Original Article

The Potential Therapeutic Effect of Liposomal Ozonated Oil on Experimentally Induced Corneal Alkali Burns in Adult Male Albino Rats (Histological and Immunohistochemical Study)

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ABSTRACT

Introduction: Corneal alkali burns are true ophthalmic emergency cases that need immediate medical intervention to reduce the severe complications and restore patients' vision. Liposomal ozonated oil is a new eye drop formulation with regenerative, anti-inflammatory and antimicrobial properties.

Aim of the Study: Evaluation of the potential therapeutic effect of liposomal ozonated oil on experimentally induced corneal alkali burns in rats by histological and immunohistochemical techniques.

Materials and Methods: Forty adult male albino rats were randomly divided into four equal groups. Group I (control group), group II (liposomal ozonated oil-treated group), group III (alkali burn group), group IV (alkali burn+ liposomal ozonated oil-treated group). At the end of this experiment, corneal specimens were obtained and provided for the light and electron microscopic examination. Morphometric and statistical studies were performed.

Results: Alkali burn group (group III) showed epithelial separation and denudation, vacuolated epithelial cells with the presence of karyolytic nuclei in some cells. There were disorganized collagen fibers with wide spaces in between, degenerated keratocytes, neovascularization and inflammatory cell infiltration. Descemet's membrane detachment was observed in some sites. Morphometric and statistical results demonstrated decreased total corneal and epithelial thickness, decreased area% of collagen fibers, decreased E-cadherin immune-expression and increased Ki-67 immune-expression compared to control group. Group IV revealed much improvement and restoration of corneal structure with nearly normal histological and immunohistochemical pictures.

Conclusion: liposomal ozonated oil highly improved the structure of the cornea burned by an alkali substance. So, it could be considered a unique and a suitable therapy for patients suffering from a corneal alkali burn.

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Key Words: Alkali burn, E-cadherin, Ki 67, liposomal ozonated oil.

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INTRODUCTION

The cornea is described as avascular transparent tissue that located in the anterior part of the eye, considered as a window to the eye. It is the strongest focusing part of the eye which transmits and concentrates light rays on retina for vision generation and its translucence should be maintained for the best visual acuity[1,2].

A wound in the cornea is one of the biggest problems in field of the ophthalmology. It may result from many conditions including mechanical or thermal trauma, burns by chemical materials, infections and immunological defect[3].

Corneal chemical burn is a common ocular injury as it induces massive corneal damage and impairment of vision[4]. Exposure to alkaline or acidic substances can get a burn to the eye, but alkaline agents tend to induce much more serious burns than acidic ones. This is attributed to the hydrophilic and lipophilic nature of the alkali substances which make their association and permeation through the eye surface more rapid[5,6].

Corneal alkali burns are an ophthalmic crisis commonly seen among males (with age range from 20 to 40 years old) working in chemical laboratories and factories. Due to the young age of patients, possible long term disabilities following ocular burns could adversely impact patients' lives[7].

The damage resulting from chemical injuries could be so severe leading to opacification, neovascularization and acute increase in the intraocular pressure due to shrinkage and contraction of the cornea. After the injury, an immediate medical intervention is required to restore the normal ocular surface & corneal clarity and prevent permanent visual impairment[8]. Although the big achievements in treating such cases, the restoration of the normal structure and function of the cornea remains challenging[9].

Ozone (O3) gas is a powerful oxidant agent expressing antifungal, antibacterial and antiviral characteristics as well as tissue-repair promoting property and anti-inflammatory activity. Ozone gas has been utilized in many diseases as skin wound, musculoskeletal disorder, diabetic ulcer, ocular pain and inflammation[10,11,12].

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The gaseous state of ozone makes it highly reactive and not always favorable for topical treatment. Interestingly, the ozone molecule can be established as ozonized oil for topical uses, an organic analog of ozone, formed by making ozone gas to react with double carbon to carbon bonds of unsaturated fatty acids. Ozonated oil is used for the topical treatment of various conditions as trophic ulcers, burns, wounds and anaerobic infections. Using ozone in specific injuries of ocular anterior segment could be advantageous because of its bactericidal, anti-inflammatory and reparative properties[13,14].

As ozonated oil causes high irritation to the tissues of cornea, a certain formulation for ophthalmic use has been recently prepared to improve the tolerability. This formulation is composed of ozonated sunflower oil, encapsulated in liposomes and included in Hypromellose solution[12,15].

The newly formed liposomal ozonated oil formulation was utilized for wound repair promotion, treating some inflammatory and infectious diseases of ocular anterior segment as persistent dystrophic corneal ulcer, granulomatous and vernal conjunctivities i.e. diseases needing adequate regenerative and anti-inflammatory treatment[13].

To our knowledge, the effect of Liposomal ozonated oil on the corneal changes induced by alkali burn is not studied histologically before. Thus, this study has been designed to assess the potential therapeutic effect of liposomal ozonated oil on experimentally induced corneal burns in adult male albino rats by histological and immunohistochemical techniques.

MATERIALS AND METHODS

Chemicals and drugs

1. **NaOH:** was obtained from El-Gomhoria chemicals company (Cairo, Egypt).
2. **Liposomal ozonated oil:** a specific eye drop formulation under trade name, Ozodrop® (FB Vision, San Benedetto del Tronto, Italy).

Animals

We used in this study forty adult male albino rats. Their weights ranged from 150 - 200 g. The rats were housed in well-ventilated cages with maintenance of strict care and hygiene to keep them in normal healthy conditions. They received a balanced diet and drank water freely in animal house of the Faculty of Medicine, Menoufia University. This experiment was done in accordance with Animal Care and Ethical Committee Guidelines of our Faculty (Approval number: 10/2022 HIST28-1).

Experimental design

We equally divided the animals into four main groups (10 rats each). The duration of the experiment was 3 weeks.

Group I (control group): left without treatment during the experiment time.

Group II (Liposomal ozonated oil-treated group): treated 6 times daily with liposomal ozonated oil eye drops, for 3 weeks[16].

Group III (Alkali burn group): Rats of this group were anesthetized with 0.5mg/ kg ketamine by intramuscular injection. Then a filter paper immersed in 1 M NaoH was put on the center of cornea of rat`s right eyes only for 25 seconds then was rinsed with 10 ml of distilled water. The left eyes were left to enable the animals to move, eat and drink. The animals were left without treatment for the rest of the experiment (3 week post-alkali burn)[17].

Group IV (alkali burn + Liposomal ozonated oil-treated group): corneal alkali burns were induced in the rats of this group as described in group III. After burn induction by 24 h, the rats were treated with liposomal ozonated oil eye drops as described in group II.

Methods

At the end of the experiment (3 weeks post-alkali burn), the animals were anesthetized by the intraperitoneal injection of pentobarbital (40 mg/kg)[18], then the right eyes were carefully enucleated and excised. Corneal specimens were collected and processed for examination by the light and electron microscopes.

A- Light microscopic study

Specimens were fixed for 3days in 10% neutral-buffered formalin and processed in the usual way to obtain the ordinary paraffin blocks. Sections of 4μm thick were cut and underwent the following studies:

1. Histological study: Hematoxylin & eosin (H&E) stain and Masson trichrome[19].
2. Immunohistochemical study: for detection of E-cadherin (responsible for corneal epithelial structural and functional integrity) and Ki-67 (cell proliferation marker).

It was performed on 4-mm thick Paraffin sections by using streptavidin–biotin complex technique[20]. The sections were incubated with Primary mouse monoclonal anti E-cadherin antibody; (NeoMarkers/Lab Vision, Fremont, California, USA) and rabbit polyclonal anti-Ki67 (ab15580; Abcam, Massachusetts, USA). The positive control of the primary antibodies was rat colon for E-cadherin and mouse spleen tissue for Ki-67. The negative control was done by adding phosphate buffer solution instead of the primary antibody.

B- Electron microscopic study

Tiny specimens of corneas (1 mm^3) were obtained, fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetraoxide, dehydrated and embedded in epoxy resin. Semi thin sections of 1 μm thick were stained by toluidine blue. Ultrathin sections (70–90 nm) were contrasted with lead citrate and uranyl acetate. All sections of all groups were examined by a JOEL (Japan) electron microscope at the EM unit of Tanta University[21].

Morphometric measurements

1. The mean total corneal thickness (μm) was measured in sections stained with H&E.
2. The mean thickness of corneal epithelium (μm) was measured in H&E-stained sections.
3. The area percentage of collagen fibers was measured in sections stained with Masson's trichrome.
4. The mean Percentage of Ki67 immuno positive cells was measured in immuno-stained sections.

All measurements were done by using a computerized image analyzer system (Leica Q 500 MC Program; Leica, Cambridge, UK) in Anatomy Department, Faculty of Medicine, Menoufia University. They were performed in 10 non-overlapped randomly chosen sections from each group at the same magnification (X 400).

Statistical analysis

Morphometric data were statistically analysed by using SPSS program, version 17, (IBM Corporation, Somers, New York, USA). The data were described as mean ± SEM (standard error of mean). We compared the mean of each group with that of the others using one-way analysis of variance (ANOVA) then "Tuckey" post hoc test. *P value* > 0.05 was regarded non-significant and *P values* < 0.05 were regarded statistically significant but *P values* < 0.001 were considered high significant[22].

RESULTS

Light microscopic results

Histological study

Haematoxylin and Eosin staining: Examination of H&E-stained sections of control group (I) and liposomal ozonated oil-treated group (II) revealed the normal well-known corneal histological structure. From outside inwards, the cornea was composed of stratified squamous non-keratinized epithelium sitting on a regular very thin acidophilic basement membrane named Bowman's membrane. The stroma was avascular and constituted most of corneal thickness. It was composed of orderly arranged collagen fibers and spindle shaped nuclei of keratocytes scattered in between the fibers. Underneath the stroma, there was a thin homogenous acidophilic layer called Descemet's membrane. Finally, a single layer of flat cells with flat nuclei named corneal endothelium was located on the posterior surface of Descemet's membrane (Figures 1A,B). On the other hand, the alkali burn group (III) showed apparent thinning of corneal epithelium with disrupted Bowman's membrane. Some areas showed epithelial separation from underlying stroma and other revealed complete denudation of the epithelium. Vacuolated epithelial cells were observed with the presence of karyolytic or hyperchromatic & apoptotic nuclei in some cells. As regard stroma, there were disorganized collagen fibres with wide space in between and irregularly dispersed keratocytes. Also, neovascularization and inflammatory cells were seen in the stroma. Descemet's membrane detachment was observed in some areas (Figures 2,3,4,5). Interestingly, sections from group IV (Alkali burn+liposomal ozonated oil) exhibited pictures almost similar to those of the control group except for few changes. Continuous corneal epithelium of uniform thickness was observed, but some vacuolated epithelial cells were still seen. The Bowman's membrane appeared intact. The stroma demonstrated regularly arranged collagen fibres with few spaces in between. Also, organized flattened keratocytes were seen. Neovascularization could be detected in some sections. The Descemet's membrane appeared acidophilic, regular, continuous and lined by endothelial cells (Figure 6).

Masson's trichrome staining: The control group (I) and liposomal ozonated oil-treated group (II) revealed green-staining orderly arranged collagen fibers of the stroma with no spaces in between (Figure 7). In contrast, the alkali burn group (III) demonestrated many spaces between disorganized collagen fibers of the stroma (Figure 8). Sections from group IV (Alkali burn+liposomal ozonated oil) exhibited regular appearance of collagen fibers with few spaces in between (Figure 9).

Immunohistochemical study

E-cadherin immunostaining: The control group (I) and liposomal ozonated oil-treated group (II) revealed strong positive E-cadherin immunoexpression in the corneal layers (Figure 10). On the other hand, sections from alkali burn group (III) demonestrated weak positive E-cadherin immunoexpression in the corneal layers (Figure 11). While sections from group IV (Alkali burn+liposomal ozonated oil) exhibited moderate positive E-cadherin immunoexpression in the corneal layers (Figure 12).

Ki-67 immunostaining: The control group (I) and liposomal ozonated oil-treated group (II) demonstrated few Ki67-immunopositive cells with a brownish nuclear coloration in the corneal epithelium (Figure 13). In contrast, the alkali burn group (III) showed numerous Ki67-immunopositive cells in the corneal epithelium (Figure 14). While sections from group IV (Alkali burn+liposomal ozonated oil) exhibited some Ki67-immunopositive cells in the corneal epithelium (Figure 15).

Electron microscopic results

Electron microscopic results of control group (I) and liposomal ozonated oil-treated group (II) revealed normal organization and typical ultrastructure of the corneal layers. The superficial layers of corneal epithelium were formed of regularly arranged squamous cells. The cells were joined together by by numerous electron-dense desmosomes. The apical surface of the outer most cells exhibited short microvilli. The cells had flat euchromatic nuclei and numerous mitochondria (Figure 16). The cells of intermediate layer were connected to each other by many desmosomes. They contained oval nuclei and

mitochondria (Figure 17). The basal layer was composed of columnar cells which connected to each other by numerous desmosomes and to the basement membrane with several electron-dense hemidesmosome. They exhibited euchromatic rounded nuclei and numerous mitochondria (Figure 18).

Regarding the stroma; it was composed of orderly arranged bundles of collagen fibers interspersed with keratocytes. Keratocytes had spindle shape with flat euchromatic nucleui and thin attenuated cytoplasm (Figure 19).

Descemet's membranes appeared regular, homogenous non-cellular and electron dense. It is lined by flattened endothelial cells. The endothelial cells showed flattened euchromatic nuclei and variable sized cytoplasmic pinocytotic vesicles (Figure 20).

The electron microscopic examination of alkali burn group reveled remarkable ultra-structure changes involving the different layers of the cornea. The superficial squamous cells of corneal epithelium were separated by wide intercellular spaces. Most cells exhibited irregular nuclei and degenerated mitochondria (Figure 21). The cells of the intermediate layer contained small shrunken nuclei and degenerated mitochondria. Most cells lost their desmosomal connections and the intercellular spaces revealed obvious expansion (Figure 22).

As for corneal stroma of this group, it displayed few disorganized collagen fibres with focal areas of separated collagen bundles. Degenerated keratocytes were noticed in stroma. The stroma contained many inflammatory cells as neutrophil, eosinophil and telocytes-educated macrophage cells. Long and thin processes (telopodes) of telocytes (a special type of interstitial cells) were noticed in the stroma besides the inflammatory cells with one process making contact with the macrophage. Dilated congested blood vessels were noticed inside stroma. (Figures 23,24,25).

Regarding the Descemet's membranes, it appeared homogenous with no obvious changes. The lining endothelial cells revealed shrunken condensed nucleus. Their cytoplasm showed rarefaction and contained large vacuoles and numerous pinocytotic vesicles (Figure 25).

Interestingly, the electron microscopic examination of group IV (Alkali burn+liposomal ozonated oil) exhibited much amelioration in the corneal ultrastructure with few changes. Most of the superficial squamous cells appeared with euchromatic nuclei, only few cells had heterochromatic condensed nucleus and small vacuoles. The desmosomal connections between the cells were restored in most sections with the presence of some intercellular spaces between the cells (Figures 26,27). Few cells of the intermediate and basal layers appeared irregular in shape and separated by small intercellular spaces. They also had shrunken nuclei, degenerating mitochondria and small vacuoles (Figure 28). In stoma, orderly arranged collagen fibers were observed with spindle-shaped keratocytes between them (Figure 29). Descemet's membrane appeared regular and homogenous. The endothelial cells appeared nearly similar to those of the control except for small vacuoles (Figure 30).

Morphometric and Statistical Results

The mean total corneal thickness

In comparison with control group, the total corneal thickness of liposomal ozonated oil-treated group (II) had non-significant difference ($P>0.05$). On the other hand, the alkali burn group (III) revealed highly significant reduction ($P<0.001$) of total corneal thickness compared to control group. While group IV (Alkali burn+liposomal ozonated oil) showed non-significant decrease ($P>0.05$) compared to control group. Comparing group IV to group III, *p value* was <0.001indicating a highly significant increase in group IV. All the above statistical data were summarized in (Table 1, Diagram 1)

The mean thickness of the corneal epithelium

The thickness of the corneal epithelium of the control group (I) and liposomal ozonated oil-treated group (II) revealed non-significant difference ($P>0.05$). The alkali burn group (III) showed highly significant decrease ($P<0.001$) in the epithelial thickness compared with control group. While group IV (Alkali burn+liposomal ozonated oil) showed significant decrease ($P<0.05$) compared to control group. Comparing group IV to group III, *p value* was <0.001indicating a highly significant increase in group IV. All the above statistical data were summarized in (Table 1 , Diagram 2).

The mean area percentage of collagen fibers

In comparison with control group, the area percentage of collagen fibers of group II did not reveal any significant difference ($P>0.05$). In contrast, the alkali burn group (III) showed highly significant decrease in area percentage of collagen fibers ($P<0.001$) compared to control. In Alkali burn+liposomal ozonated oil group (IV), the *P value* was < 0.05 reflecting significant decrease in area percentage of collagen in this group compared to control group. Comparing group IV to group III, *p value* was <0.001indicating highly significant increase in group IV. All the above statistical data were summarized in (Table 1, Diagram 3)

The mean percentage of Ki67 immuno positive cells

The percentage of Ki67 immuno positive cells of group II revealed non-significant difference ($P>0.05$) compared to control group. Compared to control group, the alkali burn group (III) and Alkali burn+liposomal ozonated oil group (IV) showed highly significant increase ($p<0.001$) in percentage of Ki67 immuno positive cells. Comparing group IV to group III, *p value* was <0.001indicating highly significant decrease in group IV. All the above statistical data were summarized in (Table 1, Diagram 4).

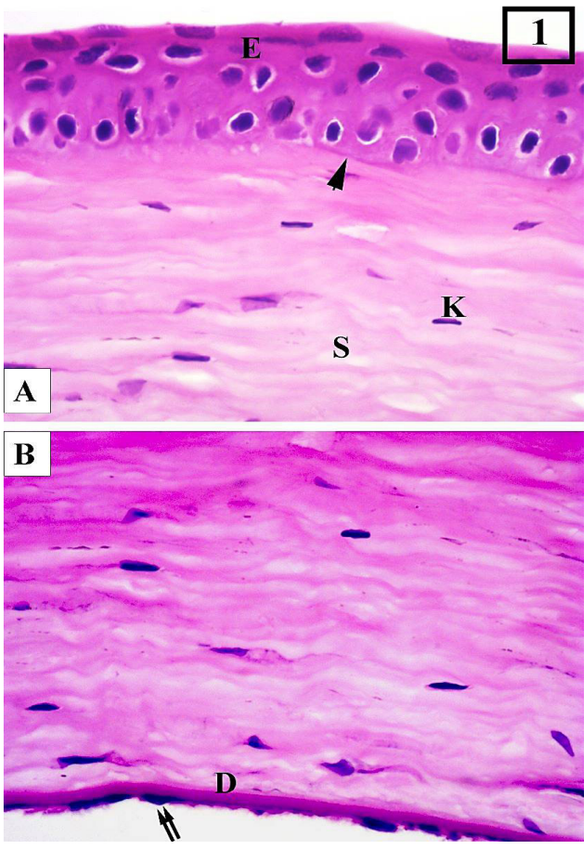


Fig. 1A: A photomicrograph of a rat`s corneal section of control group (I) showing the corneal layers. The non-keratinized stratified squamous epithelium (E) rests on a regular Bowman's membrane (arrow head). The thick stroma (S) consists of regularly arranged collagen fibers and spindle shaped nuclei of keratocytes (K). 1B: The homogenous acidophilic Descemet's membrane (D) is lined by flat endothelial cells (double arrow). H&E, X400

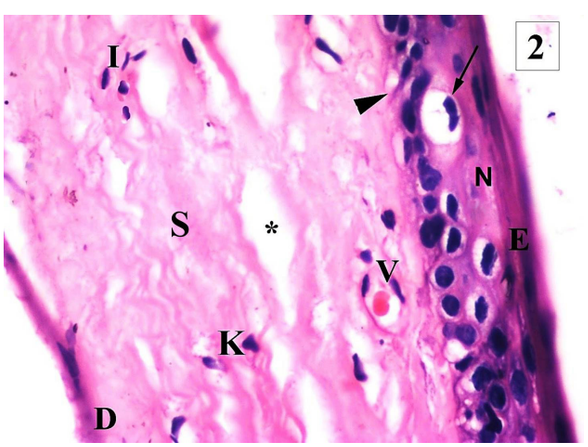


Fig. 2: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing apparent decrease in thickness of epithelium (E) with disrupted Bowman's membrane (arrow head). Some epithelial cells appear with karyolytic nuclei (N) and other with hyperchromatic and apoptotic nuclei (arrow). The stroma (S) has disorganized collagen fibers with wide spaces in between (*), irregularly dispersed keratocytes (K), neovascularization (V), and cellular infiltration (I). Descemet's membrane detachment is observed (D). H&E, × 400

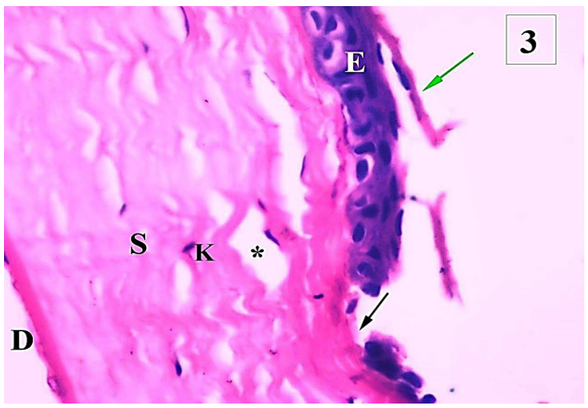


Fig. 3: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing separation of superficial epithelial cells (green arrow) from the underlying epithelium (E). An area shows complete denudation of the epithelium (black arrow) with disrupted Bowman's membrane. The stroma shows disorganized collagen fibers with wide spaces (*) in between and irregularly dispersed keratocytes (K). Notice: Descemet's membrane (D) appears normal. H&E, × 400

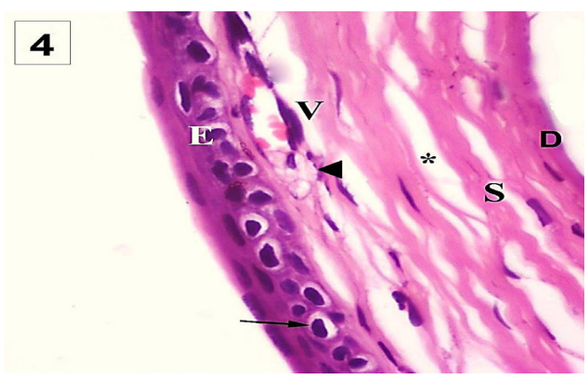


Fig 4: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing apparent thinning of the corneal epithelium (E). Some epithelial cells show vacuolated cytoplasm (arrow). The stroma (S) consists of disorganized collagen fibers and wide spaces (*). Neovascularization (V) in the stroma is also observed. A cell with foamy cytoplasm (arrow head) is noticed in the stroma, most probably macrophage. Descemet's membrane (D) appears thin and faint. H&E, × 400

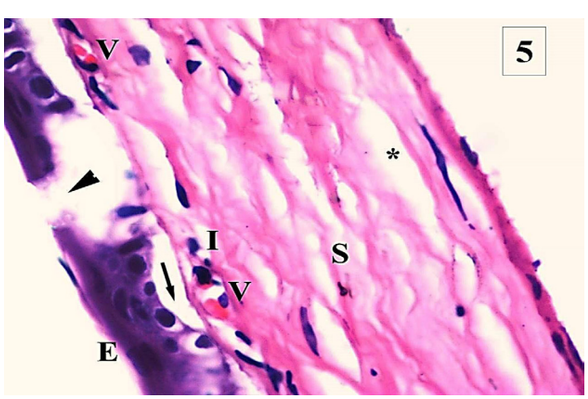


Fig. 5: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing separation of the epithelium (E) from the underlying stroma (arrow). An area shows complete denudation of the epithelium with disrupted Bowman's membrane (arrow head) .The stroma (S) shows disorganized collagen fibers and wide spaces (*). Neovascularization (V) with inflammatory cells (I) are seen. H&E, × 400

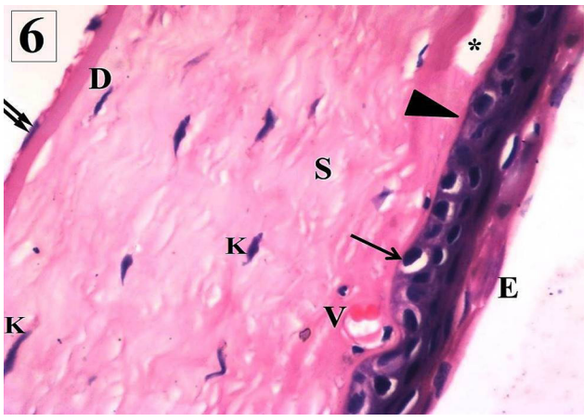


Fig. 6: A photomicrograph of a rat`s corneal section of alkali burn+Liposomal ozonated oil group (IV) showing continuous corneal epithelium (E) of uniform thickness and intact Bowman's membrane (arrow head). Some vacuolated epithelial cells (arrow) are seen. The stroma (S) shows regularly arranged collagen fibres and organized flattened keratocytes (K). Neovascularization (V) and few spaces (*) are still seen in the stroma. The Descemet's membrane (D) appears acidophilic, regular, continuous and lined by endothelial cells (double arrow). H&E, × 400

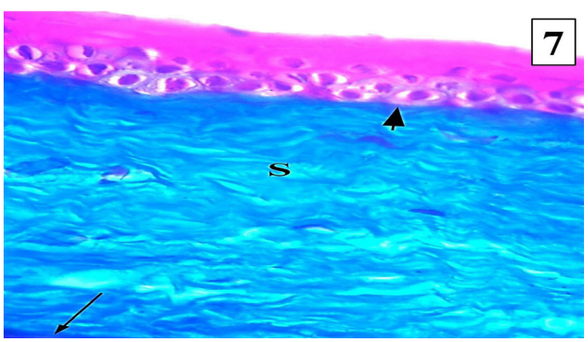


Fig. 7: A photomicrograph of a rat`s corneal section of control group (I) showing corneal stroma (S) consisted of green-staining regularly arranged collagen fibers with no spaces in between. Notice: regular continuous Bowman's membrane (short arrow) and Descemet's membrane (arrow). Masson's trichrome, x 400

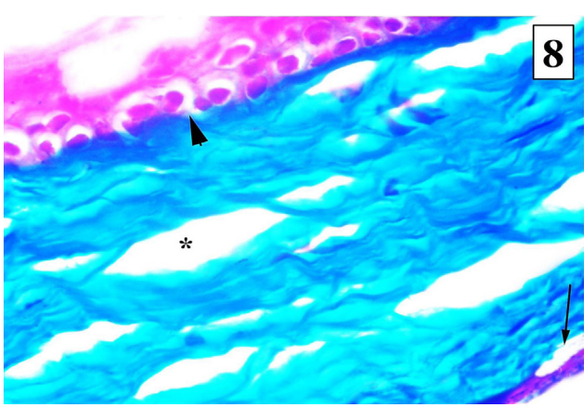


Fig. 8: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing disorganized stromal collagen fibers with many spaces (*) in between. Irregular Bowman's membrane (short arrow) is seen. Notice: Descemet's membrane (arrow) appears separated from the stroma. Masson's trichrome, x 400

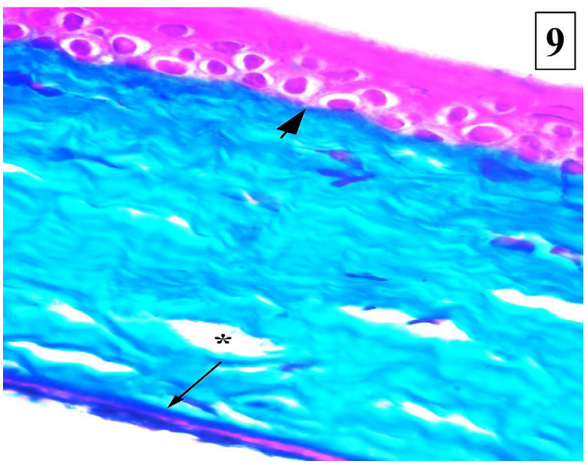


Fig. 9: A photomicrograph of a rat`s corneal section of alkali burn+Liposomal ozonated oil group (IV) showing regular appearance of collagen fibers with few spaces (*) in between. Notice: regular continuous Bowman's membrane (short arrow) and Descemet's membrane (arrow). Masson's trichrome x 400

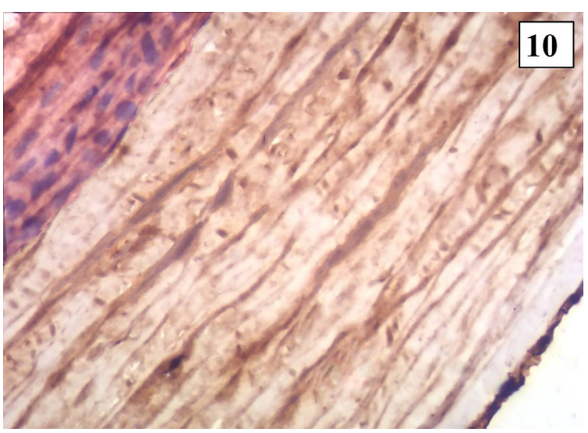


Fig 10: photomicrograph of a rat`s corneal section of control group (I) showing strong positive E-cadherin immunoexpression in the corneal layers. E-cadherin, x 400

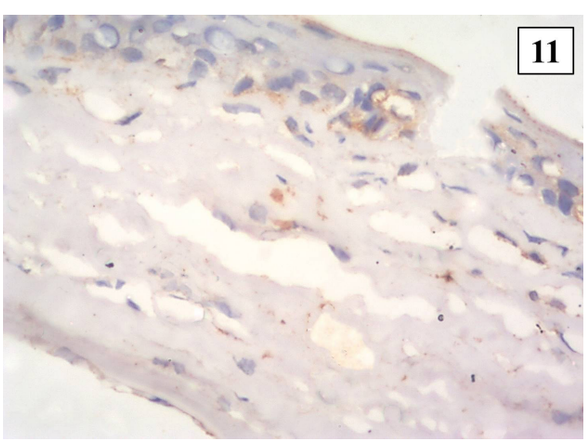


Fig 11: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing weak positive E-cadherin immunoexpression in the corneal layers. E-cadherin, x 400

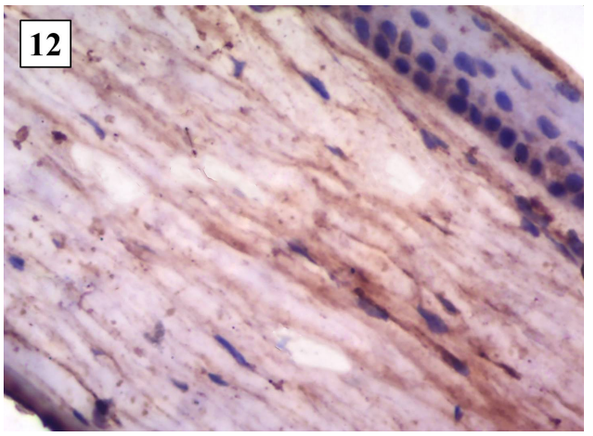


Fig 12: A photomicrograph of a rat`s corneal section of alkali burn+Liposomal ozonated oil group (IV) showing moderate positive E-cadherin immunoexpression in the corneal layers. E-cadherin, x 400

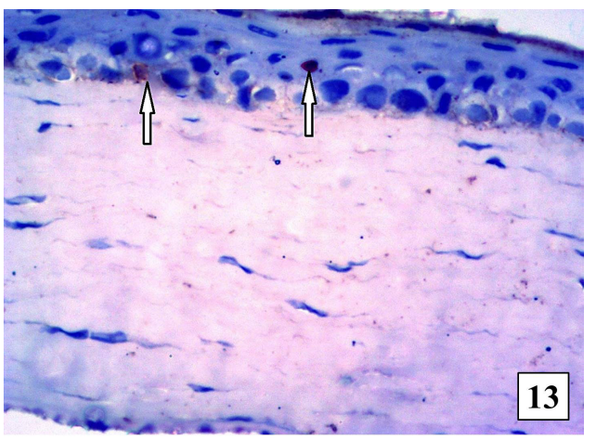


Fig 13: A photomicrograph of a rat`s corneal section of control group (I) showing few Ki67-immunopositive cells (arrow) in the corneal epithelium with a brownish nuclear coloration. Ki67, x 400

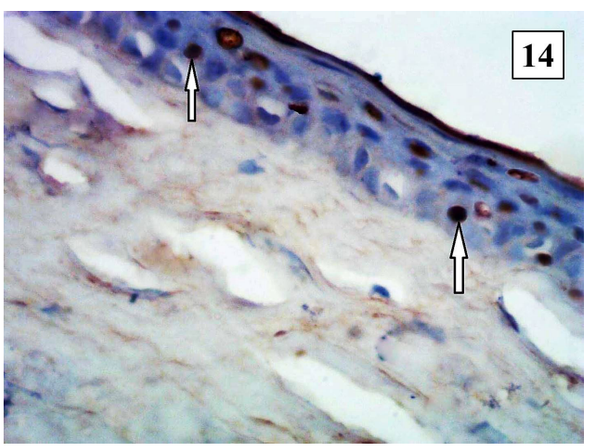


Fig 14: A photomicrograph of a rat`s corneal section of alkali burn group (III) showing numerous Ki67-immunopositive cells (arrow) in the corneal epithelium with a brownish nuclear coloration. Ki67, x 400

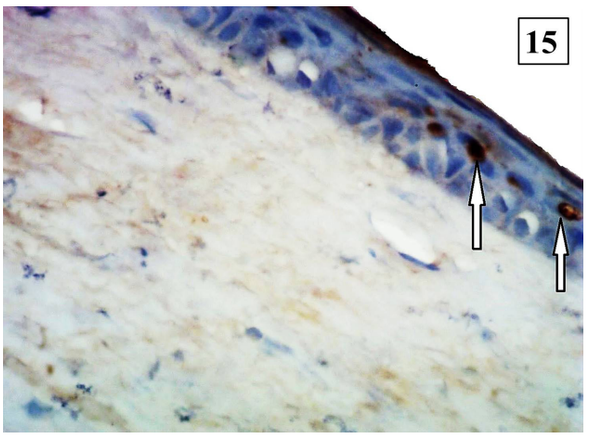


Fig 15: A photomicrograph of a rat`s corneal section of alkali burn+Liposomal ozonated oil group (VI) showing some Ki67-immunopositive cells (arrow) in the corneal epithelium with a brownish nuclear coloration. Ki67 x 400

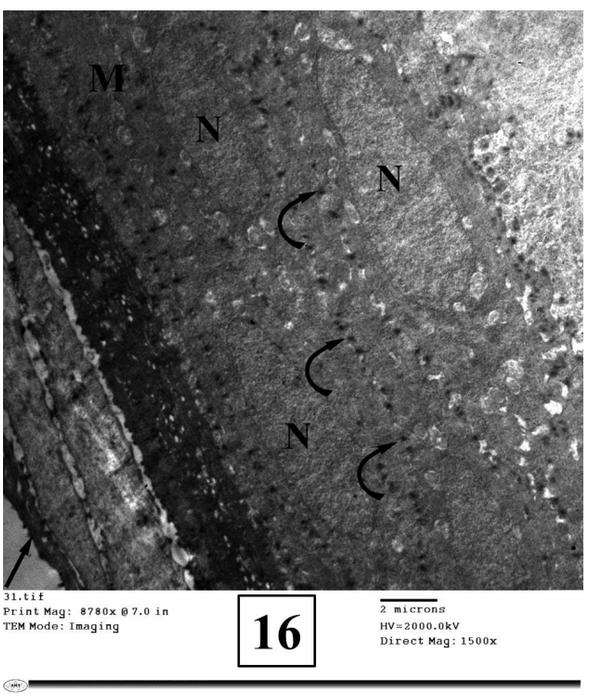


Fig. 16: An electron micrograph of a rat`s cornea of control group (I) showing the squamous cells of the superficial layer of corneal epithelium. They contain flattened euchromatic nuclei (N) and numerous mitochondria (M). The cells are connected by numerous electron-dense desmosomes (curved arrows). Notice: short apical microvilli on the surface of outermost cells (arrow). (TEM X 1500)

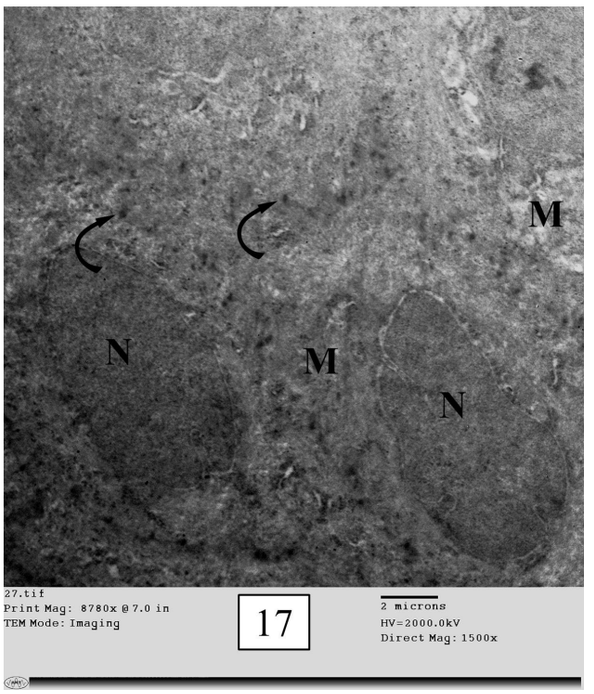


Fig. 17: An electron micrograph of a rat`s cornea of control group (I) showing the cells of the middle layer of the corneal epithelium with oval nuclei (N), mitochondria (M). The cells are connected to each other by many electron-dense desmosomes (curved arrows). (TEM X 1500)

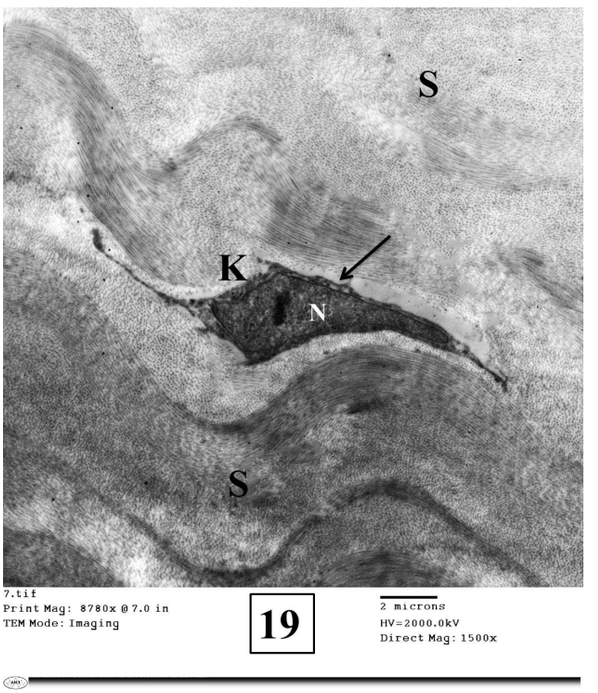


Fig. 19: An electron micrograph of a rat`s cornea of control group (I) showing regular arrangement of the collagen fibre of corneal stroma (S). Keratocyte (K) appears spindle shaped with flat euchromatic nucleus (N) and thin rim of the cytoplasm (arrow). (TEM X 1500)

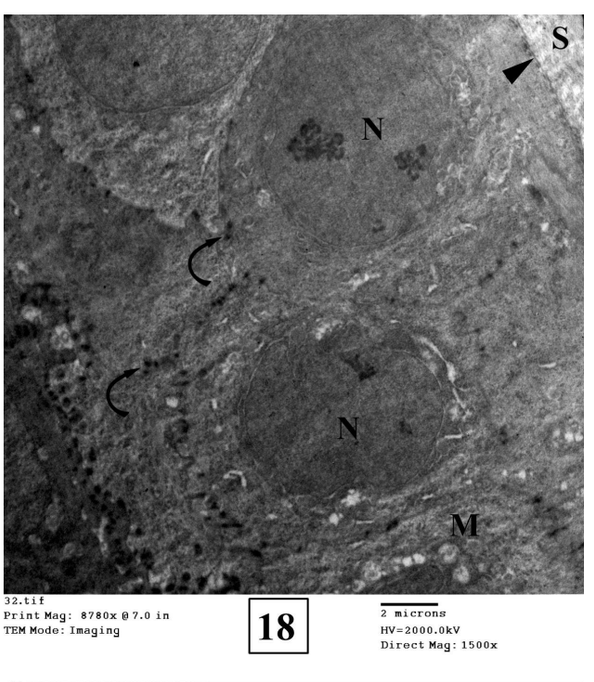


Fig. 18: An electron micrograph of a rat`s cornea of control group (I) showing the columnar cells of basal layer of corneal epithelium connected to each other by desmosomes (curved arrows) and to the basement membrane by several electron-dense hemidesmosome (arrow head). They contain euchromatic rounded nuclei (N) and numerous mitochondria (M). Notice: part of the stroma is seen (S). (TEM X 1500)

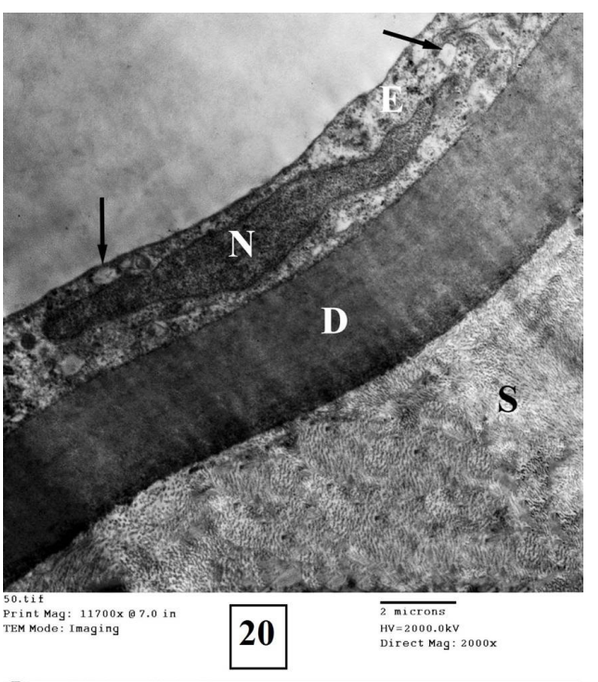


Fig. 20: An electron micrograph of a rat`s cornea of control group (I) showing regular homogenous, non-cellular electron dense Descemet's membrane (D). The lining endothelial cell (E) shows a flattened euchromatic nucleus (N) and the cytoplasm contains variable sized pinocytotic vesicles (arrow). Notice: regularly ordered collagen fibres of corneal stroma (S). (TEM X2000)

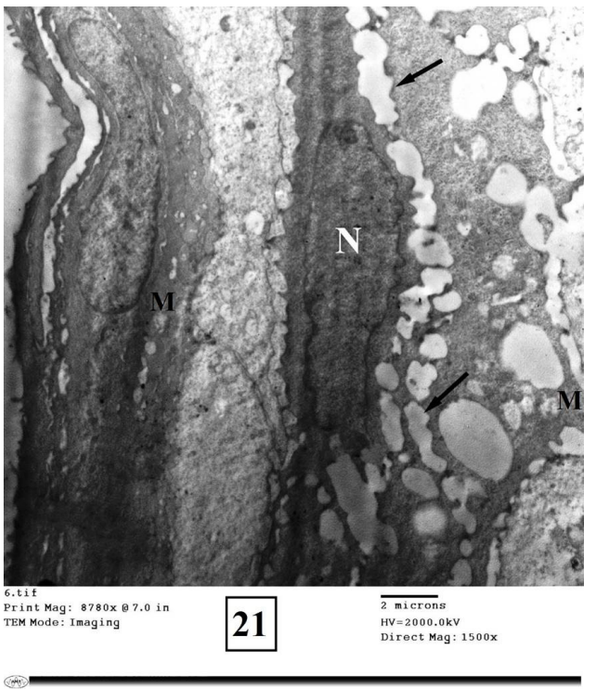


Fig. 21: An electron micrograph of a rat`s cornea of alkali burn group (III) showing the squamous cells of the superficial layer separated by wide intercellular spaces (arrows). Most cells have degenerated mitochondria (M). Some cells contain irregular nuclei (N). (TEM X 1500)

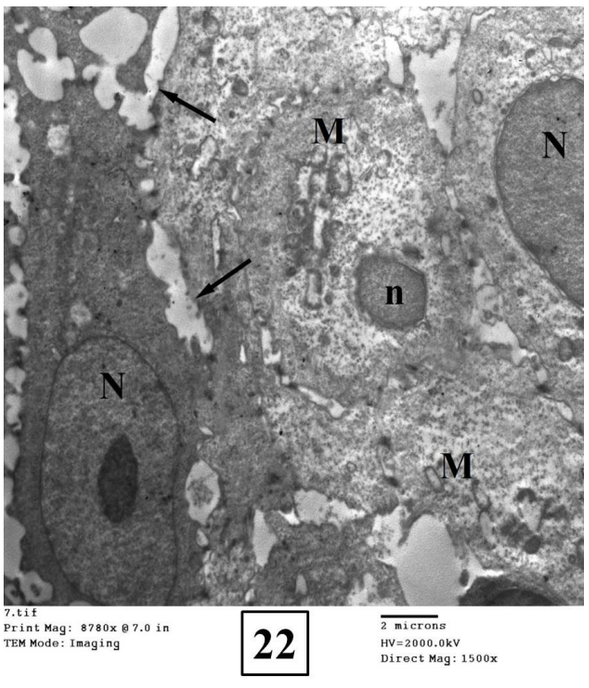


Fig. 22: An electron micrograph of a rat`s cornea of alkali burn group (III) showing some cells of the intermediate layer with small shrunken nuclei (n) and degenerated mitochondria (M). Others contain euchromatic nuclei (N). Cells lose their desmosomal connections with expansion of the intercellular spaces (arrows). (TEM X 1500)

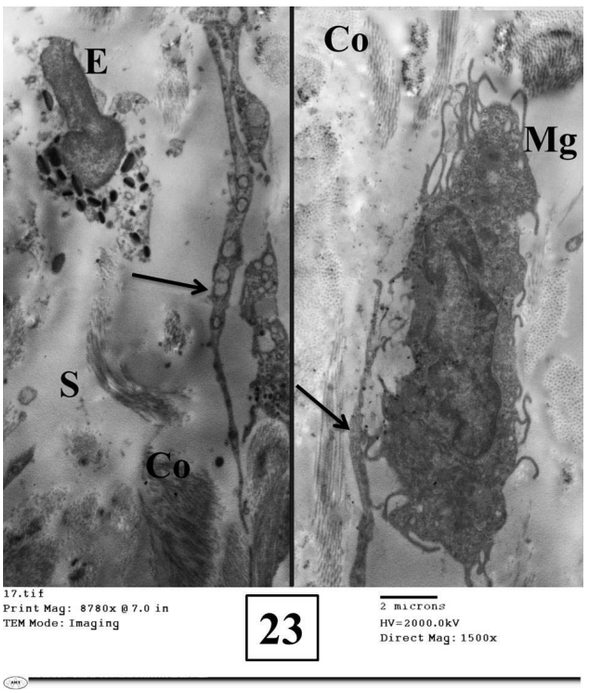


Fig. 23: An electron micrograph of a rat`s cornea of alkali burn group (III) showing corneal stroma (S) containing few disorganized collagen fibres (Co) with focal separation in some areas. Eosinophilic cell with characteristic granules (E) and telocytes-educated macrophage with characteristic nucleus and pseudopodia (Mg) are seen. Notice: long and thin processes (telopodes) of telocytes (arrows) appear beside the inflammatory cells with one process contacts the macrophage. (TEM X 1500)

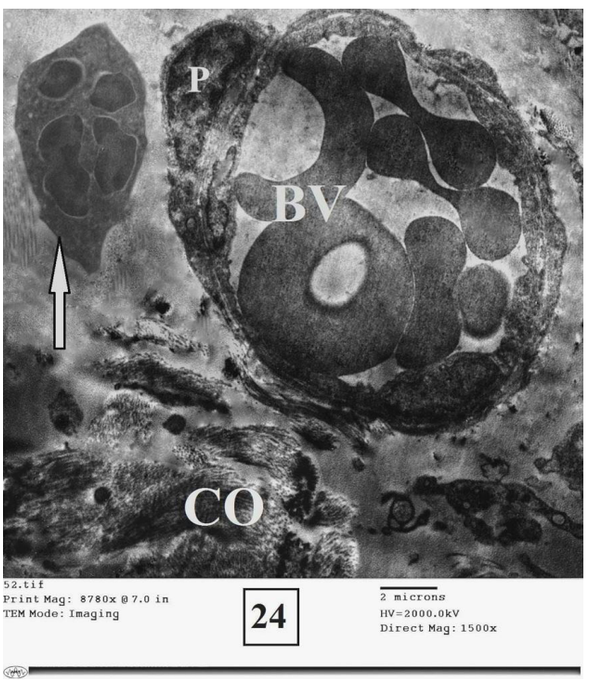


Fig. 24: An electron micrograph of a rat`s cornea of alkali burn group (III) showing a dilated congested blood vessel (BV) surrounded by the pericytes (P) in the stroma. Neutrophil (arrow) is seen near the blood vessel. Notice: disorganised collagen fibres (Co) in the stoma. (TEM X 1500)

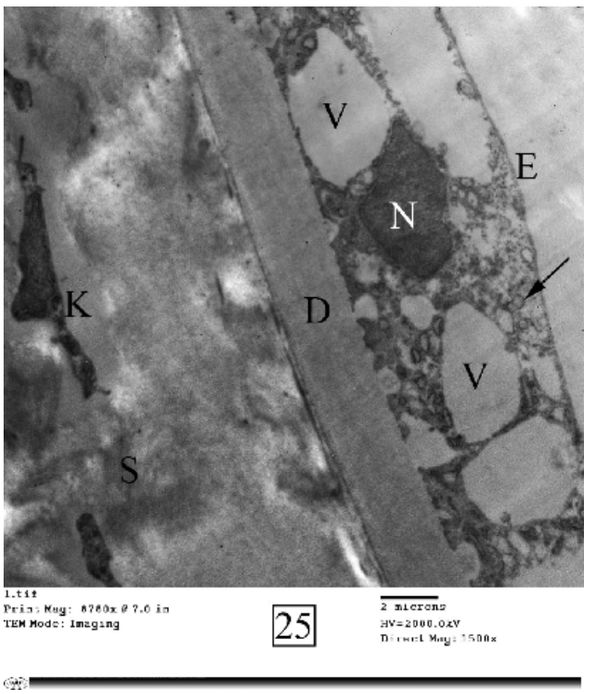


Fig. 25: An electron micrograph of a rat`s cornea of alkali burn group (III) showing homogenous Descemet's membrane (D) lined by endothelial cell (E). Endothelial cell reveals shrunken condensed nucleus (N), rarefied cytoplasm with large vacuoles (V) and numerous pinocytotic vesicles (arrow). Notice: Area of corneal śtroma (S) with degenerated keratocyte (K). (TEM X 1500)

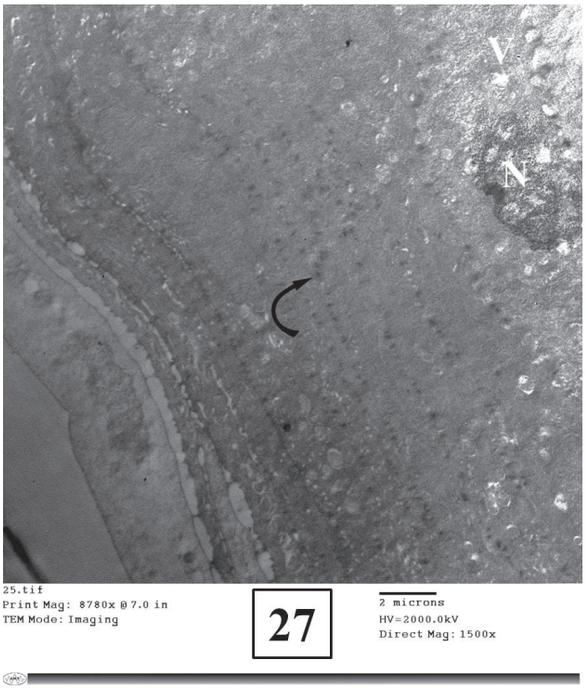


Fig. 27: An electron micrograph of a rat`s cornea of alkali burn+Liposomal ozonated oil group (IV) showing Few cells of superficial layer with heterochromatic condensed nucleus (N) and small vacuoles (V). Notice: the presence of desmosomes (curved arrow) connecting the cells to each other. (TEM X 1500)

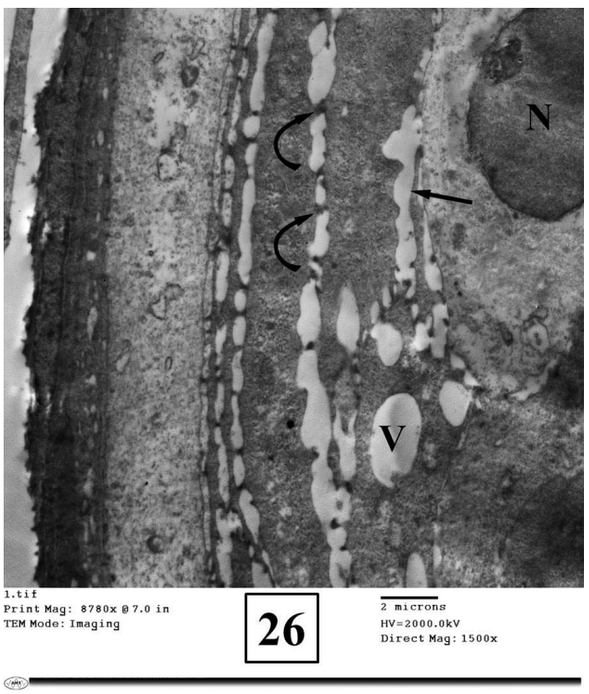


Fig. 26: An electron micrograph of a rat`s cornea of alkali burn+Liposomal ozonated oil group (IV) showing superficial squamous cells with euchromatic nuclei (N). Notice the presence of some intercellular spaces (arrow) and desmosomes (curved arrows) connecting the cells to each other. Some vacuoles (V) are seen. (TEM X 1500)

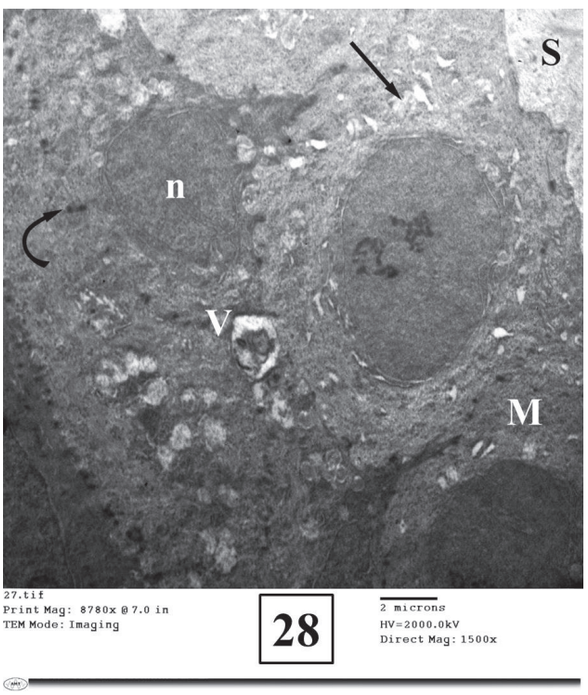


Fig. 28: An electron micrograph of a rat`s cornea of alkali burn+Liposomal ozonated oil group (IV) showing irregular shaped cells of the intermediate and basal layers separated by small intercellular spaces (arrows). Few nuclei (n) appears shrunken. The cytoplasm contains mitochondria with disrupted criśtae (M) and vacuoles (V). Part of śtroma (S) is seen. (TEM X 1500)

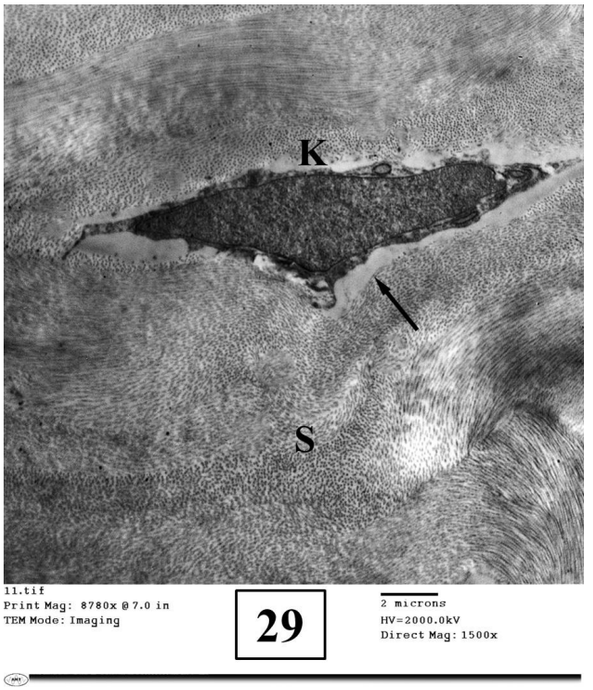


Fig. 29: An electron micrograph of a rat`s cornea of alkali burn+Liposomal ozonated oil group (IV) showing the stroma (S) with regularly arranged collagen fibres (arrow) and a spindle-shaped keratocyte (K) with an oval elongated nucleus. (TEM X 1500)

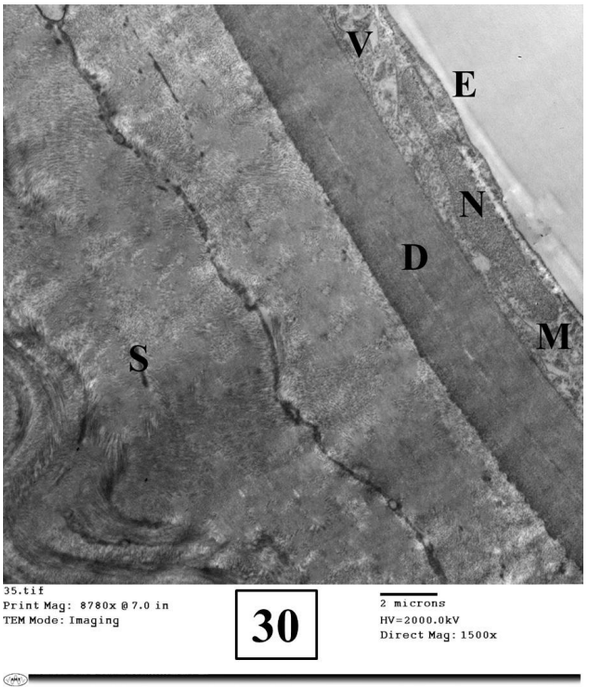


Fig. 30: An electron micrograph of a rat`s cornea of alkali burn+Liposomal ozonated oil group (IV) showing homogenous Descemet's membrane (D). The endothelial cell (E) appears attenuated with flattened nucleus (N) and cytoplasm contains mitochondria with disrupted cristae (M) and small vacuoles (V). Notice: Stroma (S) with regular arrangement of collagen fibers. (TEM X 1500)

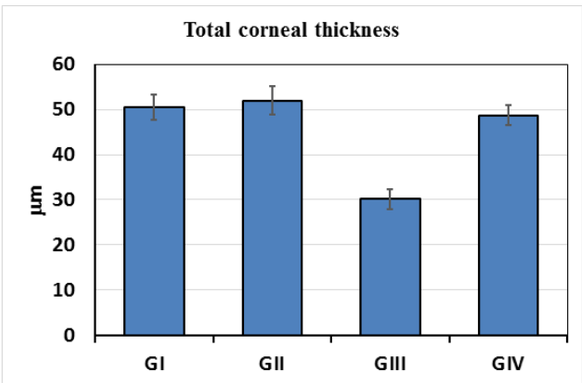
Table 1. The morphometric results in the control and experimental groups

	Group I	Group II	Group III	Group IV	*P.value*
Total corneal thickness Mean ± SD	50.5±2.71	52.01±3.1	30.15±2.17	48.7±2.19	(P1>0.05) * (P2<0.001) *** (P3>0.05) * (P4<0.001) ***
Thickness of corneal epithelium Mean ± SD	11.14±0.7	10.9±0.94	5.69±0.56	8.99±0.87	(P1>0.05) * (P2<0.001) *** (P3<0.05) ** (P4<0.001) ***
Area percentage of collagen fibers Mean ± SD	80.11.57±	78.01.97±	57.02.11±	73.11.9±	(P1>0.05) * (P2<0.001) *** (P3<0.05) ** (P4<0.001) ***
Percentage of Ki67 immuno positive cells Mean ± SD	6.73±0.89	6.010.13±	21.791.79±	11.231.88±	(P1>0.05) * (P2<0.001) *** (P3<0.001) *** (P4<0.001) ***

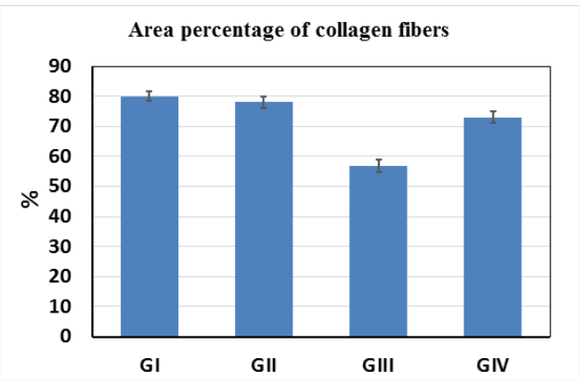
P1: Group I V Group II P2:Group I V Group III

P3: Group I V Group IV P4: Group III V Group IV

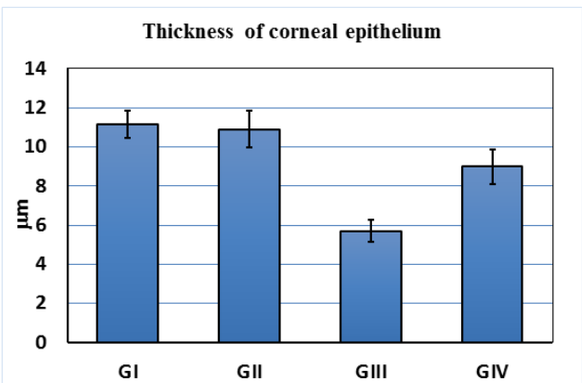
Non-significant * (*P*> 0.05) Significant** (*P*<0.05) Highly significant*** (*P*<0.001)



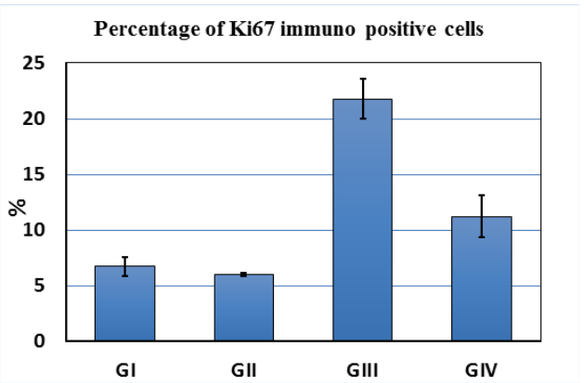
Histogram 1: The mean total corneal thickness in the control and experimental groups



Histogram 3: The mean area percentage of collagen fibers in the control and experimental groups



Histogram 2: The mean thickness of corneal epithelium in the control and experimental groups



Histogram 4: The mean percentage of Ki67 immuno positive cells in the control and experimental groups

DISCUSSION

The cornea is the anterior part of eye and it has to be translucent for perfect visual acuteness. Its accurate arrangement of collagen fibers and unique avascular characteristics prohibit light from dissipation, leading to a focused image on the retina. The corneal injury by a chemical substance can threaten its translucence by inflammation, neovascularization, and persistent scarring[23].

Corneal alkali burns are considered one of the true emergencies in the ophthalmology that demand quick and intense assessment and treatment. The sequelae of corneal burn can be severe and challenging to manage, resulting in permanent visual impairment[8,24].

Liposomal ozonated oil is a new eye drop formulation with anti-inflammatory, bactericidal and regenerative properties applied to enhance wound repair and treat some infectious and inflammatory diseases of the anterior eye segment[13].

Therefore, the purpose of our study was the evaluation of the anti-inflammatory and regenerative (therapeutic) effect of liposomal ozonated oil on the experimentally induced corneal alkali burn in rats by histological and immunohistochemical techniques.

In the current study, the light and electron microscopic examination of the cornea of alkali burn group (III) revealed several histological alterations in the whole corneal layers. There were epithelial thinning with disrupted Bowman's membrane, epithelial separation & denudation, degeneration & vacuolation of epithelial cells. Few disorganized collagen fibers, neovascularization, inflammatory cellular infiltration and degenerated Keratocytes were observed in the stroma. Also, Descemet's membrane detachment was noticed with degenerated vacuolated endothelial cells. Similar findings were reported by previous histological studies on corneal alkali burns induced by NaoH[9,25,26]. Moreover, our results were in line with a recent study using histological and imaging techniques to assess alkali injury on mouse cornea and the researchers detected stromal splitting, Descemet's membrane detachment, neovascularization and cellular infiltration in the burned cornea[27].

The effect of NaoH on the cornea was attributed to dissociation of alkali into cation and a hydroxyl ion in the eye. The hydroxyl ion saponifies cell membrane fatty acids leading to cell damage, while cation interacts with collagen and glycosaminoglycans of stroma causing their destruction. This interaction leads to deep permeation of alkali into the cornea and the deeper eye parts[28,29].

Some researchers[29] explained the deleterious effect of NaoH on cornea by the occurrence of oxidative stress with enhanced ROS production immediately after the injury. They added that oxidative disturbances in untreated burn leads to increased expression of nitrotyrosine and malondialdehyde (markers of oxidative stress and lipid peroxidation) in the epithelium of the cornea. Moreover, several inflammatory cells and neovasculature were found in the stroma with high expression of matrix proinflammatory cytokines and metalloproteinase 9.

Also, other researchers[30,31] attributed the effect of NaoH on cornea to its strong inflammatory reaction with cellular infiltration and release of cytokines oxidative derivatives and proteolytic enzymes that could cause severe loss of the extracellular matrix.

Concerning the corneal stroma of group III, the light and electron microscopic results revealed disorganization and wide separation of collagen fibers and this was supported by morphometric and statistical results of area percentage of collagen fibers in sections stained with Masson's trichrome. In our study, there was a highly significant decrease in the area percent of collagen of this group and such finding coincided with a previous study[32]. Some authors[33] attributed these changes to rapid infiltration of inflammatory cells into the cornea following alkali burn. They stated that neutrophils release matrix metalloproteinases and several proteases that cause dissolution and melting of collagen fibers and hence inhibition of the stromal regeneration process. Our results supported this explanation as there was inflammatory cells infiltration in corneal stroma including neutrophils, eosinophils and macrophages.

The macrophage appearing in our study had contact with one process of telocyte reflecting an activation of macrophage by the telocyte and hence it became telocytes-educated macrophage. Such finding was in harmony with previous studies on telocytes[34,35]. Marini *et al*.,[34] stated that the telocyte is a special type of interstitial cells recently described in the stroma of many organs (as cornea) in vertebrates including humans. Moreover, this special stromal cell is being implicated in many pathologies with possible applications in regenerative medicine. They added that the telocyte is composed of a small cell body and very long and thin prolongations, termed telopodes. Jiang *et al*.,[35] reported that the telopodes of telocytes connected to various activated immunocytes (macrophages, mast cells, eosinophils and neutrophils) via heterocellular junctions both in normal and disease-affected tissues. In addition, the telocytes can activate macrophages, trigger and maintain an immune response, through indirect paracrine effects suggesting potential immunoregulation roles of telocytes in local immuno-inflammatory processes either repression or activation of their responses. Also, they stated that telocytes can be functional players in the initiation and regulation of immune responses of macrophages.

The neovascularization observed in alkali burn group could be explained by the occurrence of inflammation which initiated tissue hypoxia leading to vascular endothelial growth factor (VEGF) release which is an angiogenic growth factors. VEGF stimulates over-proliferation of the endothelial cells of capillaries leading to new vessels formation[36]. Moreover, Goktas *et al*.[37] stated that the proteolytic enzymes MMP-9s produced by inflammatory cells in the stroma cause the destruction of connections in extracellular matrix and hence ease the transmigration of endothelial cells to other areas, leading to new vessel formation.

E-cadherin is a main transmembrane protein playing a major role in adhesion between adjacent cells, so it is one of the essential molecules for the maintenance of structural and functional integrity of the epithelial cells[38]. The decrease in E-cadherin expression mostly reflected the change in epithelial integrity with weak organization and packing of the cells[39]. This was in accordance with the results of alkali burn group which included reduction of E-cadherin expression, epithelial separation & denudation, loss of desmosomal junction with wide intercellular spaces and decreased epithelial thickness. In confirmation to our result, Ebrahim *et al*.[9] stated that that adherent's junctions and gap junctions were lost in corneal alkali burn

Ki-67, a nuclear protein, is demonstrated inside the proliferating cell, and it is used as a cellular marker to assess the extent of cell proliferation in tissues[40]. Alkali burn group demonestrated a highly significant rise in Ki-67 expression compared to the control. This was in harmony with results of Sharaf Eldin *et al*.,[25] who detected numerous Ki67-immunopositive cells in the basal and supra-basal layers of the corneal epithelium exposed to NaoH. They reported that proliferation of epithelial cells depends on mitotic activity of basal cell layer. Moreover, they added that quiescent limbal stem cells located at the periphery were stimulated by injury to proliferate and give rise to these proliferating cells.

Morphometric and statistical results of group III revealed a highly significant decrease in the total corneal thickness in comparison with the control group. Such finding might be attributed to the reduction in the epithelial thickness mentioned in our results and dissolution of stromal collagen fibers as has been discussed before in this study.

As regard group IV, the treatment of corneal alkali burn with liposomal ozonated oil eye drops (Ozodrops) started 24h after burn induction in order to promote re-epithelialization, reduce inflammation, prevent infection and limit further degeneration of the epithelium and stroma. The examination of corneal sections of this group by light and electron microscope revealed obvious regeneration in all the corneal layers to be nearly similar to control group. There were continuous and well organized epithelium, intact Bowman's membrane and regular arrangement of stromal collagen fibers with no

inflammatory cells invasion. Moreover, keratocytes appeared organized, flattened and scattered in the stroma. In addition, Descemet's membrane and endothelium were apparently regular and continuous. Slight alterations were still seen in sections including vacuolation in some epithelial & endothelial cells with few spaces between stromal collagen fibers. Such findings were supported by an increase in E-cadherin immunoexpression compared to group III. Also, morphometric and statistical results of this group revealed highly significant decrease in Ki-67, highly significant increase in total corneal thickness and epithelial thickness in comparison with corneal burn group (III). All these results may prove the regenerative, healing and anti-inflammatory effects of liposomal ozonated oil on corneal burn. This finding was in consistence with a recent clinical study[16] on corneal abrasion treated with liposomal ozonated oil eye drops and the researchers detected complete healing of epithelium after 5 days of treatment. Also, Basile *et al*.,[13] on their study on a case of Persistent dystrophic corneal ulcer, reported that the ulcer become more circumscribed with re-epithelization after one week of treatment with topic Ozodrop. In addition, patel *et al*.,[41] reported significant improvement in the size of the wound and healing of epithelium after topical ozonated oil application on palatal wounds. Moreover, topical ozonated oil was found to enhance wound healing in the skin of Guinea pigs through stimulating fibroblast proliferation and collagen production at the site of injury[11]. Also kim *et al*.,[11] reported that topical ozonated oil could activate NF-κB (transcription factor) which is capable of regulating inflammatory reaction and hence the whole wound-repair process.

The beneficial therapeutic effects of liposomal ozonated oil eye drops could be attributed to it content of ozone as has been previously reported by Basile *et al*.,[13] who reported that Ozone can improve the inflammation response pathways (through reduction of inflammatory cytokines) and modulate tissue repair (through the upregulation of the growth factors, transforming growth factor-β and platelet-derived growth factor) with improvement of corneal transparency. Furthermore, they added that ozone can help wound repair by rising the oxygen tension in the wound site by ozone exposure, reducing bacterial load in the wound, and aiding in progressive adaptation to oxidative stress through enhancement of anti-oxidant systems (Superoxide dismutase, Glutathione reductase, Catalase). Paduch *et al*.,[42] stated that the Ozodrop has the ability to scavenge free oxygen radicals that can induce damaging of the eye surface cells.

In addition, liposomal ozonated oil has antimicrobial (antifungal, antibacterial and antiviral) property and can be used to prevent & treat local infections. Moreover, it has the advantage over the antibiotic drugs because it is able to prohibit development of antimicrobial resistance & allergic responses[16].

The enhanced epithelial healing of wounds could be also attributed to the antimicrobial property of ozone. Increased bacterial load in the wound leads to increased area of granulation tissue & inflammation which may extend the healing time[41]. Megahed *et al*.,[43] attributed antibacterial property of liposomal ozonated oil to ozone which kills bacteria by attacking the cell membrane glycolipids and glycoproteins resulting in cell destruction. In our study, the prevention of infection is needed for proper burn healing as the infection can hinder tissue repair.

Interestingly, in our study we demonstrated that Ozodrop application on the corneal burn is capable of up-regulating E-cadherin expression and this was in agreement with the result of some researcher[s][44] in their study on the effect of ozonated oil on a skin disease. They stated that during tissue healing, cell to cell adhesion is mediated by E cadherin which shares in the reconstitution of epithelial barrier, and takes part in re-epithelialization through modification of cell polarity, differentiation, growth, and migration.

As regard group IV, there was highly significant decrease in Ki-67 immunoexpression in comparison with alkali burn group and this was in harmony with a previous study on the effect of ozone therapy on acute colitis in which the researchers detected a decrease in Ki-67 immunostaining in ozone therapy group in a comparison with acute colitis group and became closer to control[45].

CONCLUSIONS

From all the mentioned data and results, it is concluded that the application of liposomal ozonated oil eye drops on a corneal alkali burn could improve the histological, immunohistochemical and morphometric abnormalities of the burned cornea. This proved the regenerative, anti-inflammatory and antimicrobial properties of this new eye drop formulation. For all these beneficial therapeutic effects, it is considered a unique therapy for patients suffering from a corneal alkali burn and showing non-cooperation in receiving medications. Further studies are recommended to explore the effect of this new eye preparation on other ocular pathological conditions.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير العلاجي المحتمل للزيت الدهني المعالج بالأوزون على حروق القرنية القلوية المستحدثة تجريبياً في ذكور الجرذان البيضاء البالغة (دراسة هستولوجية و هستوكيميائية مناعية)

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الخلفية: حروق القرنية القلوية هي حالات طارئة حقيقية للعين تحتاج إلى تدخل طبي فوري للحد من المضاعفات الشديدة واستعادة رؤية المريض. الزيت الدهني المعالج بالأوزون هو تركيبة جديدة لقطرة العين ذات خصائص تجديدية ومضادة للالتهابات ومضادة للميكروبات.

هدف الدراسة: تقييم التأثير العلاجي المحتمل للزيت الدهني المعالج بالأوزون على حروق القرنية القلوية المستحدثة تجريبياً في الجرذان باستخدام تقنيات هستولوجية و هستوكيميائية مناعية.

مواد وطرق البحث: تم تقسيم أربعين من ذكور الجرذان البيضاء البالغة بشكل عشوائي إلى أربع مجموعات متساوية. المجموعة الأولى (المجموعة الضابطة) ، المجموعة الثانية (المجموعة المعالجة بالزيت الدهني المعالج بالأوزون)، المجموعة الثالثة (مجموعة الحرق القلوي) ، المجموعة الرابعة (مجموعة الحرق القلوي و المعالجة بالزيت الدهني المعالج بالأوزون). في نهاية التجربة ، تم الحصول على عينات القرنية وتحضيرها من أجل الفحص بالمجهر الضوئى والإلكتروني. تم عمل دراسات مورفومترية وإحصائية.

النتائج: أظهرت مجموعة الحرق القلوية (المجموعة الثالثة) انفصال وتعرية للنسيج الطلائي ، وخلايا طلائية مفرغة مع وجود أنوية متحلله في بعض الخلايا. كان يوجد ألياف كولاجين غير منتظمة مع وجود مسافات واسعة بينها، خلايا كيراتوسيت متحلله ،أوعية دموية جديدة ، تسلل الخلايا الالتهابية. لوحظ انفصال غشاء ديسمت في بعض الأماكن. أظهرت النتائج المورفومترية والإحصائية انخفاض سمك القرنية و النسيج الطلائي ، وانخفاض نسبة ألياف الكولاجين ، وانخفاض مستوي التفاعل المناعي لـ E-cadherin وزيادة مستوي التفاعل المناعي لـ Ki-٦٧ مقارنة بالمجموعة الضابطة. كشفت المجموعة الرابعة عن تحسن كبير في بنية القرنية وترميمها مع صور هستولوجية و هستوكيميائية مناعية شبه طبيعية.

الخلاصة: أدى الزيت الدهني المعالج بالأوزون بصورة كبيرة إلى تحسين بنية القرنية المحروقة بواسطة مادة قلوية. لذلك ، يمكن اعتباره علاجًا فريدًا ومناسبًا للمرضى الذين يعانون من حروق القرنية القلوية.