Effects of In-vivo Application of Cold Atmospheric Plasma on Corneal Wound Healing in New Zealand White Rabbits

Rashed Alhabshan1, David Belyea2, Mary Ann Stepp3, Jeffrey Barratt4, Sanjeev Grewal1, Alexey Shashurin1 and Michael Keidar5

Abstract
Cold Atmospheric Plasma (CAP) has been shown to influence tissue wound healing but little is known about the impact of CAP on healthy corneal tissues and their ability to respond to injuries. The objective of this study is to examine the effect of CAP on wound healing after corneal epithelial and basement membrane ablation in New Zealand white rabbits. The rabbits were assigned into three groups. Ten Rabbits from two groups underwent a 6 mm corneal ablation to the right eyes. After ablation, five rabbits in group (A) received 2 minutes of CAP whereas the other five rabbits (B) were not treated with CAP. A third group (C) included two rabbits and received CAP without ablation. Eyes monitored for corneal haze, epithelial healing, lens clarity and any signs of inflammation. At 24 hours, two rabbits from group A and two from group B were sacrificed to harvest the corneas. Twenty days, all remaining rabbits in groups A, B, and C were sacrificed and corneas were harvested. Corneas were fixed in formalin and stained with H&E or used for immunofluorescence microscopy to assess scar formation using antibodies against fibronectin and α-smooth muscle actin. At 24 hours, corneas from group A had average epithelial defect of 9.25 mm² on day 1 whereas those from group B had average defect of 12.05 mm² (P=0.57). H & E stained corneal sections didn’t show abnormal responses to injury at 24 hours and 20 days. Epithelial thickness and stromal cell counts 20 days after injury showed no significant differences. Analysis of immunofluorescence microscopy images showed no differences between all groups. In conclusion, CAP application to cornea doesn’t appear to have obvious adverse effects. CAP does not interfere with rate of wound closure or induce increased inflammation. CAP did not have an effect on corneal wound healing or lead to scar formation.

Keywords
Corneal epithelium; Corneal stroma; Corneal scar; Keratocytce; Fibroblast; Cold atmospheric Plasma; Corneal wound healing

Introduction
Plasma is the fourth state of matter and is produced by the constant supply of energy in a mixture of air and a noble gas, eg. Helium [1,2]. This process results in ionization of the atoms in the air and the production of plasma which contains positive and negative ions, free electrons, free radicals, ozone and ultraviolet radiation [2]. While heat can be used to create plasma, electrical current is usually used to produce plasma at ambient temperatures. This type of plasma is referred to as either non-equilibrium (non-thermal) plasma or Cold Atmospheric Plasma (CAP) and it has the same properties of high-temperature plasma but without heat production [3]. The potential applications of plasma have created new areas of study. The field of plasma medicine is relatively new when compared to the other fields of plasma [4]. Some of the earlier applications of plasma in medicine relied mainly on the thermal effects of plasma while the non-thermal effects of CAP have been investigated only recently in biology and in medicine [3]. CAP has been experimented in dentistry as a tooth bleaching agent [5] and in dental plaque treatment [6], as an alternative therapy for cancer [7,8], in disinfection and sterilization[9,10], in wound care[11,12] and in blood coagulation [13].

The major focus of CAP in medicine has been the antimicrobial property of plasma [14]. Although some recent studies have touted the effects of CAP on wound healing [15,16], Tiptop and Kroesen demonstrated that plasma treatment can stimulate the proliferation of fibroblast cells in a wound model of cell culture and thus accelerating the wound healing process. Grigoras et al. [16] assessed the re-epithelialization and healing of skin wounds inflicted on Wistar rat skin. They noticed accelerated re-epithelialization in the plasma treated skin lesions. However, the effect of CAP on wound healing, as on any other biological system, depends largely on the cell and tissue targeted, the composition of plasma used, discharge dose (power/time) and on the shape of the voltage applied to the discharge [3].

While the effect of cold atmospheric plasma on various biological systems and on wound healing has been studied, plasma effects on the eye and its potential applications in Ophthalmology remains to be investigated [9,17-20]. Due to the transparent nature of the cornea, controlled wound healing is essential to ensuring ideal corneal clarity and optimum vision following corneal injury. Haze formation following refractive surgery and improper bleb formation following glaucoma surgery are examples of some of the challenges that face both the ophthalmologists and their patients during the post-operative management. Modern trabeculectomy surgery for glaucoma involves using antimetabolites to modify post surgical wound healing at an attempt to prevent fibrosis of subconjunctival and episcleral tissues [21]. Using stringent criteria, Fontana et al. showed that the success rate of trabeculectomy procedure with the use of antimetabolite to be 80% at 1 year and 45% at 3 years [22]. However, the use of antimetabolites has been shown to increase the risk of bleb leakage and infection [23]. CAP may provide a novel approach to modify improper wound healing following ocular surface surgeries and could possibly increase their success rate. In addition, CAP can be used as an alternative to or in conjunction with antibiotics for treating corneal bacterial infections because of its antibacterial properties. Before considering the use of CAP in Ophthalmology, researchers have to first investigate the potential toxicity from CAP application to the ocular structures in order to determine the safety profile for CAP. This study investigates the effects of CAP therapy...
on corneal wound healing. We look also for any adverse effects from CAP application to the cornea.

**Methods**

This was an experimental animal pilot study involving Twelve New Zealand white rabbits. The study was approved through George Washington University Animal Care and Use Committee (IACUC) and was conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Visual Research. Only right eyes underwent treatment with no treatment to left eyes. The rabbits were divided into three groups. Group A (5 rabbits), Group B (5 rabbits) and Group C (2 rabbits). All rabbits in groups A and B underwent surgical intervention in the form of Epithelial/Basement Membrane (E/BM) ablation applied to the central 6 mm of the cornea using an Alger brush with 0.5 mm burr after receiving 92 mg/IM ketamine and topical anesthetic eye drops. After ablation, rabbits in Group A received CAP treatment for 2 minutes. The CAP application was limited to the area of ablation and spared the normal unwounded corneal epithelium. Group C received CAP treatment to the central 6 mm of the corneas of the right eyes for 2 minutes without ablation. In addition, two normal corneas from left eyes of two rabbits represented control group.

The cold plasma source was developed at the George Washington University. The plasma gun (Figure 1) is made of a Pyrex pipet through which helium [He] flow is supplied (the inner diameter of the thinner part of the pipet is about 4 mm, He flow rate is about 11.5 L/min). The gun is equipped with a pair of high HV electrodes—a central electrode (whose end was in direct contact with the plasma) and an outer ring electrode. The electrodes are connected to the secondary coil of a high-voltage transformer, generating sinusoidal high-voltage with amplitude 5 kV and a frequency of about 17 kHz. The plasma gun produced about 5 cm long plasma jet. When applied to the cornea, the tip of the plasma gun was kept about 2 cm away from the cornea and was moved in a fixed mode in a horizontal zig-zag form.

Figure 2 shows spectroscopic measurements of the cold atmospheric plasma jet along the plasma jet conducted in earlier work at similar experimental conditions, namely voltage ~ 4.6 kV, frequency ~ 26 kHz and He flow rate ~ 11.5 L/min [24]. Assigned spectrum measured at the jet’s end is shown in Figure 2A (this spectrum characterizes radiation from the whole length of plasma column including the discharge inside the Pyrex tube). The dependences of the intensities of O, N, N+, He, O along the plasma jet are shown in the Figure 1B (logarithmic scale). Based on these measurements, it was concluded that the optimal condition for maximization of radiation intensity would be 2-3 cm from the nozzle.

Rabbit eyes were evaluated clinically for corneal haze, lens clarity, E/BM defect size at day one and anterior chamber inflammation. Two rabbits from group A and two from group B were sacrificed 24 hr after the intervention and their corneas harvested to look for any inflammatory reaction and early reparative processes. The remaining eight rabbits in groups A, B, and C were sacrificed after 20 days and the corneas harvested to look for disorganized corneal wound healing or scar formation from CAP intervention. The corneas were cut at the limbus and then cut in half and put in formalin. The corneas were stained later with Hematoxylin and Eosin (H/E) and then examined under the light microscope to look for any inflammatory reaction, disorganized wound healing or scar formation. Four retinas (two from group A and two from group B) were sacrificed 24 hr after the intervention and their corneas harvested to look for any possible effects of CAP on the retina.

Immunofluorescence microscopy was performed for all groups to assess corneal fibroblast stimulation and scar formation using antibodies against a-smooth muscle actin (a-SMA)[Sigma-Aldrich Inc., St Louis, MO] and fibronectin [Chemicon International Inc., Temecula, CA], respectively. DAPI (4',6-diamidino-2-phenylindole) and antibodies to CD45 were also used to stain for the nuclei and to look for non-specific inflammatory cellular responses, respectively.

Images were taken for the corneal H/E sections at a 20x magnification and the average corneal epithelial thickness was calculated in both groups by taking multiple thickness measurements from 8 contiguous areas of the epithelium using Image J program [25]. Using the same images, the stromal cells were counted in both groups over an average stromal area of 0.08 mm² that covers the anterior...
and posterior stroma equally. Images of immunofluorescence stained corneal sections were also taken for further qualitative analysis.

Statistical analysis of stromal cell count, corneal epithelial thickness and epithelial defect healing velocity was performed with the student t-test to get p values with a level of significance at α=0.05, using Microsoft Excel® 2008 and StatPlus®.

Results

The clinical observations during the first 24 hours revealed similar corneal epithelial defects in the first 24 hours in group A (ablated with CAP treatment) compared to group B (ablated with no CAP treatment). Calculated average healing velocity was 0.79 mm/24 hr in the former group compared to 0.67 mm/24 hr in the later group, a difference of 0.12 mm/24 hr (95% CI, 0.12+/-.044; P=0.285). No other significant clinical observations were found between the groups. The corneas and lenses remained clear in all groups and there were no signs of corneal or anterior chamber inflammation noticed as a result from treatment with CAP. Table 1 summarizes the results of the clinical observations.

Histological exam under light microscopy using H/E stain of the corneas harvested 24 hr from intervention(Figure 3A) showed the presence of scant Polymorphonuclear cells (PMN) in the anterior stroma of Group A. PMN cells were absent in group B (Figure 3D). Keratocytes were absent in the anterior stroma of both groups, especially in areas where there was bare stroma from wounding. No other obvious differences were noticed in the structural integrity of the corneas between the two groups. The histological exam under light microscopy for the corneal sections stained with H/E stain and harvested 20 days from intervention showed no differences between groups A, B, and C (Figure 3B, C and E) when compared to control (Figure 3F). There were no signs of any late inflammatory responses, nor any disorganized healing processes or any epithelial detachments. No obvious adverse effects were noticed under the light microscopy from treatment with CAP. The histological evaluation for the H/E stained retinal sections was normal (data not shown). Tables 2 and 3 summarize the results of the H/E examination of the cornea at 24 hr and 20 days respectively.

Statistical analysis showed no significant difference between groups A and B at 20 days using the parameters of total stromal cell count (mean cell count of 87 cells/0.08 mm² in Group A vs. 90 cells/0.08 mm² in Group B, a difference of 3 cells/0.08 mm²: 95%CI, 3+/-.11.16; P=0.73) or corneal epithelial thickness (mean thickness of 0.03 mm in both groups and a difference of zero). Cell count at 20 days was higher in the anterior stroma than in the posterior stromal in both groups (a difference of 27 cells between the means; 95% CI 27+/-.97; P=0.002).

Table 1: Clinical Observations. A summary results of clinical examination for groups A and B.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Defect Area at Day 1</td>
<td>9.25 mm²</td>
<td>12.04 mm²</td>
<td>P=0.57</td>
</tr>
<tr>
<td>Epithelial Healing Velocity in the first 24 hr</td>
<td>0.79 mm²/hr</td>
<td>0.67 mm²/hr</td>
<td>P=0.28</td>
</tr>
<tr>
<td>Corneal Examination</td>
<td>Clear (No Haze)</td>
<td>Clear (No Haze)</td>
<td></td>
</tr>
<tr>
<td>Anterior Chamber Examination</td>
<td>Quiet/Clear</td>
<td>Quiet/Clear</td>
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*Group A: Corneas received ablation and CAP treatment
*Group B: Corneas received ablation without CAP treatment
CAP: Cold Atmospheric Plasma

Immunofluorescence microscopy analysis of stained corneal sections using antibodies against a-SMA [Sigma-Aldrich Inc.] and fibronectin [Chemicon International Inc.] showed no difference between the groups at 20 days post CAP treatment (Figure 4). However, analysis at 24 hours was inconclusive. CD-45 cell marker analysis were done to the corneas at 20 days post-injury and showed
Discussion

Our results showed that the in-vivo application of CAP to the exposed stroma of rabbit corneas at the assigned parameters in our experiment didn’t have any obvious adverse effects. The application of CAP to rabbit corneas after corneal ablation didn’t have an effect on the velocity of re-epithelialization in the first 24 hr when compared to the corneas which underwent ablation without CAP treatment. Grigoras et al. [16] showed on their experiment on the effect of plasma on the Wistar rat skin lesions that CAP increases the speed of re-epithelialization. The mechanism behind this observed effect remains unclear. In our experiment, CAP was applied to the corneal wound while sparing the surrounding normal epithelium. The limited power of our study could have also hindered the effect from showing at a significant statistical difference. More studies will be needed to study the effect of CAP on epithelial cell migration and investigate its possible mechanisms.

While Misyun et al. observed significant changes to the rabbit corneas after a very short in-vivo application of CAP in the form of corneal opacities, edema and epithelium, the dose of plasma used wasn’t mentioned which poses difficulties in evaluating and comparing their experiment to our study. In our experiment, CAP intervention didn’t induce any visible corneal inflammation or haze, and the corneas remained clear both at 24 hr and 20 days post intervention. We speculate that the plasma power utilized in the experiment by Misyun et al. was significantly higher than the one utilized in this study [18]. Fridman et al. [13] did a series of experiments on cadaveric tissues and on SKH1 mouse models to define the maximum acceptable dose that doesn’t cause any visible or histological changes. They concluded that a dose of 0.6 W/cm² for 10 minutes is deemed maximum acceptable prolonged treatment and a dose of 2.3 W/cm² for 40 seconds is deemed maximum acceptable high-power treatment. Higher doses produce visible changes in the form of partial to full thickness damage to the skin.

At 24 hours post intervention, some inflammatory response in all groups (Data not shown).

Conclusion

Plasma medicine is a rapidly growing field that might offer solutions to many of the modern challenges that face health care practitioners. However, a global effort is needed to further define the effective plasma components, to build certain standards and guidelines for plasma applications in biology and medicine and to clearly identify the hazards of plasma applications on living cells. Our study shows that the in-vivo application of CAP to the cornea of New Zealand White Rabbits doesn’t appear to have obvious adverse effects using our settings of Helium flow rate 11.5 L/min, Voltage 5 kV and Frequency 17 kHz. CAP treatment at the same settings following corneal injury does not interfere with rate of wound closure or induce increased inflammation. CAP did not have an effect on corneal wound healing or lead to scar formation based on final image analysis of a-SMA and fibronectin stained rabbit corneas. In this pilot study, the small number of rabbits reduced the power of the study.

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More studies are needed to evaluate the potential effects of CAP on the cornea and its possible applications in the field of Ophthalmology.

Acknowledgment

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References


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