory cells. Descemet's membrane cellularly infiltrates **(Fig.2c).**

Group III: Rat corneal sections show multilayered, organized epithelium. Apparently flat cells overlap the superficial epithelial layer. The collagen fibers in the stroma are regularly arranged in posterior portion of cornea and disturbed in the anterior part with wide spaces are still present. Descemet’s membrane appeared with focal loss of some endothelial cells **(Fig.2d)**.

Group IV: Rat corneal sections show that the layers of the cornea can be easily identified. The corneal epithelium appears continuous with very thin intact Bowman's membrane. The corneal stroma is formed of regularly arranged collagen fibers. Descemet’s membrane and endothelium are apparently normal **(Fig.2e)**.

**EM results**

***Group I:*** The top layer of corneal epithelium is formed of flat cells with apical microvilli. The nucleus is flattened with a fine, light and scattered condensed chromatin granulation. The numerous electron-dense desmosomes are present at cell membranes connecting cells together **(Fig.3a)**.

The basal layer is formed of columnar cells having spherical nucleus with fine granular chromatin. They are attached to the straight basement membrane by well-apparent hemidesmosomal junctions. Columnar cells are connected with their neighbors by desmosomes **(Fig.3b)**.

The collagen fibers of corneal stroma within each lamella are arranged parallel to one another arranged in longitudinal and transverse planes. In between fibers, there is long, spindle shaped keratocyte with thin and long cytoplasmic processes. Its nucleus is occupied the most central area with thin rim of heterochromatin at the periphery. The cell has many stacks of rough endoplasmic reticulum and mitochondria within the cytoplasm **(Fig.3c)**.

Descemet’s membrane appears as a thick homogenous electron-dense non-cellular membrane. The endothelial cell is seen as single layer of flat cell with flat nucleus which has a moderate electron-dense chromatin. Its cytoplasm shows pinocytotic vesicle **(Fig.3d).**

***Subgroup IIa:*** superficial layers of the corneal epithelium show that epithelial cells are separated from each other by wide intercellular spaces with loss of desmosomes in between. These cells have marked and heavily stained chromatin patches in their nuclei with irregularity in their shape**.** Wide intercellular spaces have showed mononuclear cell infiltration in between the cells **(Fig.4a).**

The cells of the basal layer have irregular contour with loss of their columnar shape. Some nuclei of these cells have condensed chromatin and other with pale chromatin. The basement membrane appears irregular and ill defined. Wide intercellular spaces are also noticed **(Fig.4b).**

The stromal keratocytes show darkely stained nuclei and many vacuoles in its cytoplasm with short cytoplasmic processes **(Fig.4c).**

Descemet’s membrane appears regular and homogenous; however, the endothelial cells are swollen with large cytoplasmic. The endothelial cell has elongated nucleus with light stained chromatin **(Fig.4d).**

***Subgroup IIb:*** it shows loss of microvilli on the free surface of the superficial layer of the corneal epithelium. Their nuclei have light stained chromatin. Wide intercellular spaces present between them. Mononuclear cell infiltrations are obvious in the superficial layer of cornea **(Fig.5a)**.

Cells of the basal layer appear irregular in shape containing mitochondria with partial lysis of their cristia. Their nuclei have heavily stained chromatin patch. They rest on an irregular basement membrane. They show loss of desmosomes with narrow intercellular spaces **(Fig.5b)**.

Corneal stroma shows an irregular arrangement of the collagen fibers. keratocyte has shrunken nuclei with condensed chromatin with little vacuolated cytoplasm and degenerated cytoplasmic processes **(Fig.5c)**.There is congested blood vessel can be seen **(Fig.5d).**

Descemet’s membrane is lightly stained and lined with endothelial cell which has cytoplasmic vacuoles and many swollen degenerated mitochondria **(Fig.5e)**.

***Group III***: it reveals flat cell with a relatively homogeneous chromatin material in the nucleus. The free surface shows irregularity with numerous apical microvilli. They are connected by desmosomes with wide intercellular gaps **(Fig.6a)**.

The basal cells are columnar in shape with fine granular chromatin of their nucleus and prominent nucleolus connected together with desmosomes. Regular basement membrane is noticed **(Fig.6b)**.

The collagen fibers of stroma are regular with normal Keratocytes with rough endoplasmic reticulum in its cytoplasm in between **(Fig.6c)**.

The Descemet’s membrane is homogenous moderate electron dense membrane and the endothelial cell has nucleus with irregular electron dense chromatin and multiple cytoplasmic vacuoles **(Fig.6d)**.

***Group IV***: it shows nearly normal superficial squamous cell with superficial microvilli. The nucleus has normal configuration. They are connected by electron-dense desmosomes **(Fig.7a).**

The basal layer has tall columnar cells with euchromatic nuclei resting on regular basement membrane joining with it by hemidesmosomes **(Fig.7b).**

Corneal stroma has apparently regular collagen fibers and normal spindle shaped keratocyte with euchromatic nucleus in-betweenfibers **(Fig.7c).**

Descemet’s membrane is homogenous electron-dense membrane lined with a single layer of flat endothelial cell that has moderate electron-dense elongated nucleus, mitochondria and pinocytotic vesicles **(Fig.7d).**

**Immunohistochemical study:**

Sections of control rat cornea have shown negative reaction for Anti-CD44 in both epithelial and stromal cells **(Fig.8a)**. While positive cells within the stroma and epithelium are detected in Sections of group IV. It is suggested that the MSCs can differentiate into epithelial cells and keratocytes **(Fig.8b)**.

Weak positive immune reaction for vimentin is detected in Sections of groupI and subgroup IIa **(Fig.8c & 8d).** While corneal sections of subgroup IIb show moderate positive reaction for vimentin in the stroma which has indicated regeneration and proliferation of cells **(Fig.8e)**.

Sections of groups III and IV stained by vimentin have shown strong positive cytoplasmic reaction in stromal cells which means more regeneration and proliferation of cells **(Fig.8f & 8g)**.

**Morphometric and statistical results**

The mean and SD of corneal epithelial, stroma and Descemet’s membrane thicknesses for all groups were represented in Table 1.

In group II, the mean value of the epithelial, stromal and Descemet’s membrane thicknesses showed a highly significant differences (P˂0.01) comparing with control group.

In group III, the mean value of corneal epithelial and stromal thicknesses showed a highly significant changes (P˂0.01) comparing with control group. The mean value of corneal Descemet’s membrane thickness showed non-significant changes comparing with control group.

In group IV, the mean value of corneal epithelial, stroma and Descemet’s membrane thicknesses showed non-significant difference comparing with control group.

**Discussion**

 A chemical burn of cornea is one the dangerous ocular injuries resulting in visual impairment. Therefore, it is necessary to use proper management during accurate duration.

In this work, corneal sections for subgroup IIa showed that alkali burn induced corneal injury with desquamation of surface epithelium and disturbance with spaces in between collagen fibers of stroma. Ultrastructural observations showed marked disfigurement in their appearance and heterochromatic nuclei. Stroma showed degenerated keratocytes. The endothelial cell over it had vacuolated cytoplasm. These results are in agreement with other investigators(2,9) who observed desquamation and degeneration of superficial cells, with regularly arranged collagen fibers in some parts of the stroma but other parts showed separated and disturbed collagen fibers.

Anotherinvestigator(10) stated that the guinea pig corneal sections Two days after alkali burn revealed the epithelial cells and Bowman’s membrane were destroyed, leaving necrotic debris with regular collagen bundles in the stroma.

In the present study, examination of corneal sections of subgroup IIb showed variability in thickness of the corneal epithelium with areas of focal thinning. The epithelial cells were disorganized and some showed vacuolated cytoplasm. The stromal fibers showed wide spaces in between and congested blood vessels. Descemet’s membrane was detected with an interrupted layer of endothelial cells. However the cells of the superficial layer had flat euchromatic nucleus with mononuclear cell infiltrations.

These results are similar to the previous reports(2,9,10,11) which showed marked ultrastructural changes in the form of separation of the superficial cells from underlying cells and showed multiples cytoplasmic vacuoles with wide intercellular spaces with disfigurement in their shapes and degenerated nuclei, while stroma showed an irregularly arranged collagen fibers with degenerated and neovascularization. The endothelial cell showed cytoplasmic vacuoles.

Some researchers(10,12) found that new corneal blood vessels were variable in size and shape as evidenced by both light and electron microscopy two weeks after alkali burn. This blood vessel had the loosest endothelial junctions that participated in inflammatory process and exchange of cells.

The new blood vessels can induce leakage of plasma, increasing corneal edema which in turn reduces cornea oxygenation and delays wound healing. (13)

Alkali burns induced ulcers were accompanied with inflammatory cells infiltration that damaged the normal corneal architecture due to liberation of Matrix Metalloproteinases and several proteolytic enzymes.(14,15,16)

In the recurrent study, Rats of group III showed organized epithelium and disturbed collagen fibers in anterior part of the stroma. The electrondense desmosomes appeared connecting cells together with narrow intercellular gap between them without inflammatory cell infiltrations. The keratocytes had thin cytoplasmic processes. The endothelial cell was still degenerated.

Many studies(2,17,18) explained that rats treated by HA for two weeks had a significantly enhanced rate of epithelial defect healing with a multilayered epithelium, morphological regularity, and intercellular adhesion in epithelial cells compared with untreated eyes.

In the present study, alkali burn treated with MSC group showed homing of PKH26 labeled MSCs to the site of injury in cornea which corroborates the reports(9,19,20,21) on MSCs permeation after systemic and other reports(22,23) on MSCs permeation after sub-conjunctival administration.

In this work, the corneal sections of group IV showed nearly uniform thickness with very thin intact Bowman's. Normal nuclear configuration in the corneal epithelial cells was observed. They were connected by electron dense desmosomes. There were long normal keratocytes in between regular collagen fibers. The endothelial cell appeared normal.

These results agreed with other investigators(9,11,20,24) who provided evidence that intravenous or topical application of BM-MSCs could be used to reconstruct corneal surfaces under light and electron microscopes. An investigator(25) added that MSCs could differentiate into corneal epithelial cells in vitro.

MSCs enhance corneal epithelial and stromal wound healing after alkali burns injuries through their anti-inflammatory effects and modulating immunity.(22,26,27,28)

In the current study, Immunohistochemical examinationofcorneal section using Anti-CD44 in control group showed absence of positive cells. This was in agreement with some researchers(20,29)who reported that CD44 positive cells were absent in corneal epithelium. Another researchers(30,31) detected positive CD44 expression in normal cornea and during corneal epithelial wound healing.

In this work, corneal sections of group IV using Anti-CD44 showed positive cells in epithelium and stroma that was agreed with previous works(9,20,31) showing CD44 positive cells in groups treated with MSCs.

Some reports(32,33,34) revealed CD44 expression is one of the characteristics of MSCs in both humans and mice.

In the current work, Immunohistochemical staining of alkali burn group showed relatively increased immune-reaction for vimetnin compared to those of control group which showed faint brown cytoplasmic reaction. The vimentin immune-reaction was more expressed in group III and group IV.

These findings were in agreement with previous work(20)reported that rabbits treated with BM-MSCs after corneal alkali burn showed a significant increase in vimentin expression compared to control group and pathological group.

Also, other studies(35,36) showed high levels of vimentin expressed in keratocytes at alkali-injured stroma.

Another researcher (37) observed high levels of vimentin in the corneal stroma after MSCs transplantation confirming the differentiating ability of MSCs. the MSC population shares many properties of keratocytes and expresses myofibroblastic cell markers a-SMA and vimentin.(38)

These results have been supported by the statistical results. The mean value of corneal epithelial, stroma and Descemet’s membrane thicknesses of group II showed highly significant difference comparing with control group in agreement with results of other works.(2,11)

Corneal alkali burn caused increasing of the thickness of the cornea due to edema, neovascularization, and inflammatory cell infiltration in the stroma and exudation attached to the endothelium.(39)

The mean value of corneal epithelial and stroma thicknesses of group III showed highly significant changes comparing with control group. This statistical results were disagreed with another researcher(2) who showed HA-treated corneas had a non-significant change in these thicknesses compared with the control group.

In group IV, the mean value of three thicknesses showed non-significant changes comparing with control group in agreement with results of other researchers.(11,20,40) It was due to restoration of corneal keratocytes and endothelial cells by MSCs.(41)

Our results demonstrate that the use of MSCs early in corneal chemical trauma is quicker and better healing of the wounded cornea than use of HA.

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