

Original research article

Safety evaluation of the plasma on ocular surface tissue: An animal study and histopathological findings

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ABSTRACT

Purpose: The plasma soft surgery is as an alternative to invasive surgical cosmetic procedures that can reduce the recovery time and possible complications after surgery. Due to the sensitivity of ocular surface tissue and the potential of the plasma, it can be evaluated to treat some ocular surface disorders. Accordingly, we evaluated the safety of the cold plasma on the ocular surface tissue in three areas located in the cornea, limbus, and conjunctiva. **Methods:** Nine adult male New Zealand albino rabbits which divided into three groups were used for experiments. The left eye of each rabbit was chosen for test and the right eye was as the control. Experiments were performed on three ocular surface areas under the influence of the plasma. For this purpose, the plasma was created by Plexr device in continues mode and low power level (white handpiece; 0.7 W) was utilized at 0.7 s intervals using a 22-gauge needle. For evaluation of plasma safety, ocular surface integrity and histopathological changes were assessed after 24 h (A), one week (B), and one month (C) exposure to plasma.

Results: According to the external and slit-lamp examinations, after 24 h (A) only epithelial defect was visible while ocular surface in other groups (B & C) was healthy had completely recovered. OCT imaging and histologic examination also confirmed these results. After 24 h conjunctival and corneal sections showed a localized area of epithelial loss together with infiltration of inflammatory cells in the subepithelial stroma, but during the one-month period, recovery was evident. Generally, after the first week, the loss of integrity of corneal epithelium has not been completely improved yet, while it was recovered nearly to be normal in group C. It was noteworthy that in all groups the loss of endothelium cells was not apparent which confirming the lack of damage to the deep layers of the eye.

Conclusion: According to the plasma safety results, it seems that plasma soft surgery has the potential to be used as an alternative method for treatment of some of the ocular surface disorders without needing to conventional surgical procedures.

1. Introduction

Plasma is one of the four fundamental states of matter and was first described by chemist Irving Langmuir in 1927 [1]. Unlike the other three states including solid, liquid, and gas; plasma does not exist freely on the Earth's surface under normal conditions. Plasma can only be artificially generated by heating or subjecting a neutral gas to a strong electromagnetic field to the point an ionized gaseous substance becomes increasingly electrically conductive, and long-range electromagnetic fields dominate the behavior of the matter [2]. Plasma has a variety of different types depending on how electrodes are placed, the intensity of electrical potential, and environmental conditions [3]. In the general plasma is classified into two types including thermal and non-thermal plasma that their difference is based on mechanisms of generation. For thermal

plasma generation, high pressure and temperature are required while non-thermal plasma is generated under atmospheric pressure at low temperature using low energy. In thermal plasma, electrons and heavy particles (neutrals, ions, and radicals) are at the same temperature (some tens of thousands of Kelvin when measured on a temperature scale) but non-thermal plasma has electrons at a hotter temperature than the heavy particles that are at room temperature (30–60 °C). This non-equilibrium nature of non-thermal plasma facilitates chemical and physical reactions with high temperatures at low gas temperatures [4, 5]. During the process of plasma production, various chemical compounds are produced including positive/negative ions and electrons; free radicals, stable reactive atoms and molecules such as ozone, hydrogen, peroxide, metastable atomic species, and also highly energetic photons, e.g. UV [4]. These ions and metastable atomic species react rapidly with biological

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compounds which leads to their breakdown and eventual removal. Accordingly, in plasma, the atoms are excited and there are free electrons, and ions which have caused the plasma to be used extensively in medical science so that different types of plasma have been used for various medical applications [6]. The first applications of the plasma in medicine were started in the 19th century by using carbon arc lamps or “violet ray” machines for electrotherapy [7]. Its modern approaches have been made in the 1970s with so-called electrosurgical applications for cauterization and tissue removal [8]. Generally based on the plasma production method three types of plasma are used in the medical field including Spark Plasma, Cold Plasma Jet, and Floating Electrode-Dielectric Barrier Discharge (FE-DBD) [9]. Spark plasma is created as a result of the high potential difference between the electrode and target surface such as skin. Despite the high temperature of this type of plasma, the controlled form creates micro burn on the surface of the skin and has many uses for skin restoration and implant such as skin rejuvenation, mole removal, skin tightening, removal of telangiectasia, removal of striae-stretch mark, removing acne scars and gum tissue ablation [10]. The cold plasma jet is formed as a result of applying a high potential difference to an inert gas stream, such as helium, which is passing through the plasma jet nozzle [11]. The FE-DBD plasma is also caused by applying a high potential difference to the electrode covered by a dielectric layer between the dielectric surface and target surface such as the skin [12]. Since in the plasma jet and FE-DBD, plasma temperature is about room temperature so are called cold plasma (non-thermal plasma) and have a variety of applications including skin restoration, increase skin permeability, chronic wound healing, inactivation of microorganisms, blood coagulation, tooth bleaching, and elimination of cancer cells [13]. As it mentioned cold plasmas are operated under atmospheric and low-temperature condition and allow painless in vivo applications without damaging the surrounding tissue. Cold atmospheric plasma (CAP) devices can be generally classified into three different types including direct, indirect and hybrid plasma sources. Direct plasma sources use the target area as a counter electrode. One limitation of the direct technology is the need for a more or less constant distance to the treatment area, therefore, only the use of a relatively small treatment area can solve this distance problem [14, 15]. Today, doctors tend to have conventional surgeries outcomes without wounds, hemorrhage, necrosis of the adjacent tissue, stitches and other side effects. Accordingly, in recent years, the use of plasma-based soft surgery technology especially using CAP has been considered in medicine [16, 17]. In the late 2000s, several CAP sources were approved for use in medicine. For example, in 2008 the US FDA approved the Rhytec Portrait® device as a plasma jet for use in dermatology or in Germany the kINPen® device received certification class IIa in 2013, and the PlasmaDerm® device (CINOGY GmbH) was also approved for skin and wound surface [18]. In addition, the Plexr device with DBD-based plasma technology was approved and launched in the UK marketplace in 2014 [16]. This device has CE approved for dermatological and plastic microsurgery and electrical stimulation. In the last decade, the use of plasma-based devices has grown significantly. Generally, plasma-based devices are as micro-surgical hand operated system that transfers concentrated heat to the treated tissues. This technology offers an alternative to invasive surgical procedures, reducing recovery time and any complications that can arise before and after surgery and so is as an

innovative development in aesthetic medicine and rejuvenating treatments by utilizing the plasma [19–21]. In addition, in this technology heat is not spreading to the surrounding area, meaning it is perfect to operate on areas (such as eyelids), that are not suitable for other devices like radiofrequency scalpels or lasers. Considering the potential of the plasma in soft surgery of small areas, in this study as the first step, we evaluated the safety of the atmospheric pressure cold plasma (APCP) on the ocular surface tissue to develop a plasma-based therapy to treat ocular surface disorders. For this purpose, three areas of the ocular surface were under the influence of the plasma and the surface integrity and histopathological changes were evaluated.

2. Materials and methods

2.1. Animals

Nine healthy adult male New Zealand albino rabbits weighing 2–2.5 kg and aged 3–3.5 months were used for experiments. Rabbits were individually housed in separate cages under standard conditions including temperature (25–28°C), humidity (50%–60%) and light (12 h light-dark). They were fed the standard diet and water. Before starting the experiments, each rabbit was checked for corneal or conjunctival disorders and rabbits with corneal or conjunctival abnormalities were excluded. All procedures were performed with adherence to the tenets of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) “Statement for the use of Animals in Ophthalmic and Visual Research” [22].

2.2. Experiments

The 9 rabbits were divided into three groups which each group composed of 3 rabbits. All rabbits were anesthetized through intraperitoneal injection of a cocktail containing Ketamine (44 mg/Kg) and xylazine (6–8 mg/kg) in sterile normal saline. The left eye of each rabbit was chosen for test and the right eye was as the control. Experiments were performed by the Plexr device (GMV, Rocca Priora, RM, Italy) on three areas of the ocular surface including the superotemporal cornea, superonasal limbus and inferior conjunctiva (at a distance of 4 mm from the limbus) (Fig. 1). The Plexr device was in continuous mode and low power level (white handpiece; $V_{pp} = 500$ V, Power = 0.7 W, and Frequency = 75 kHz) was utilized at 0.7 s intervals using a 22-gauge needle (Table 1). In order to prevent eye infections, rabbits were treated with chloramphenicol as eye drops every 6 h for one week. During the examinations, rabbits are monitored for abnormal behavior every day.

2.3. Examinations

2.3.1. External examinations

At first, to investigate external effects of the plasma on ocular surface, the eyes of rabbits were examined for associated symptoms including corneal opacity, and conjunctival chemosis, redness, and discharge, and also lid swelling after 24 h (group A), one week (group B), and one month (group C) exposure to plasma. In order to assess the



Fig. 1. External effects of the plasma on the ocular surface. The eyes of rabbits were examined for associated symptoms including corneal opacity, and conjunctival chemosis, redness, discharge, and lid swelling after 24 h (a), one week (b), and one month (c) exposure to plasma. According to the observations redness was only observed in the first 24 h with vessels definitely injected above normal. Ocular surface areas which were under influence of the plasma shown with red arrows.

Table 1
Technical features of the Plexr device.

Parameters	Values
Working gas	Air
Power supply	Docking station = 24 V Handpieces: embedded inductive charger = 5 V
Handpieces:	
Max output	≤ 2 W
Max working voltage	≤ 1,3kVPP
Output frequency	(70–80) kHz
Handpiece types:	
White*	V peak to peak = 500 V, Power = 0.7 W, Frequency = 75 kHz
Green	V peak to peak = 600 V, Power = 1 W, Frequency = 75 kHz
Red	V peak to peak = 700 V, Power = 2 W, Frequency = 75 kHz
Maximum absorbed power (Docking station)	120 W
Applicator electrode	Stainless steel sterile disposable needle
Risk classification of the device	IIb** (Medium-high risk)

* In current study, the white handpiece was used.

** This classification relates to the Non-invasive medical devices within the field of dermatology.

Table 2
The scale of weighted scores for grading the severity of ocular lesions based on the Draize protocol.

Lesion	Score
<i>Cornea</i>	
<i>i. Opacity</i>	
Scattered or diffuse area – details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
<i>Conjunctivae</i>	
<i>i. Redness</i>	
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
<i>ii. Chemosis</i>	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
<i>iii. Discharge</i>	
Any amount different from normal (does not include small amount observed in inner canthus of normal rabbits)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3

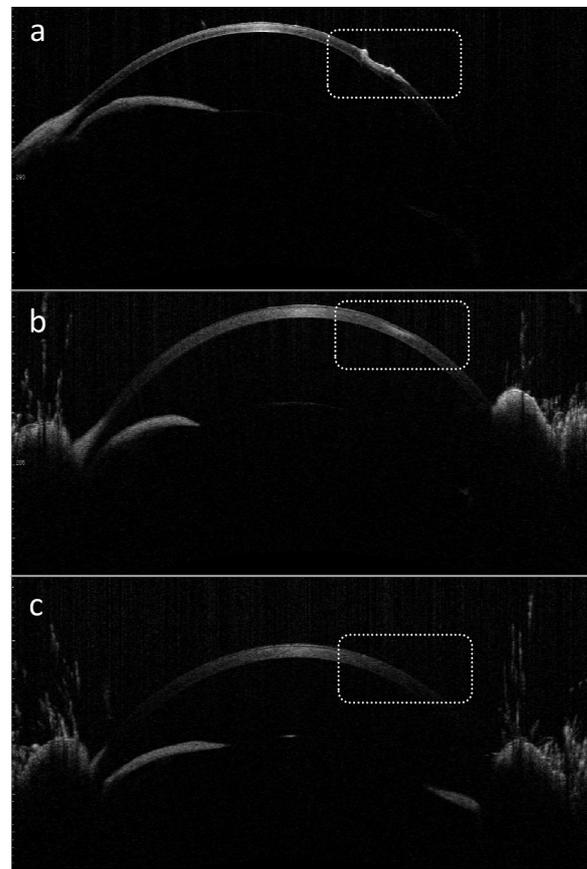


Fig. 3. Evaluation of plasma effects on ocular surface by anterior-segment optical coherence tomography imaging after 24 h (a), one week (b), and one month (c) exposure to plasma. (a) Epithelial defect along with the destruction of the superficial stroma is visible; (b) The image shows the restoration of damaged areas with a significant reduction in epithelial defect; (c) Epithelial and superficial stroma are completely improved and cornea is normal.

level of injuries to the cornea and conjunctiva, we used Draize scoring. According to the Draize protocol, scores for the observed ocular injuries are from 1 to 3 for conjunctival redness and discharge, and from 1 to 4 for corneal effects and conjunctival chemosis. A score of zero is also assigned when the ocular surface is normal and no adverse effects are observed (Table 2) [23].

2.3.2. Slit-lamp examination

Surface abnormalities such as infiltration, inflammation, and epithelial defect were also examined after 24 h (A), one week (B), and one month (C) exposure to plasma by slit-lamp microscope (Topcon Corporation, Tokyo, Japan). The corneal epithelial defect was evaluated using a cobalt blue filter 3 min after applying a standard fluorescein strip (Jingming, China) on the ocular surface. The areas of positive staining showed the epithelial



Fig. 2. Evaluation of surface abnormalities such as infiltration, inflammation, and epithelial defect by Slit-lamp examination after 24 h (a), one week (b), and one month (c) exposure to plasma. According to the fluorescein staining, the corneal and conjunctival epithelial defect (about 0.8 mm) was only visible in group A (a) at locations which were under the influence of the plasma. There was no epithelial defect in corneal and conjunctival after one week (b) and one month (c). Ocular surface areas which were under influence of the plasma shown with red arrows.

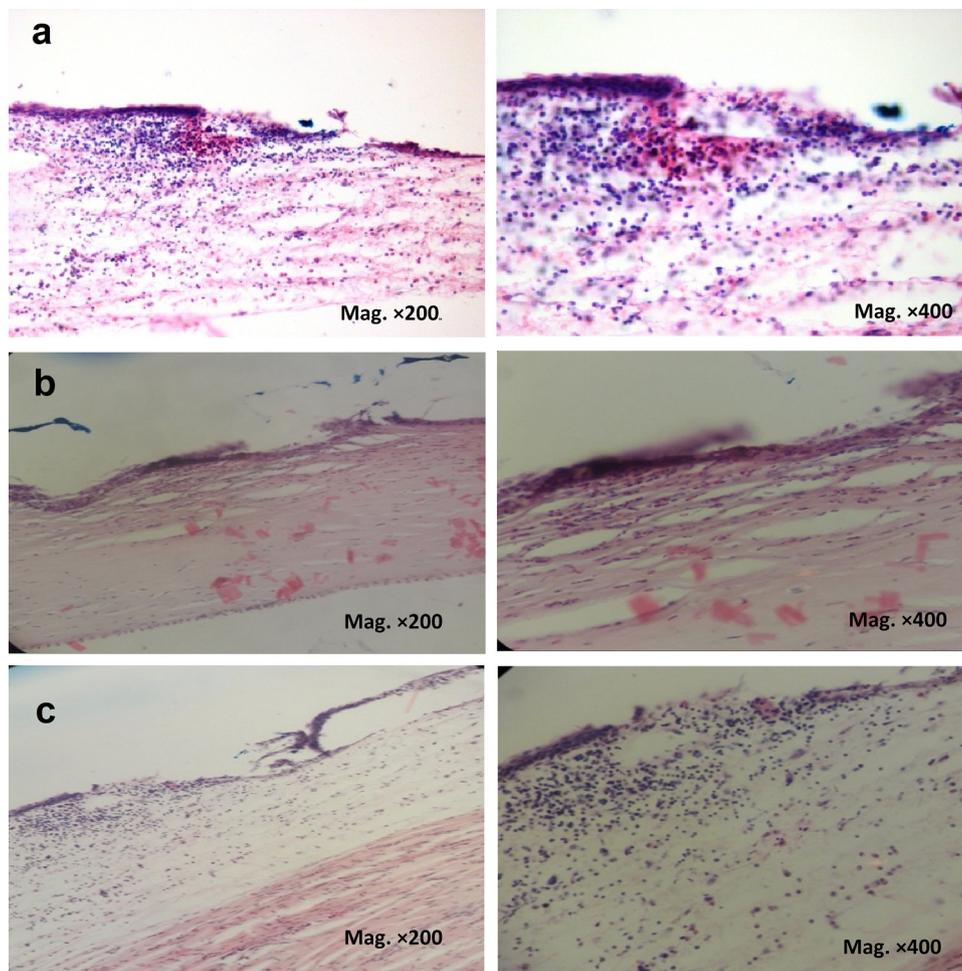


Fig. 4. Effects of the plasma on histological changes (H & E staining) in the conjunctival (a), cornea (b), and limbus (c) tissues after 24 h exposure to plasma. (a) H & E conjunctival sections show a localized area of epithelial loss together with infiltration of inflammatory cells in subepithelial stroma; (b) H & E sections of the cornea show ulcerated tissue with denuded epithelium and mild to moderate inflammatory cells including neutrophils and lymphocytes infiltration; (c) H & E sections of the limbus show ulcerated tissue with denuded epithelium and mild to moderate inflammatory cells including neutrophils and lymphocytes infiltration.

defect, therefore, in order to assess the level of injuries, the corneal and conjunctival surface staining was measured. The same ophthalmologist performed all ophthalmologic evaluations.

2.3.3. Anterior-segment optical coherence tomography (OCT) imaging

OCT imaging was performed on all corneas in three groups after 24 h (A), one week (B), and one month (C) exposure to plasma. Images were obtained with an anterior segment OCT system (Casia SS-1000 OCT, Tomey, Nagoya, Japan). For OCT, 30 000 axial scans (A-scans) per second with axial resolution 10 μm and wider resolution 30 μm were acquired. The same ophthalmologist performed all OCT imaging and their processing.

2.3.4. Histopathological examination

Before histological analysis, animals were euthanized with an overdose of 200 mg/kg intravenous ketamine. The eyes of rabbits were enucleated after 24 h (group A), one week (group B), and one month (group C) exposure to plasma, respectively, and subjected to histopathological examination. Specimens were taken from the affected area of cornea, limbus, and conjunctiva. Tissues were fixed in 10% formalin and processed in paraffin blocks. Sections were obtained and stained with hematoxylin and eosin (H&E), then, examined by optical microscopy to assess the histopathological changes.

2.4. Statistical analysis

Statistical values were calculated in each group and compared with

Student's t-tests (control samples). Data were analyzed using the SPSS software and reported as means \pm SD. *P* value < 0.05 was considered statistically significant.

3. Results

3.1. External examination

According to the observations, the ocular surface was healthy in three groups for conjunctival chemosis, discharge and also corneal opacity (Score 0) but the redness was observed in the first 24 h with vessels definitely injected above normal (Score 1) (Fig. 1a). In addition, eyelid swelling was observed during the first 48 h which was mild. After plasma treatment and during the first 72 h the rabbits showed photophobia but after this time, rabbits did not have light sensitivity.

3.2. Slit-lamp examination

According to the fluorescein staining, the corneal and conjunctival epithelial defect (about 0.8 mm) was visible in group A at locations which were under the influence of the plasma (Fig. 2a). On the other hand, after exposure to plasma, there was no epithelial defect in corneal and conjunctival during the course of the study in group B and C (Fig. 2b and c). In addition, no infiltration and inflammation were observed during the study period. In addition, the anterior chamber (AC) reaction was not seen in all groups.

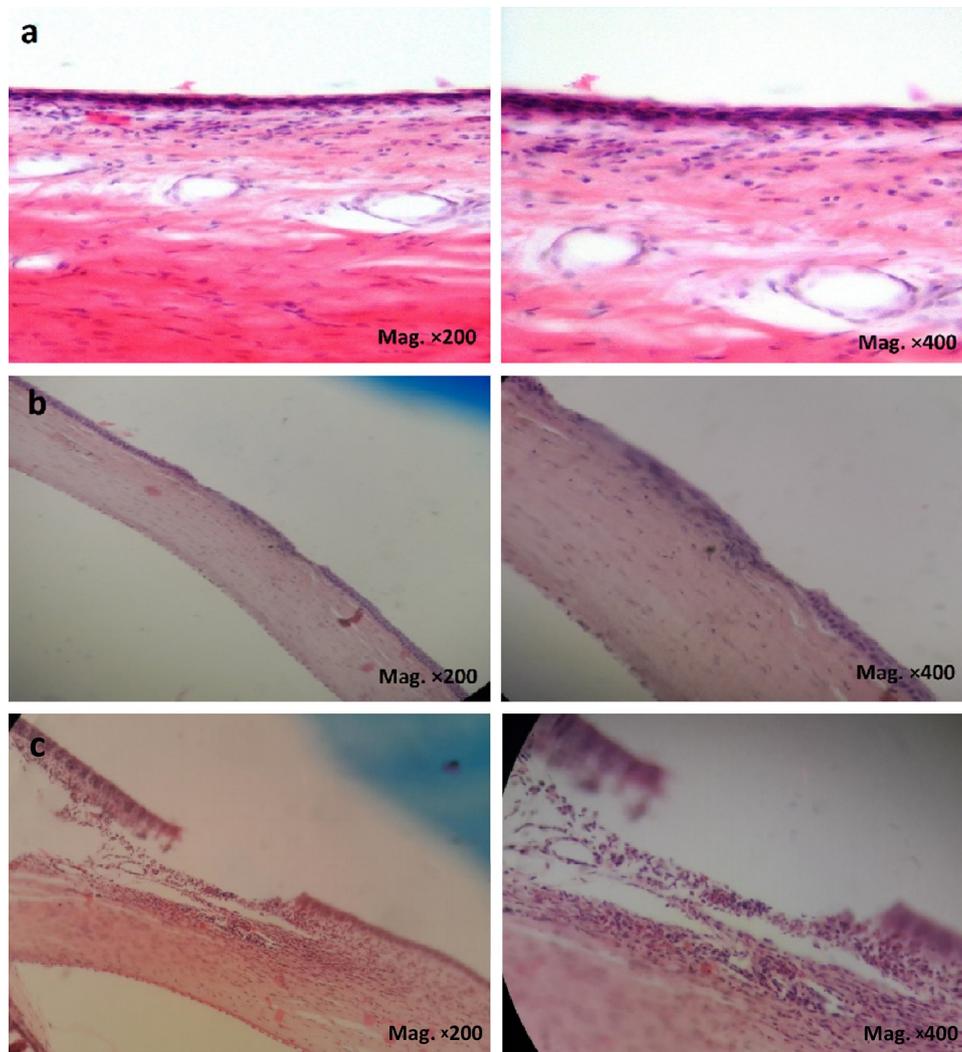


Fig. 5. Effects of the plasma on histological changes (H & E staining) in the conjunctival (a), cornea (b), and limbus (c) tissues after one week exposure to plasma. (a) No prominent pathologic findings were evident in conjunctiva after one week; (b) H & E sections of the cornea show tissue with regenerated epithelial cells lining with minimal lymphocytic infiltration within stroma; (c) H & E sections of the limbus show tissue with regenerated epithelial cell lining, with cytoplasmic vacuolization.

3.3. OCT imaging

Based on OCT analysis, epithelial defect along with the destruction of the superficial corneal stroma was visible after 24 h (Fig. 3a). However, after one week the images showed the restoration of damaged areas with a significant reduction in the epithelial defect (Fig. 3b). As shown in Fig. 3c, after one month, epithelial and superficial stromal were completely improved and the cornea was normal.

3.4. Histopathology

According to the histological results, after 24 h (group A), conjunctival sections which were affected by the plasma showed a localized area of epithelial loss together with infiltration of inflammatory cells in the sub-epithelial stroma (Fig. 4a). On the other hand, corneal sections disclosed an area of epithelial and superficial stromal loss associated with infiltration of inflammatory cells (Fig. 4b). In addition, peripheral cornea H & E sections showed ulcerated tissue with denuded epithelium and mild to moderate inflammatory cells including neutrophils and lymphocytes infiltration (Fig. 4c). After the first week (group B), the loss of integrity of corneal epithelium has not been completely improved yet, while it was recovered nearly to be normal in group C. In addition, no prominent pathologic findings were evident in conjunctiva after one month (Figs. 5 and 6). In

general, after one month, pathological observation showed a localized area of epithelial irregularity, mild depression, and mild haphazard arrangement of superficial corneal stromal collagen lamellae (Fig. 6). It was noteworthy that in all groups the loss of endothelium cells was not apparent which confirming the lack of damage to the endothelial layer of the cornea. According to the histological results, there was no inflammation and necrosis in the sclera and its integrity was preserved.

4. Discussion

Ocular surface diseases are commonly associated with corneal scarring and vascularization, conjunctival inflammation, symblepharon, and forniceal shortening. Any surgical intervention in the ocular surface environment may lead to exacerbation of the disease, which may result in visual deterioration. Therefore, achieving non-invasive and safe methods for the treatment of ocular surface abnormalities can be important, although these methods may also aggravate the disease [24, 25]. In recent years, many studies with positive results have been done about the efficacy of the plasma for the treatment of excess skin on the upper eyelid, perioral rhytides, acne, and removal of skin and ocular microbial infections [9, 15-17, 19-21, 26]. Accordingly, some studies have been done using cold plasma to remove ocular infections and especially assess its effects on the ocular surface. For instant, Reiterberger et al., (2018) evaluated the potential of argon cold

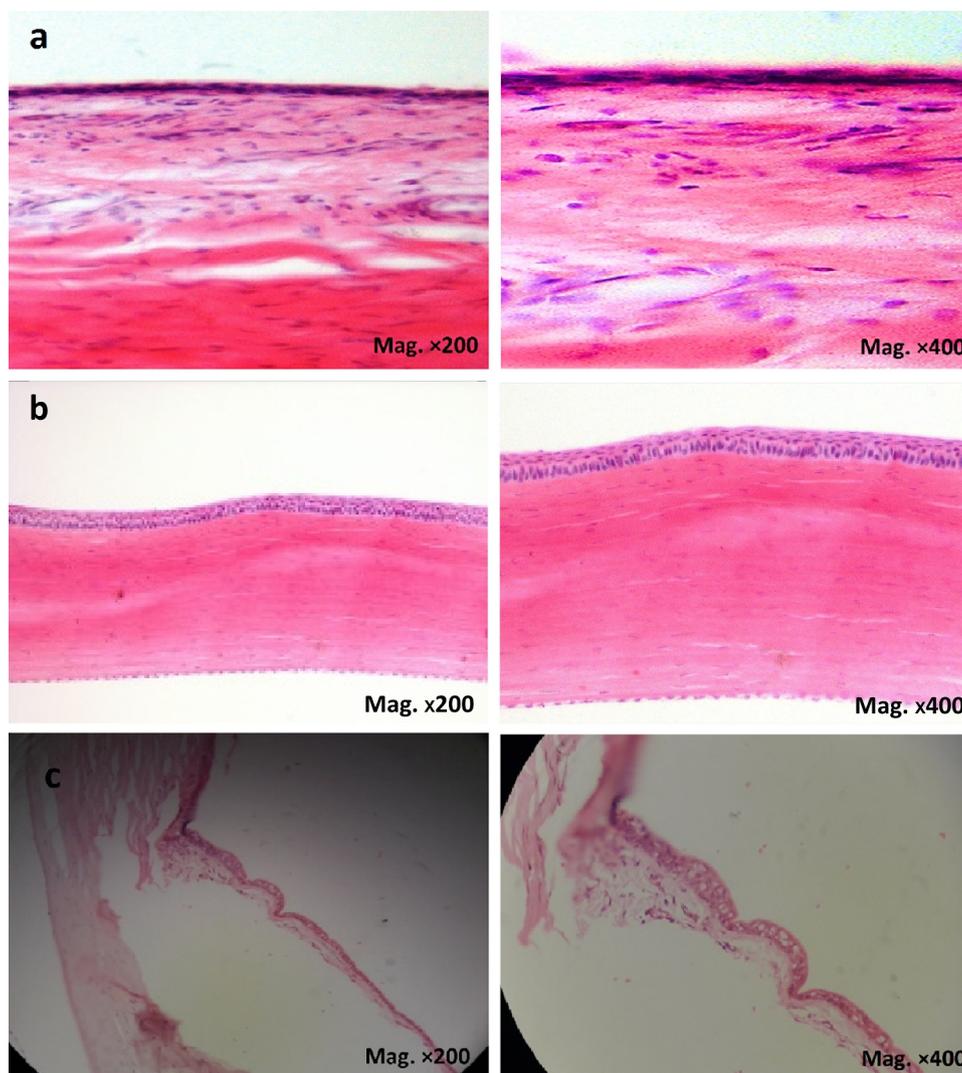


Fig. 6. Effects of the plasma on histological changes (H & E staining) in the conjunctival (a), cornea (b), and limbus (c) tissues after one month exposure to plasma. (a) No prominent pathologic findings were evident in conjunctiva after one month; (b) H & E sections of the cornea shown repaired corneal epithelial lining without inflammation; (c) Similar to group B, H & E sections of the limbus show tissue with regenerated epithelial cell lining, with cytoplasmic vacuolization.

plasma to treat corneal infections using exposure times from 0.5 to 10 min and its side effect on the viability of primary human corneal limbal epithelial cells [27]. Their results showed cells that were treated for 0.5 to 5 min completely recovered after 24 h without changes in morphology, only 10 min treatment impaired the cells permanently. In addition, there was no oxidative stress, apoptosis, or corneal damage. Their study showed that argon cold plasma can be used on the ocular surface without impairing corneal epithelial cells *in vitro*, *ex vivo* and in the direct application on patients' eyes. In another study, Brun et al., (2012) investigated whether atmospheric pressure cold plasma (APCP) generated by helium gas can inactivate ocular pathogens without causing significant tissue damage [28]. For safety evaluation, human ocular cells including conjunctival fibroblasts and keratocytes and *ex-vivo* corneas were exposed to the plasma for 0.5–5 min. According to their results, exposure to plasma for 0.5–5 min was not effective on cell viability. Although a minimal reduction in cell viability was found only in cells of some donors exposed to APCP for 5 min but flow cytometry confirmed that after a 2 min exposure to APCP, the transitory increase of apoptotic cells almost completely disappeared after 24 h. On the other hand, increased levels of intracellular reactive oxygen species (ROS) in exposed cells were found. In addition, a transient increased expression of some genes related to oxidative stress was determined in ocular cells and corneas. However, immunoassays confirmed no induction of thymine dimers in corneal tissues and cell cultures. Their findings showed that short-

term application of APCP could be used as an efficient and rapid ocular disinfectant for bacteria and fungi without significant damage on ocular cells and tissues. Similar to this study, Rosani et al., (2015) evaluated morphological and functional changes in human corneas exposed to APCP for 2 min [29]. Analyses of corneal tissues collected at 6 h post-APCP treatment by immunohistochemistry and western blotting demonstrated no morphological tissue changes but a transient increased expression of some genes related to oxidative stress was determined that returned to control levels in 24 h which is in accordance with the results reported by Brun et al. Generally, these findings indicate that the effects of cold plasma on the cells of a target tissue are depended on the exposure time to plasma.

Based on these studies and the potential of the plasma in soft surgery of small areas, we hypothesized that plasma technology can be used for the treatment of some ocular surface abnormalities such as pinguecula. Since there is not a similar report in this regard, therefore in the first stage we investigated the safety of the plasma on the ocular surface tissue in three areas including the cornea, limbus, and conjunctivae via low power level of the Plexr device (white handpiece). According to the results, in daily control of all groups serious complications such as conjunctival chemosis, and discharge were not observed, although redness (Score 1) was visible at first 24. In addition, histological results showed that after one month the loss of integrity of corneal epithelium has been completely improved and was recovered nearly to be normal, which is in accordance with the OCT imaging

results (Fig. 3). On the other hand, the loss of endothelium cells was not apparent which confirms the ineffectiveness of the plasma on the deep corneal layers such as the endothelial layer of the cornea. As seen in the pathological images of the corneal and limbus (Figs. 4–6), after encountering plasma, over time, these changes undergo the repair process and ultimately it leads to the formation of the scar in the area. It should be noted that according to the studies which some of them were also mentioned, all effects of cold plasma on cells is dependent on plasma dosage and treatment time. Short plasma treatment times/low plasma doses have stimulating effects such as increase of proliferation and migration, induction of DNA repair while long plasma treatment times/high plasma doses induce lethal effects like cell death by apoptosis, stop of proliferation, DNA damage, cell cycle arrest [26, 30]. Accordingly, in comparison with the similar studies described above, in the current study, the duration of use of the plasma was less than 5 s including 3 spots with a period of about 1 s, and it is unlikely couldn't lead to serious damages. However, although our macroscopic and microscopic studies show that the plasma has no inappropriate effects on the target tissue, but analyses of plasma effects in molecular levels, such as the investigation of pro-inflammatory cytokines, can confirm these findings. In addition, in order to demonstrate the safety of this method in the limbus area, there is require specific evaluation in terms of number and function of limbal stem cells. Also, to confirm the safety of the plasma for use in the ocular surgery on patients' eyes, the effect of the corneal scar on the visual acuity and the subsequent corneal aberrations should be evaluated.

5. Conclusions

According to the safety results in the animal model, it seems that plasma soft surgery has the potential to be used as an alternative method for treatment of some of the ocular surface disorders without needing to conventional surgical procedures, although more studies should be done.

Ethical statement

All animal processes were performed by the Principles of Laboratory Animal Care (NIH publication), as well as the specific rules provided by the Animal Care and Use Committee of National Medical and Health Service.

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Conflict of interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpme.2019.100084.

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