OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Effect of Topical application of Lyophilized Xenogenous Mesenchymal Stem Cell-Derived Extracellular Vesicles on Central and Peripheral Corneal Ulcers Healing in Rabbits

Mohamed M. Bahr[1], Mohamed S. Amer[1*], Khaled Abo-EL-Sooud[2], Shaimaa M. Kamel[3], Marwa A. Fouly[4], Ahmed N. Abdallah[5], Ashraf A. Shamaa[1], Omar S. El-Tookhy[1]

[1]Surgery, Anesthesiology and Radiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt; [2]Pharmacology department, Faculty of Veterinary Medicine, Cairo University, Egypt; [3]Biochemistry and Molecular Biology Department, Faculty of Veterinary Medicine, Cairo University, Egypt; [4]Research Institute of Ophthalmology; [5]Hormones Department, Medical Researcher and Clinical Studies Institute, National Research Center.

Abstract | Background: Corneal ulcer is the discontinuation of the surface epithelial layer of the cornea with variable amounts of the stroma affected. Recent research suggests that Extracellular Vesicles (EVs) of stem cells have the same effects on target cells that their parental stem cells act, these effects are brought on by paracrine signaling involves the release of cytokines and nucleic acids (DNA, mRNA, and miRNA) which may lead to alteration of the gene expression, proliferation, and differentiation of the recipient cells. Methodology: 24 male New Zealand albino rabbits were used in this study, divided randomly into 4 groups (6 rabbits per group), induction of corneal ulcers centrally in groups (I, II) and peripherally in (III, IV). Treated groups are (I, III) using topical lyophilized canine Mesenchymal Stem Cells derived EVs & control groups are (II, IV) using topical normal saline. Result: both treated groups appeared fluorescein negative at the 5th day and are characterized by rapid reepithelization significantly earlier than control groups which were still fluorescein positive. AS-OCT (Anterior Segment Optical Coherence Tomography) showed well-healed epithelium with intact and homogenous stroma on the 5th day in both treated groups, but control groups showed irregular arrangements of epithelial plaques and presented some abnormalities as stromal fibrosis and sub-epithelial cyst formation. Histopathology in both treated groups showed the development of a complete layer of epithelium within 1week which increased in rows at 2nd and 3rd weeks of treatment and the stromal edema decreased with time unlike control groups that showed weak fragmented epithelium development, sub-epithelial hemorrhage and vascularization. These findings prove that EVs have the ability to heal corneal wounds even if in the peripheral parts of the cornea that are deficient in limbal stem cells. Conclusion: In terms of safety, quality, regulatory concerns, and cost, EVs are superior to corneal transplantation and live stem cell therapy.

Keywords | Mesenchymal-stem-cell, Xeno-Exosomes, corneal-ulcer, histopathology, corneal-transplantation, AS-OCT, New-Zealand-rabbits, paracrine-signaling.

Received | September 29, 2022; **Accepted** | October 20, 2022; **Published** | December 20, 2022
***Correspondence** | Mohamed S Amer, Surgery, Anesthesiology and Radiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt; **Email:** andyhasan13@gmail.com
Citation | Bahr MM, Amer MS, Abo-El-Sooud K, Kamel Sm, Fouly MA, Abdallah AN, Shamaa AA, El-Tookhy OS (2023). Effect of topical application of lyophilized xenogenous mesenchymal stem cell-derived extracellular vesicles on central and peripheral corneal ulcers healing in rabbits. Adv. Anim. Vet. Sci. 11(1): 132-140.
DOI | http://dx.doi.org/10.17582/journal.aavs/2023/11.1.132.140
ISSN (Online) | 2307-8316

INTRODUCTION

The cornea is frequently referred to as the "front window of the eye," and eyesight depends on its optical transparency. In addition to protecting the eye from external harm, the transparent cornea also contributes 60% of the refractive power needed to focus images on the retina. The stroma, which mostly consists of mesenchymal extracellular matrix and keratocytes, makes up around 90% of the cornea's thickness. A transparent optical channel with excellent light transmission is provided by the highly structured collagen matrix. To preserve the form of the corneal surface and produce a steady refractive power, the stroma has a particular degree of stiffness and elasticity.

The epithelium and the endothelium are two cellular layers that bound the cornea, the epithelium rests on a basement membrane rather than the endothelium which rests on a Descemet membrane. Between these cellular layers, The stroma, a thicker, cellular layer of connective tissue, and Bowman's layer, a thin layer of acellular connective tissue that are sandwiched.

A corneal ulcer is a damaged epithelial layer of the cornea with variable amounts of stroma. The epithelial layer acts as a barrier to normal commensal bacteria in the conjunctiva, once epithelium is damaged these commensals becomes pathogenic . Trauma such as extensive rubbing the eye is the most prevalent cause of corneal ulcers , diseases associated with eyelid as entropion, distichiasis, and trichiasis lead to epithelial damage and ulcer.

Extracellular vesicles (EVs) are membrane-bound lipid bilayer vesicles that cells expel as a way of cellular communication. The three most prevalent subtypes of EVs are exosomes (40–200 nm), Microvesicles (50–1000 nm), and apoptotic bodies (500–2000 nm). EVs can be classified according to their biogenesis, size, molecular make-up, purpose, or manner of separation. The biggest vesicles that develop from programmed cell death are called apoptotic bodies . Microvesicles formation depends on cell membrane budding but, exosomes are produced when endosomes budding intracellularly are released into the extracellular compartment.

Exosomes are essential for cell-to-cell communication, it interacts with surrounding cells as both endocrine and paracrine mediator. Recent research suggests that EVs derived from stem cells have the same kinds of effects on target cells that their parental stem cells do, these effects are brought on by paracrine signaling and changing the host's microenvironment. Fresh exosomal solutions have the disadvantage of must be collected and reinjected into the same individual, and they cannot be stored for a long time, not to forget the prolonged preparation time . As a result, it was necessary to use preserved EVs to be suitable for clinical application.

Freeze drying (lyophilization) is a method for preserving perishable items by dehydration of samples to delay degradation which water is essential for the growth of microorganisms and for enzyme activity. lyophilization is a two-step preservation procedure that involves freezing water and then removing it under pressure (4.579 mm of Hg) and at a temperature below 0.0099°C so the best method for preserving thermo-sensitive substances such as EVs, vaccines, viruses, proteins, peptides, and colloidal carriers is lyophilization .

Aim of the present study is evaluation of lyophilized xeno-Mesenchymal Stem Cell Extracellular Vesicles as regenerative therapy in Central and Peripheral corneal ulcer treatment.

MATERIALS AND METHODS

This Experimental study design was approved by Institutional Animal Care and Use Committee Cairo University (CU-II-F-25-21), this study was achieved at the animal house of the Research Institute of Ophthalmology, Egypt.

Experimental animals

Twenty-four adult male New Zealand albino rabbits (3kg (+/-200gm) average weight, 6 months age) were used in this study. Rabbits were housed in batteries at 25O C and 60:70 % relative humidity with 12hrs light/dark, Rabbits were free to access food (dry pellet ration) and water. Before starting the experiment, all rabbits were kept in quarantine for 2 weeks for monitoring and adapting to laboratory conditions, each rabbit was exposed to visual, ophthalmoscopic examination and fluoresceine examination to exclude rabbits suffering from any ophthalmic conditions.

Experimental design

Rabbits were divided randomly into 4 groups (6/each group), group I, II, III, IV. The route of drug application was topical in all groups, a central corneal ulcer was created in groups I and II and a peripheral corneal ulcer was created in III and IV. Groups number (I, III) were the treated groups & groups number (II, IV) were the control groups.

Induction of corneal wound

Rabbits were anesthetized by intramuscular injection of xylazine 5mg/kg and ketamine 50mg/kg. Using of 6-mm diameter Vacuum Trephine to gently mark the border of the corneal ulcer (central or peripheral) and drilling groove in the epithelial layer then scrapped by keratome Kinfe,

one complete rotation of vacuum trephine reached to 250 µm of corneal depth, so that one third to one-half rotation of vacuum trephine is sufficient to reach superficial lamellae of stroma through epithelium and bowmen layer.

Source of lyophilized canine mesenchymal stem cell-derived extracellular vesicles

Lyophilized canine mesenchymal stem cells (MSCs)-derived EVs powder purchased from (Bioluga ® Canada) which was reconstituted in 5ml distilled water and mixed till obtaining a homogenous gel, each 1ml of the gel contained exosomes derived from 0.5×106 MSCs.

Treatment

Treated groups I and III were treated by EVs solution applied as 0.1ml once daily topically for one week. Control groups II and IV were treated using Normal saline 0.9% applied as 0.1ml once daily topically for one week.

Evaluation of treatment:

1- **Fluorescein test:** using sodium fluorescein 0.5% for ulcer examination day after day till fluorescein was negative, the most significant diagnostic method for corneal ulcers is topical fluorescein dye staining. The normal corneal epithelium is a lipid-soluble barrier that prevents topical sodium fluorescein dye from penetrating the stroma. Fluorescein dye diffuses into the corneal stroma when a corneal epithelial defect is present. which appears green under the cobalt blue filter of the ophthalmoscope.

2- **Anterior segment optical coherence tomography (AS-OCT):** is a non-contact ocular imaging technology used for monitoring and diagnosing ocular disease. This technology depends on using low coherence interferometry and measuring the echo time of light backscattered delay from different tissues. Anterior segment imaging needed long wavelengths (1050-1310 nm) , Examination of the cornea was operated by (AS-OCT) day after day after fluorescein test till fluorescein negative.

3- **Histopathological analysis:** two rabbits from each Group were euthanatized by decapitation weekly for 3 successive weeks for histopathological examination. 10% neutral buffer formalin used for fixation the corneal button, then washed by water after trimming, ascending grade of ethyl alcohol was used for dehydrating the sample, using xylene for cleaning and embedded in paraffin. 4-6 micrometer sections were processed then stained by hematoxylin and eosin. Masson's trichrome stain (produced by sigma) for fibrous connective tissue staining.

4- **Statistical analysis:** performing two-way repeated measures ANOVA for analyzing treatment effect (between-subjects factor) and time (within-subjects factor) on the healing of epithelium in case of corneal ulcer Time in weeks was analyzed at three levels (Week 1, Week 2, and Week 3). Summarizing data as mean epithelial thickness ± standard deviation (SD). Performed statistical analysis using PASW Statistics, Version 18.0 software (SPSS Inc., Chicago, IL, USA). Significance was set at $P< 0.05$.

Result

Fluorescein test and (AS-OCT): Both treated groups I & III were characterized by very rapid reepithelization of the epithelial layer of the cornea and was significantly earlier than control groups II & IV, treated groups appear fluorescein negative on the 5th day but ulcer in the cornea was still present as fluorescein +ve in control groups (fig.1, 2). Using Heidelberg Engineering SPECTRALIS®, AS-OCT showed stromal thickness that gradually increased with thin regular epithelium layer formed at 3rd day then epithelial layer appeared well developed with intact and homogenous stromal layer on the 5th day in both treated groups I & III (fig.1, 2) on the other hand, control groups II & IV OCT images at 3rd day appeared as irregular arrangements of clusters of epithelial layers and present some hyperreflective lesion with different densities at the 5th day which was found in the whole corneal thickness as stromal fibrosis (fig.1) and cyst formation in the sub-epithelium with thin stroma (fig. 2).

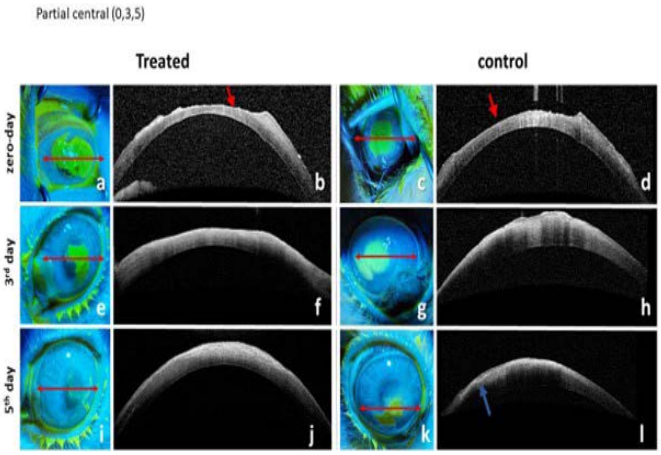

Figure 1: The (0) day of ulcer induction (a, c); showing +ve fluorescein test at the center of cornea of both treated and control groups, (b, d); OCT images showing thin hyperreflective corneal stroma with absence of epithelial layer and upper corneal stroma over ulcered area (red arrow). (e, g) fluorescein test photos at the 3rd day, notice quicker reepithelization of corneal epithelium in the treated group compared to control group, (f); OCT images at the 3rd day of treatment showing increase of stromal thickness with formation of thin regular layer of epithelium, (h) OCT images At the 3rd day of control group showing irregular plaques of epithelium. (i, k); fluorescein test photos at 5th day treated group which appeared fluorescein -ve rather

than control group the corneal ulcer which was still present as fluorescein +ve, (j) OCT image of treated group at 5th day showing well developed epithelial layer and homogenous intact stromal layer, (l); OCT image of control group at 5th day showing hyperreflective lesion with different densities affecting the whole corneal thickness (blue arrow) (stromal fibrosis).

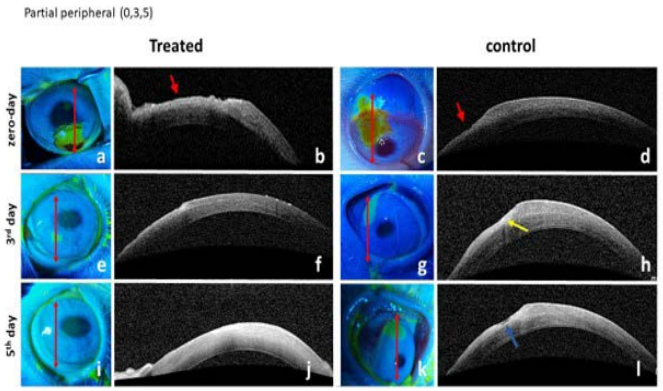

Figure 2: (a, c); the day of ulcer creation photos showing +ve fluorescein test at the peripheral cornea of both treated and control groups, (b, d); At the day of ulcer creation OCT images showing thin hyperreflective corneal stroma with absence of epithelial layer and upper corneal stroma over ulcer area (red arrow). (e, g); fluorescein test photos on the 3rd day notice quicker reepithelization of corneal epithelium in the treated group compared to the control group, (f); OCT images on the 3rd day of the treatment showing the early healing pattern of stroma with the formation of a thin layer of epithelium, (h); OCT images on the 3rd day, the control group showed of heterogenous reflectivity of stroma with hyperreflective lesion (yellow arrow). (i, k); fluorescein test photos at 5th day treated group which appeared fluorescein -ve rather than control group the corneal ulcer which was still presents as fluorescein +ve, (j); OCT image of treated group on the 5th day showing well developed epithelial layer and homogenous intact stromal layer, (l); OCT image of control group on the 5th day of induction showing a thin epithelial layer formation and a thin stromal layer with sub epithelial cyst (blue arrow).

Histopathology

Corneal histological sections of both treated groups I & III after one week of ulcer induction showed the formation a thin layer of epithelium with wide separation of collagenous lamellae which was indicated to stromal edema in addition to infiltration of mononuclear inflammatory cells. After two weeks layers of epithelium became more than one row and showed a gradual decrease of the edema in stroma. On the 3rd week, examination showed the development of several rows of epithelium with minimal stromal edema, little mononuclear cells infiltration and some vascularization. Unlike control groups II & IV at first week no epithelium was formed and wide separation of collagenous lamellae with vascularization and infiltration of mononuclear inflammatory cells, on the 2nd week the epithelium formed partially with persistant edema in stroma and congestion of stromal blood vessels with infiltration of mononuclear inflammatory cells. On the 3rd week, the epithelial layer was still partially developed and stromal edema was still present with hemorrhage (Figure 3 and 4).

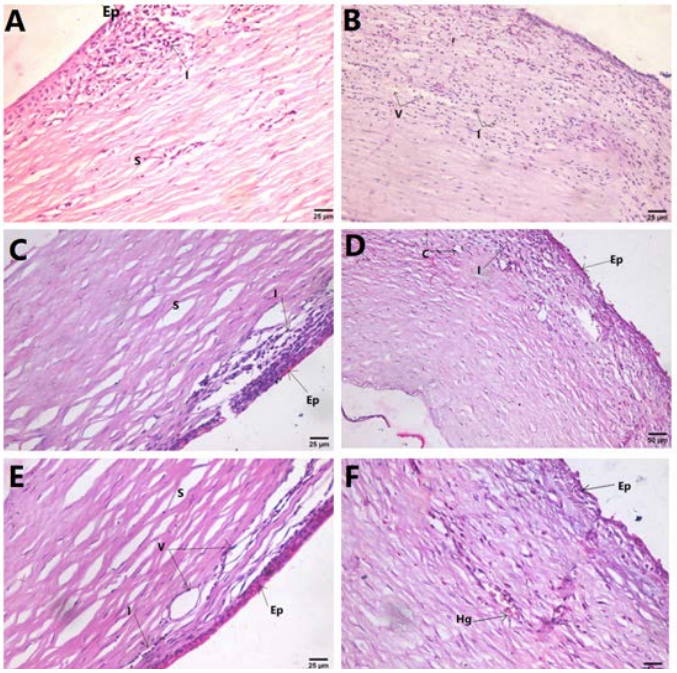

Figure 3: Showing (A); formation of epithelium (Ep) and presence of mononuclear inflammatory cells infiltration (I), stromal edema with thin collagenous lamellae separation giving a waving appearance (S) after one week of treatment in partial central ulcer treated group H&E X 400. (B); Presence of mononuclear inflammatory cells infiltration in stroma (I) and vascularization (V) without formation of epithelium after one week in partial central ulcer control group H&E X 400. C); Formation of more than one raw of epithelium (Ep) with mononuclear inflammatory cells infiltration in stroma (I) Presence of stromal edema with thin collagenous lamellae separation giving a waving appearance (S) after two weeks of treatment in partial central ulcer treated group H&E X 400. D) partial formation of epithelial layer (Ep) with mononuclear inflammatory cells infiltration in stroma (I) and congestion of stromal blood vessels after two weeks in partial central ulcer control group H&E X 200. (E); Showing formation of several layer of epithelium (Ep), stromal edema with thin collagenous lamellae separation giving a waving appearance (S) and presence of low number of mononuclear infiltration (I) and vascularization after three weeks of treatment in partial central ulcer treated group H&E X 400. (F); partial formation of epithelial layer (Ep) with hemorrhage in stroma partial central ulcer control group (Hg) H&E X 400

Table 1: Mean Epithelial Thickness ± SE (µm)

Type of corneal ulcer		Mean Epithelial Thickness ± SD (µm)			
	Groups	Week 1	Week 2	Week 3	P-value (time)
Peripheral partial corneal ulcer	Control	0.00 [b]	10.57±2.22 [a]	18.70±3.50 [a]	<0.001
	Treated	7.93±3.53 [b]*	14.70±2.50 [b]	28.57±2.47 [a]*	<0.001
	P-value (groups)	0.018	NS	0.016	
Central partial corneal ulcer	Control	0.00 [b]	8.14±2.10 [a]	14.46±2.36 [a]	<0.001
	Treated	10.12±1.99 [c]*	24.21±4.11 [b]*	35.33±4.13 [a]*	<0.001
	P-value (groups)	0.001	0.004	0.002	

Table 2: Mean Epithelial Thickness ± SE (µm)

Type of corneal ulcer		Mean Epithelial Thickness ± SD (µm)		
	Groups	Week 1	Week 2	Week 3
Partial corneal ulcer	Peripheral	7.93±3.53 [c]	14.70±2.50 [b]	28.57±2.47 [a]
	Central	10.12±1.99 [c]	24.21±4.11 [b]*	35.33±4.13 [a]
	P-value	NS	0.027	NS

* Asterisk in the same column indicates significant difference between control and treated groups ($P<0.05$).
[a,b,c] Different superscripts in the same row indicate significant differences between different time points (weeks) in the same group (Bonferroni test, $P<0.05$); NS: Not significant; SD: Standard deviation.

Statistical analysis

A two-way repeated measures ANOVA of results of peripheral partial corneal ulcer cases revealed no significant interaction between the effects of treatment and time ($F(2,8) = 1.99$, $P = 0.199$, $\eta p2 = 0.332$). However, main effects analyses showed that treatment ($P = 0.006$) and time (weeks) ($F(2,8) = 90.85$, $P < 0.0001$, $\eta p2 = 0.958$) did have statistically significant effects on average corneal ulcer epithelial thickness (Table 1).

For central partial corneal ulcer cases there was a statistically significant interaction between the effects of treatment and time ($F(2,8) = 13.42$, $P = 0.003$, $\eta p2 = 0.77$). The main effects analyses of both treatment ($P = 0.001$) and time ($F(2,8) = 183.07$, $P < 0.0001$, $\eta p2 = 0.979$) did have statistically significant effects on average corneal ulcer epithelial thickness (Table 1).

Central partial corneal ulcer cases revealed significant higher thickness over time compared to peripheral cases ($P = 0.05$) (Table 2) (Figure 5). On the other hand, time ($F(2,8) = 169.60$, $P < 0.0001$, $\eta p2 = 0.977$) showed statistically significant effects on average corneal ulcer epithelial thickness. No significant interaction between the effects of treatments and time was indicated ($F(2,8) = 4.40$, $P = 0.052$, $\eta p2 = 0.52$).

Results in this study revealed that time (weeks) and treatment of various corneal ulcers did have a statistically significant effects on average corneal ulcer epithelial thickness, which was indicated by the progressively increased corneal epithelial thickness across the testing weeks.

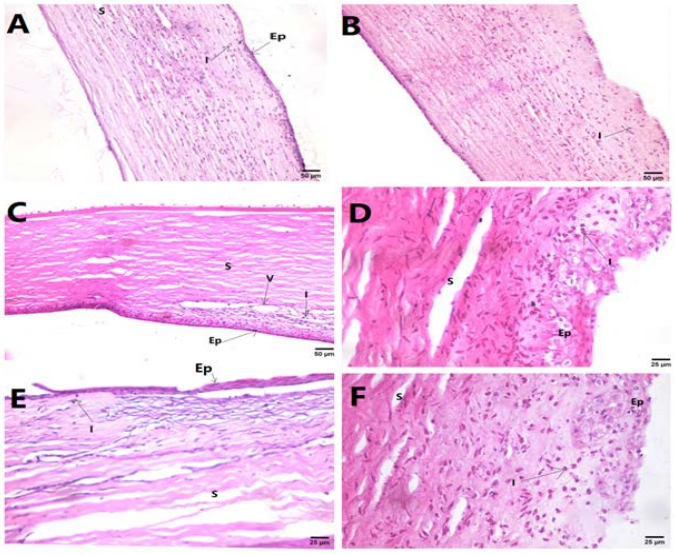

Figure 4: Showing (A); formation of one layer of epithelium (Ep) and presence of mononuclear inflammatory cells infiltration (I), stromal edema with thin collagenous lamellae separation giving a waving appearance (S) after one week of treatment in partial peripheral ulcer treated group H&E X 200. (B); Presence of mononuclear inflammatory cells infiltration in stroma (I) without formation of epithelium after one week in partial peripheral ulcer control group H&E X 200. C); Formation of more than one raw of epithelium (Ep) with mononuclear inflammatory cells infiltration in stroma (I) and vascularization (V). Presence of stromal edema with thin collagenous lamellae separation giving a waving appearance (S) after two weeks

of treatment in partial peripheral ulcer treated group H&E X 200. (D); partial formation of epithelial layer (Ep) with mononuclear inflammatory cells infiltration in stroma (I) Presence of stromal edema with thin collagenous lamellae separation giving a waving appearance (S) after two weeks in partial peripheral ulcer control group H&E X 400. (E); Showing formation of several layer of epithelium (Ep), stromal edema with thin collagenous lamellae separation giving a waving appearance (S) and presence of low number of mononuclear infiltration (I) after three weeks of treatment in partial peripheral ulcer treated group H&E X 400. (F); partial formation of epithelial layer (Ep) with mononuclear inflammatory cells infiltration in stroma (I) Presence of stromal edema with thin collagenous lamellae separation giving a waving appearance (S) after three weeks in partial peripheral ulcer control group H&E X 400.

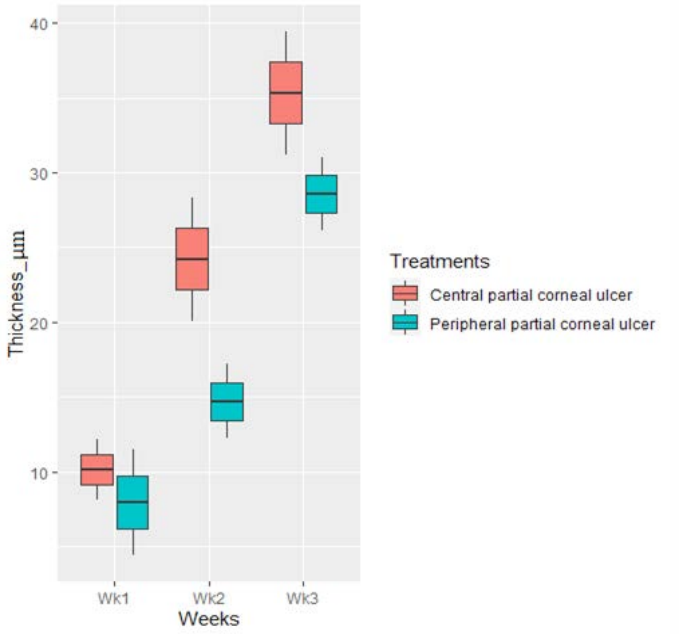

Figure 5: Central partial corneal ulcer cases revealed significant higher thickness over time compared to peripheral cases

DISCUSSION

The cornea, which is the structure of the eye that is primarily exposed to the environment, is particularly susceptible to injury which may be a partial or a complete absence of a portion of corneal tissue from burns, abrasions, inadequate tear production, infections, various medical conditions, and refractive procedures. Such wounds frequently result from such injuries, which start the tissue's healing process. Therefore, corneal wound healing is both a fundamental scientific issue and a major therapeutic challenge.

A single layer of basal cells and four to six layers of stratified squamous epithelial cells make up the corneal epithelium . With the assistance of Limbal stem cell multiplication, immigration, and specialization these layers are regularly shed and replaced to maintain corneal homeostasis . Adult limbal epithelial stem cells, which are situated at corneo-scleral junction's periphery, are necessary for corneal epithelial regeneration . Continuous cells turn over maintain a constant structure and thickness which prevents corneal transparency loss.

In the case of corneal wounds, sheets of epithelial cells start to migrate to close the defect, after that, epithelial cells start to differentiate and stratify. Although it has been conclusively demonstrated that corneal epithelial stem cells play a role in wound healing, the role of stromal and endothelial stem cells in this process is still up for debate . healing of the cornea is a very complicated process starting by cell death then progenitor cell migration, proliferation, myofibroblast differentiation, and modification of the extracellular matrix. Regardless of the type of injury, the inflammatory process and formation of new blood capillaries are usually associated with a fibrotic process in the cornea . The regenerative and reparative abilities of stem cell therapies have increased in interest during the recent years. As previously mentioned, the inflammatory response is crucial to the healing and fibrosis of corneal wounds. Only MSCs among the other stem cell types have the capacity to modulate the immune system. The ability of MSCs derived from various tissues to cure corneal wounds and restoration of corneal transparency has been studied . The therapeutic impact of the transplanted MSCs may be related to the suppression of angiogenesis and inflammation rather than the MSCs' epithelial differentiation Recent researches suggests that EVs which derived from stem cells have the same effects on target tissues that their parental stem cells do.

Stem cell-derived EVs are being investigated for their potential in regenerative medicine and tissue repair. For example, in the treatment of cardiovascular and neurological illnesses . Due to the fact that EVs produced from MSCs have both regenerative and anti-inflammatory effects the area of research became increasingly interested in how MSC-derived EVs affect corneal wound healing and regeneration.

The cargos of EVs, which includes micro RNAs, certain proteins, lipids, and metabolites, control how they behave. Immunomodulatory/anti-inflammatory, antiangiogenic, and antiapoptotic capabilities of MSCs-derived EVs are anticipated to interact during the healing of corneal wounds in a way that favorably shifts the fibrotic process to a regenerative pathway. However, it is still unclear how exactly MSCs and their EVs promote corneal epithelial and stromal wound healing as well as corneal regeneration.

In this study we use New Zealand White rabbits albino rabbit as an experimental model which has the large cornea which acts approximately 30% of the globe , dimensions of the cornea is 14mm vertically & 15mm horizontally, its thickness is 407_20 µm The normal cornea in rabbits is composed of 4 layers, the external epithelial layer with average thickness is 30:40 µm, stroma the thickest part of cornea which is composed of parallel collagen bundles, Descemet membrane's thickness is 7:8 µm which increases in old ages to 15 µm, and the inner layer endothelium as a single layer of the cell which have sodium ATPase pump responsible for keeping the cornea clear. Two types of corneal ulcers were induced in this experiment, central and peripheral ulcers. Usually thermal, mechanical, and chemical injuries, chronic diseases, or genetic defects found near to the limbus lead to Limbal Epithelial Stem Cells loss which causes limbal stem cell deficiency leading to retardation in the corneal healing process and allowing ingrowth of conjunctiva and neovascularization, and corneal opacity . Cultivated Limbal Epithelial Stem Cells or an autologous or allogeneic limbal graft is typically used to repair limbal stem cell deficiency in clinical situations.

Lyophilized xeno-Mesenchymal Stem Cell Extracellular Vesicle which were isolated from canine MSCs were used for the treatment of both central & peripheral corneal ulcers. EVs promoted not only corneal epithelial cell migration and proliferation but also epithelial wound closure. Both treated groups at the 5th day of corneal wound induction appeared fluorescein -ve by fluorescein test that indicates the epithelial wound closure andAS-OCT images showed the appearence of a well-developed epithelial layer and a homogenous intact stroma unlike the control groups at the 5th day appear fluorescein +ve and AS-OCT image that showed irregular plaques of epithelium and hyperreflective lesion with different densities affecting the whole corneal thickness with some lesions appear as stromal fibrosis and thin stromal layer with sub epithelial cyst. Histopathological examination by H&E staining for evaluation of the quality of healing for 3 successive weeks showed that both treated groups had a complete layer of epithelium appearing within 1week which increased in the number of rows in the 2nd and 3rd weeks and stromal edema decreased by time unlike control groups which had weak fragmented epithelium and sub-epithelial hemorrhage and vascularization.

Statistical analysis of epithelial layer thickness revealed that time (weeks) and treatment of various corneal ulcers did have a statistically significant effect on average corneal ulcer epithelial thickness, which was indicated by the progressively increased corneal epithelial thickness across the testing weeks, Central partial corneal ulcer cases revealed significant higher thickness over time compared to peripheral cases.

This finding offers preliminary evidence that Xenogenous EVs have the ability to heal corneal wounds even if in peripheral corneal ulcer with deficiency of limbal stem cell, EVs was able to stimulate the remnant limbal stem cell and accelerate corneal defect healing than control group.

CONCLUSION AND RECOMMENDATIONS

EV as Cell-free therapy is seeming to be a promising therapy due to its advantages over traditional therapies for numerous diseases, lyophilized EVs can be applied topically in outpatient settings and don't need as strict of storage requirements as live tissues and cells do. EVs maintain their potency after lyophilization and storage at room temperature that dramatically expands the number of patients who can get them globally. In terms of safety, complications, quality, regulatory concerns, and cost, EVs are superior to corneal transplantation and live stem cell therapy.

Using EV as Cell-free therapy in treatment of different ocular diseases.

ACKNOWLEDGEMENTS

Dr/ Eslam Elgohary in preparation of histopathological sample. Availability of data and material. All data are available (data transparency).

CONFLICT OF INTEREST

The authors have no conflicting financial interest.

AUTHORS CONTRIBUTIONS

Mohamed Bahr: was responsible for the preparation of the stromal/stem cells and EVs.
Ahmed Abdallah was responsible for histopathology.
Mohamed S. Amer: was responsible for surgical induction of the ulcers and the statistical analysis.
Khaled Abo-EL-Sooud was responsible for histopathology.
Shaimaa M. Kamel: was responsible for the preparation of the stromal/stem cells and EVs.
Marwa A. Fouly: was responsible for AS-OCT evaluation before and after the treatment.
Ashraf Shamaa and Omar EL-Tookhy: were responsible for AS-OCT evaluation before and after the treatment.
All authors read and approved the final manuscript.

REFERENCES

Afsharkhamseh N., Movahedan A., Gidfar S., Huvard M., Wasielewski L., Milani B.Y., Eslani M., Djalilian A.R. (2016). Stability of limbal stem cell deficiency after mechanical and thermal injuries in mice. Exp. Eye Res. 145: 88–92. https://doi.org/10.1016/j.exer.2015.11.012

Andrew S.E. (2002). Corneal diseases of rabbits. Veterinary Clinics: Exotic Anim. Pract. 5: 341–356. https://doi.org/10.1016/S1094-9194(01)00003-2

Bahr M.M., Amer M.S., Abo-El-Sooud K., Abdallah A.N., El-Tookhy O.S. (2020). Preservation techniques of stem cells extracellular vesicles: A gate for manufacturing of clinical grade therapeutic extracellular vesicles and long-term clinical trials. Int. J. Vet. Sci. Med. 8: 1–8. https://doi.org/10.1080/23144599.2019.1704992

Bahr M.M., Amer M.S., Abo-EL-Sooud K., Abdallah A.N., Shehab G.G., El-Tookhy O.S. (2021). Proficiency of Carboxymethylcellulose as a Cryoprotectant. Clinical and Histological Evaluation of Cryopreserved Heterogenous Mesenchymal Stem Cell-Exosomal Hydrogel on Critical Size Skin Wounds in Dogs. Int. J. Hematol. Oncol. Stem Cell Res. 15: 178. https://doi.org/10.18502/ijhoscr.v15i3.6848

Ballim, S., (2013). Corneal ulcers: For the general practitioner. CME: Your SA J. CPD. 31: 153–155.

Biancone L., Bruno S., Deregibus M.C., Tetta C., Camussi G. (2012). Therapeutic potential of mesenchymal stem cell-derived microvesicles. Nephrol. Dialy. Transplant. 27: 3037–3042. https://doi.org/10.1093/ndt/gfs168

Biber J.M., Holland E.J., Neff K.D. (2010). Management of ocular stem cell disease. Int, Ophthalmol, Clin. 50: 25–34. https://doi.org/10.1097/IIO.0b013e3181e20d64

Camussi G., Deregibus M.C., Cantaluppi V. (2013). Role of stem-cell-derived microvesicles in the paracrine action of stem cells. Biochem. Soc. Trans. 41: 283–287. https://doi.org/10.1042/BST20120192

Deng S.X., dos Santos A., Gee S. (2020). Therapeutic potential of extracellular vesicles for the treatment of corneal injuries and scars. Transl. Vis. Sci. Technol. 9: 1. https://doi.org/10.1167/tvst.9.12.1

Dennis D., Abidin T., Tarigan R., Zudhistira C. (2018). Anti-inflammatory effect of mangosteen rind extract in rabbit teeth with reversible pulpitis (*In vivo* study). Int. J. Clin. Dent. 11: 79–87.

Di Girolamo N., Bobba S., Raviraj V., Delic N.C., Slapetova I., Nicovich P.R., Halliday G.M., Wakefield D., Whan R., Lyons J.G. (2015). Tracing the fate of limbal epithelial progenitor cells in the murine cornea. Stem Cells. 33: 157–169. https://doi.org/10.1002/stem.1769

Doyle L.M., Wang M.Z. (2019). Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. Cells. 8: 727. https://doi.org/10.3390/cells8070727

Eslani M., Putra I., Shen X., Hamouie J., Afsharkhamseh N., Besharat S., Rosenblatt M.I., Dana R., Hematti P., Djalilian A.R. (2017). Corneal mesenchymal stromal cells are directly antiangiogenic via PEDF and sFLT-1. Invest. Ophthalmol. Vis. Sci. 58: 5507–5517. https://doi.org/10.1167/iovs.17-22680

Flecknell P.A., Richardson C.A., Popovic A., Carpenter R.E., Brunson D.B. (2011). Anesthesia and immobilization of small mammals. Essentials of small animal anesthesia and analgesia. 2nd edition. Wiley-Blackwell. 300–325.

Gregory C.D., Dransfield I. (2018). Apoptotic tumor cell-derived extracellular vesicles as important regulators of the onco-regenerative niche. Front Immunol. 9: 1111. https://doi.org/10.3389/fimmu.2018.01111

Gupta N., Varshney A., Ramappa M., Basu S., Romano V., Acharya M., Gaur A., Kapur N., Singh A., Shah G. (2022). Role of AS-OCT in Managing Corneal Disorders. Diagnost. 12: 918. https://doi.org/10.3390/diagnostics12040918

Hassell J.R., Birk D.E. (2010). The molecular basis of corneal transparency. Exp. Eye Res. 91: 326–335. https://doi.org/10.1016/j.exer.2010.06.021

Khairnar S., Kini R., Harwalkar M., Chaudhari, S.R., 2014. A Review on Freeze Drying Process Pharmaceut. Int. J. Res. Pharm. Sci. 4.

Kinnaird T., Stabile E., Burnett M.S., Lee C.W., Barr S., Fuchs S., Epstein S.E. (2004). Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. Circ. Res. 94: 678–685. https://doi.org/10.1161/01.RES.0000118601.37875.AC

Le Q., Chauhan T., Yung M., Tseng C.-H., Deng S.X. (2020). Outcomes of limbal stem cell transplant: a meta-analysis. JAMA Ophthalmol. 138: 660–670. https://doi.org/10.1001/jamaophthalmol.2020.1120

Lopez de la Fuente C., Sánchez-Cano A., Segura F., Hospital E.O., Pinilla I. (2016). Evaluation of total corneal thickness and corneal layers with spectral-domain optical coherence tomography. J. Refract. Surg. 32: 27–32. https://doi.org/10.3928/1081597X-20151207-03

Ma Y., Xu Y., Xiao Z., Yang W., Zhang C., Song E., Du Y., Li L. (2006). Reconstruction of chemically burned rat corneal surface by bone marrow–derived human mesenchymal stem cells. Stem Cells. 24: 315–321. https://doi.org/10.1634/stemcells.2005-0046

Mohammadpour M., Khorrami-Nejad M., Naghdi T. (2022). Modified big bubble technique for deep anterior lamellar keratoplasty in keratoconus. Eur. J. Ophthalmol. 11206721221125292. https://doi.org/10.1177/11206721221125293

Oriá A.P., Gomes Junior D.C., Arraes E.A., Estrela-Lima A., Pinna M.H., Meneses Í.D.S., Martins Filho E.F. (2014). Tear production, intraocular pressure and conjunctival microbiota, cytology and histology of New Zealand rabbits (*Oryctolagus cuniculus*). Pesquisa Vet. Brasil. 34 1024–1028. https://doi.org/10.1590/S0100-736X2014001000016

Phinney D.G., Pittenger M.F. (2017). Concise review: MSC-derived exosomes for cell-free therapy. Stem Cells. 35: 851–858. https://doi.org/10.1002/stem.2575

Qiu G., Zheng G., Ge M., Wang J., Huang R., Shu Q., Xu J. (2018). Mesenchymal stem cell-derived extracellular vesicles affect disease outcomes via transfer of microRNAs. Stem Cell Res Ther 9, 1–9. https://doi.org/10.1186/s13287-018-1069-9

Sacchetti M., Rama P., Bruscolini A., Lambiase A. (2018). Limbal stem cell transplantation: clinical results, limits, and perspectives. Stem Cells Int. 2018. https://doi.org/10.1155/2018/8086269

Saghizadeh M., Kramerov A.A., Svendsen C.N., Ljubimov A. V (2017). Concise review: stem cells for corneal wound healing. Stem Cells 35, 2105–2114. https://doi.org/10.1002/stem.2667

Shojaati G., Khandaker I., Funderburgh M.L., Mann M.M., Basu R., Stolz D.B., Geary M.L., dos Santos A., Deng S.X.,

Funderburgh J.L. (2019). Mesenchymal stem cells reduce corneal fibrosis and inflammation via extracellular vesicle-mediated delivery of miRNA. Stem Cells Transl. Med. 8: 1192–1201. https://doi.org/10.1002/sctm.18-0297

Simons M., Raposo G. (2009). Exosomes–vesicular carriers for intercellular communication. Curr. Opin. Cell Biol. 21: 575–581. https://doi.org/10.1016/j.ceb.2009.03.007

Suvarna K.S., Layton C., Bancroft J.D. (2018). Bancroft's theory and practice of histological techniques E-Book. Elsevier health sciences.

Wang M., Yuan Q., Xie L., (2018). Mesenchymal stem cell-based immunomodulation: properties and clinical application. Stem Cells Int. 2018. https://doi.org/10.1155/2018/3057624

Wang X., Gericke A., Ackermann M., Wang S., Neufurth M., Schröder H.C., Pfeiffer N., Müller W.E.G. (2019). Polyphosphate, the physiological metabolic fuel for corneal cells: A potential biomaterial for ocular surface repair. Biomater. Sci. 7: 5506–5515. https://doi.org/10.1039/C9BM01289C

Waring III G.O., Charles S.B. (2010). A matrix of pathologic responses in the cornea. Cornea. St. Louis: Mosby. 47–79. https://doi.org/10.1016/B978-0-323-06387-6.00012-X

Wipperman J., Dorsch J.N. (2013). Evaluation and management of corneal abrasions. Am. Fam. Physician. 87: 114–120.

Yazdanpanah G., Jabbehdari S., Djalilian A.R. (2017). Limbal and corneal epithelial homeostasis. Curr. Opin. Ophthalmol. 28: 348. https://doi.org/10.1097/ICU.0000000000000378

Zhang Y., Hu Y.-W., Zheng L., Wang Q. (2017). Characteristics and roles of exosomes in cardiovascular disease. DNA Cell Biol. 36: 202–211. https://doi.org/10.1089/dna.2016.3496