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Research Article

Evaluation of the Effects of Plasma-Assisted Non-invasive Surgery on the Concentration of Candidate Cytokines in Tear and Serum Samples of Conjunctivochalasis Patients

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Abstract

Purpose: The present study aimed to determine whether treating symptomatic Conjunctivochalasis (CCh) patients with Atmospheric Low-Temperature Plasma (ALTP) can stimulate inflammatory responses or not; if yes, are they in a stable state and how long do they take back to the normal range? **Methods:** In this study, 20 patients with definite CCh -with a grade of 3 and 4 -were recruited (identifier: IRCT20181229042160N3). ALTP was generated and used to undertake the surgery; the digital camera examinations were used to follow up the clinical outcomes after surgery. Tear and serum samples were isolated from each patient at 1 week, 1 month, and 3 months after treatment, and the concentration of candidate pro-inflammatory (i.e. IL-2, IL-6, IL-17A, IFN-γ, and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) was measured using flow cytometry. **Results:** In all patients, grades 3 and 4 of CCh were substantially improved to grades 1 and 2, respectively; clinically, after one month, no complications were reported in the eyes that were subjected to ALTP-based surgical procedure. Besides, the analyses revealed that the ALTP-based treatment could improve the 'Ocular Surface Disease Index' score. Interestingly, regarding the concentration of the candidate cytokines in the tear and serum samples, no differences were observed at pre- and post-surgery follow-ups. **Conclusion:** In sum, we reintroduced using the Plasma-Assisted Noninvasive Surgery method as a novel approach to treat CCh because of its accurate handy nature with no stimulated inflammatory responses.

Keywords: Plasma-Assisted Noninvasive Surgery; Conjunctivochalasis (CCh); Tear and serum samples; Candidate cytokines; Ocular surface disorder; Plasma medicine; Atmospheric Low-Temperature Plasma; Inflammatory responses; Interleukins

Introduction

Plasma, as the fourth state of matter, has been used in a span of decades for sterilization of medical equipment and sterile packaging in the food industry [1]; however, its potential applications in medical sciences have been recently boosted, especially for therapeutic purposes in cancer therapy [2], disinfection and blood coagulation [3], impaired tissue ablation [4], and ophthalmology [5]. According to the previous studies, atmospheric low-temperature plasma (ALTP), produced at atmospheric pressure, is supposed to be safe for treating different complications [4,6], among which, using this technique to treat the Conjunctivochalasis (CCh) has been recently documented [4].

As a chronic conjunctival disorder, CCh is mainly manifested by redundant, loose, and nonedematous conjunctival folds [7]. It can appear anywhere on the bulbar conjunctiva, but it is most

commonly found overlying the inferior eyelid margin in both eyes' inferior bulbar conjunctiva [8,9]. CCh causes ocular surface irritation and/or pain in people of all ages (reviewed in [7]), although it most commonly affects the elderly [10]. Even though the exact prevalence of this disease yet still remains blanketed in mystery, a pilot study conducted on the Iranian population showed that only 6.2% among participants aged 45–69 years manifested CCh and the prevalence of unilateral and bilateral CCh was reported 0.7% and 5.5%, respectively [11]. This disease tends to be manifested bilaterally, but in some cases, it can be detected in only one eye [12].

For asymptomatic CCh patients, containing the most portion of all CCh cases, no treatment is required; on the other hand, patients with symptomatic CCh may receive a variety of medical or surgical treatments. Based on the CCh grade in symptomatic patients, different approaches -i.e. medical and surgical therapies -can be undertaken [7,9,13]. The thrust of CCh medical therapy is to enhance tear film function and inhibit ocular surface inflammation, hence assuaging patients' ocular irritation [14]; for example, in CCh patients, topical corticosteroids may be used to treat the associated ocular surface inflammation [15]. Remarkably, those who do not respond to medical treatment may be subjected to surgical interventions [16].

Although a variety of surgical approaches have been developed, surgeons more often use conjunctival cauterization or surgical excision with or without tissue graft [17]. Importantly, these kinds of methods do not result in the removal of normal conjunctival tissue, significant scar formation, or motility restriction [17]. Furthermore, in some cases, the procedures should be repeated to obtain the favorite outcome. Refractory CCh has long been treated with simple excision of the redundant conjunctiva and direct closure [7,18]. It is somehow difficult to assess the exact amount of conjunctiva that should be excised during this process. Moreover, the need for suture positioning extends the operating period and causes healing to be delayed [19]. Besides, suture-related complications, such as postoperative pain and foreign body sensation, pyogenic granuloma formation, giant papillary conjunctivitis, and inflammation induction are additional concerns [7].

In sum, there is an urgent need for introducing an effective therapeutic approach with substantially decreased side-effects such as ulceration, extended inflammation, and surrounded tissue necrosis [20]. According to our previous studies, Plasma-Assisted Noninvasive Surgery (PANIS) which uses the atmospheric low-temperature plasma (ALTP) can be successfully considered as a novel approach to eliminate the implications of the ocular surface tissues and CCh treatment. In that research Nejat et al. have proved the efficacy of plasma treatment for cch with comparison of examinations such as best-corrected visual acuity (BCVA), tear meniscus height (TMH), Corneal staining zone, ocular surface disease index (OSDI), intraocular pressure (IOP), Contrast sensitivity (CS) before and 6 months after procedure. However, before becoming a common-used procedure and evaluating the safety of this method, understanding the underlying molecular mechanisms influenced by ALTP-treatment and also its effects on inflammatory responses are of importance. In fact, plasma treatment has been documented to probably stimulate the expression profiles of pro-inflammatory cytokines [21]. Although in the previous study, we confirmed the safety of the ALTP used for conjunctival cyst ablation [20], there is a snippet of information about the effects of ALTP on the expression profiles of pro- and/or anti-inflammatory cytokines in CCh patients, who were subjected to surgery using this technique. We take the view that the findings of this study aid us not only to grasp the importance of drug intervention to minimize the side effects (e.g. in case of taking antibiotics and anti-inflammatory drugs for a long-time after surgery) but to immunologically assess PANIS application in CCh patients. Additionally, this study verifies whether ALTP-treatment is safe and handy to treat CCh. In the present study, we conducted an investigation into whether ALTP surgery can change the expression of pro-/anti-inflammatory cytokines in the patients' tear and serum or not.

Methods

Patients

This research fulfilled a procedure that was accepted by the ethics committee of Semnan University of Medical Science, Semnan, Iran, and was also in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from each participant and they also provided signed written consent to publish all personal and medical details included in this study. All of the patients' clinical data and medical histories were gathered at the Vision Health Research Center, Semnan, Iran (identifier: IRCT20181229042160N3). This center has also collected detailed patient histories including disease onset, signs, progression, and family history.

We enrolled CCh patients that their disease had been earlier confirmed by ophthalmic examinations such as best-corrected visual acuity (BCVA), Tear meniscus height (TMH), Corneal staining zone, Intraocular pressure (IOP), Contrast sensitivity (CS), Ocular surface disease index (OSDI) and Slit-Lamp Biomicroscopy (i.e. digital camera examinations) before, 1 week, 1month and 6 months after plasma exposure in addition to the typical ocular symptoms containing ocular discomfort, pain, foreign body sensation, and episodic tearing. Patients who wear contact lenses were disqualified, as were those with some ocular background, including ocular surgery, ocular cancer, allergy, or autoimmune disorder, even if it was only in one eye. We also excluded the patients who were using a punctual plug or topical

ophthalmic medications. We instructed the patients who used artificial tears not to use them for at least 12 hours prior to the test. All of the patients underwent comprehensive ophthalmologic examination to reconfirm their disease; the CCh grading was carried out according to the 'Meller And Tseng Grading System' [8]. According to this grading system, CCh was categorized into 4 grades including grade 1: patients with no persistent fold, grade 2: A single/small fold was observed in the CCh patients, grade 3: patients with > two folds and no higher than the tear meniscus, and grade 4: patients who showed multiple folds and higher than the tear meniscus. Remarkably, symptomatic CCh patients with grade 3/4 were subjected to procedure.

Experiment

The eye with CCh was selected for the test. Anesthesia was performed using the application of one drop of tetracaine 0.5% eye drops (Sina Daro, Tehran, Iran), used 3 times with 5 min intervals. The procedure was accomplished using the white handpiece of the PLEXR PLUS device (GMV Srl, Rome, Italy) by targeting ~15-40 spots of the lower conjunctiva to remove CCh at a distance of 4 mm from the inferior limbus (Supplementary Video 1). Every spot will take less than one second to apply so that, the entire procedure time will be short. In detail, for CCh with grade 3, two rows of plasma spots were placed at a distance of 4 mm of the limbus, while for CCh grade 4, three rows of spots were implicated. In fact, the number and plasma rows depended on the CCh grade (Figure 1).

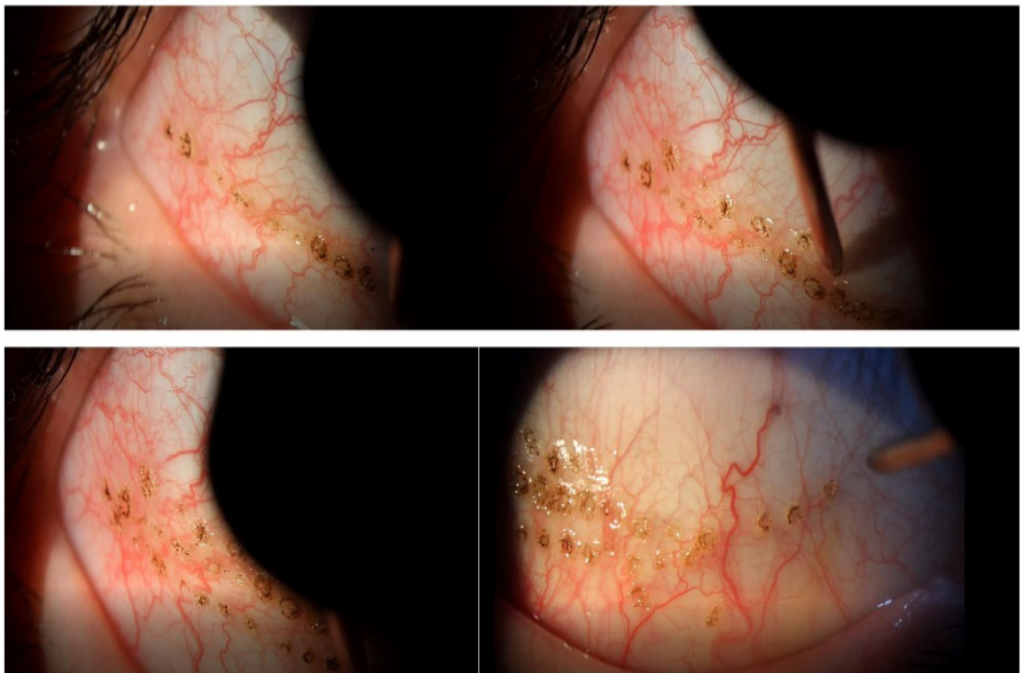

Figure 1: Plasma spots. Different rows of plasma spots can be applied depend on grade of conjunctivochalasis.

To eliminate the risk of conjunctivitis after ALTP-based surgery, the patients were subjected to take chlobiotic 0.5% (Sina Daro, Iran) using one drop every 6 hours in the first week and also betamethasone 0.1% (Sina Daro, Iran) for three weeks after surgery in tapered off mode. To detect any abnormality, the patients were followed up for a period of 6 months at a specified time (i.e. 1 day, 1 week, 1 month, 3 months, and 6 months). The Plexr device was in a continuous mode and also in the lowest power level (White handpiece; Vpp = 500 V, Power = 0.7 W, and Frequency = 75 kHz). It was used at less than 1-sec intervals using a 22-gauge needle; all detailed information is put forth in Table 1. It should be noticed that the Plexr used the sublimation process -i.e. direct conversion of the solid phase to gas without any transitional liquid phases -so thermal damage to the surrounding tissues was unlikely.

Characteristics		Values
Gas type		Air
Power supply		Docking station = 24 V
		Handpieces: embedded inductive charger = 5 V
Handpieces	Max output	≤ 2 W
	Max working voltage	≤ 1.3k VPP
	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
† in the present study, we used the 'White' handpiece.		

Table 1: Technical features of the Plexr device that was used in this study.

The ocular surface disease index (OSDI) and contrast sensitivity were assessed in a span of a 6-month follow-up. According to OSDI, the final score is in a range of 0 to 100, in which 0 to 12 refer to normal, 13 to 22 indicate mild dry eye disease, 23 to 32 show moderate dry eye disease, and >33 demonstrate severe dry eye disease [22, 23]. Moreover, the contrast sensitivity was also scored in the patients whereby score 2 refers to normal, while scores of ≥1.5 signify poor contrast sensitivity. The obtained scores between 1-1.5 were also categorized the patients into the visual impairment group, whereas scores <1 indicate a visual disability.

To assess whether the PANIS method can affect the visual acuity or not, the Best-Corrected Visual Acuity (BCVA) was measured for each participant. We also used corneal fluorescein staining to show any improvement after surgery. In order to show whether intraocular pressure can be changed due to ALTP-based surgery, an Icare tonometer was performed.

Tear Fluid and Serum Collection

For experiments comparing the concentrations of proteins in tear fluid, before and after ALTP-based surgery, the samples were taken from the inferior tear meniscus of both eyes from 20 patients with primary CCh. This caused the least possible irritation. After pre-washing the patients' eyes, tear samples were collected using the end of a micropipette tip and immediately the tears were located within a 0.5 mL tube. Tear samples were then stored at −80°C until further examination. To extract the serum sample, blood samples were taken in sterile Vacutainer tubes (Becton Dickinson, IL, USA). After a maximum storage time of 2 h at room temperature, blood was centrifuged at 1,000×g for 15 min and the supernatant was aliquoted in 300 µl polypropylene tubes and stored at -80º C until further experiments.

Flow-cytometry

The concentration of the candidate cytokines embracing the pro-inflammatory (interferon-γ: IFN-γ, interleukin-2: IL-2, IL-6, IL-17A, and tumor necrosis factor-α: TNF-α) and anti-inflammatory ones (IL-4 and IL-10) were measured using the BD Cytometric Bead Array (BD Bioscience, San Jose, California, USA). The concentration of each cytokine was calculated based on pg/mL. In brief, 100 µL of tear/serum sample was thawed and transferred to a 50 µL of each capture antibody-bead reagent. Then, 50 µL of sterile PBS was added into the samples and the cocktail was incubated for 1h at room temperature, a process that was followed by adding the antibody-phycoerythrin reagent to each sample and incubation for 2 h at 23°C. In order to remove the unbound antibodies, the samples were washed at least 3 times. Flow cytometry was carried out using a FACSCalibur® flow-cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) according to the previous studies 26.

The following antibodies were used: IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, and IFN-γ (all from R&D Systems Biotech Co. Ltd., Emeryville, USA).

Statistical analysis

All results were reported as mean ± Standard Division (SD) of three independent experiments. All statistical tests were analyzed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). A one-way ANOVA test was used, where applicable, and the level of statistical significance was set to P-value <0.05.

Results

Patients

The main characteristics of 20 affected individuals are summarized in Tables 2 and 3. According to the meticulous examinations, neither intraoperative nor postoperative complications were detected. To be on the safe side, after doing ALTP-based surgery, the patients' eyes were evaluated 1 day after surgery for ophthalmic parameters. Additionally, in a span of 6 months, we followed all patients up (Table 3).

The BCVA score calculated for each participant was almost the same before and after surgery, indicating that the visual acuity had not changed; for example, the average of BCVA calculated for the patients before and 1 week after surgery was 0.91±0.07 and 0.95±0.07, respectively. Moreover, no changes in meniscus height were reported (P-value >0.05); for instance, the average score for the pre-treated group was 0.22±0.04, and 1 week after surgery was calculated 0.19±0.04 (P-value >0.05) (Table 2). Corneal fluorescein staining was carried out in the patients so as to show the improvement; no abnormality was detected by this method as well. We also showed that the Contrast Sensitivity was unchanged, before and after performing the surgery (Table 2).

Follow up	Patients	Parameters						
-	n=20	Severity based on OSDI	BCVA (logMAR)	Tear Meniscus Height†(mm)	Corneal staining zone†	IOP (mmHg)	Contrast sensitivity (logMAR)	CCh grade
Pre	-	32.44±3.49	0.91±0.07	0.22±0.04	1/3 Inferior	11.83±1.50	1.55±0.1	Grade 3/4
1 Week	-	12.73±3.49	0.95±0.07	0.19±0.04	No staining	12.16±1.50	1.50±0.1	
1 Month	-	15.25±3.49	0.93±0.07	0.21±0.04	No staining	12.50±1.50	1.51±0.1	
3 Month	-	6.13±3.49	0.95±0.07	0.17±0.04	No staining	13.66±1.50	1.58±0.1	Grade 1/2
6 Month	-	5.14±3.49	0.95±0.07	0.25±0.04	No staining	13.00±1.50	1.57±0.1	
†Measured by Tearscope (SBM Co.)								

Table 2: the most important features of the participants.

Patients	Parameters									
	Pain scale (0-10)†	Tearing (0-5)	Redness (0-5)	Photophobia	Foreign body sensation	Chemosis (0-2)	Injection (1-10)	Anterior chamber reaction	Eyelid edema ‡	Corneal involvement §
P1	4	2	3	Yes	No	2+	2+	0	0	0
P2	0	1	1	No	No	1+	2+	0	0	0
P3	4	0	1	No	No	1+	1+	0	0	0
P4	3	1	1	No	No	1+	1+	0	0	0
P5	4	2	2	No	Yes	1+	2+	0	0	0
P6	2	1	1	No	No	1+	1+	0	0	0
P7	3	1	2	Yes	No	2+	2+	0	0	0
P8	0	2	2	No	No	1+	2+	0	0	0
P9	5	1	2	No	No	1+	2+	0	0	0
P10	3	0	3	No	No	1+	1+	0	0	0
P11	4	2	2	No	Yes	1+	2+	0	0	0
P12	2	1	3	No	No	1+	1+	0	0	0
P13	0	1	2	No	No	1+	1+	0	0	0
P14	4	1	2	No	No	2+	1+	0	0	0
P15	4	1	1	No	No	1+	2+	0	0	0

P16	3	2	1	No	No	1+	2+	0	0	0
P17	2	0	1	No	Yes	1+	1+	0	0	0
P18	4	0	1	No	No	1+	1+	0	0	0
P19	5	2	1	No	No	1+	2+	0	0	0
P20	0	1	1	Yes	No	2+	2+	0	0	0

†Score/Scale
‡ Scores, 0 = Absent; 1 = Mild edema; 2 = Moderate edema; 3 = Severe edema.
§ Scores, 0= none involved; 1 = equal or less than 50% of the conjunctiva; 2 = more than 50% of the conjunctiva; 3 = the sclera or orbital tissue is also involved, in addition to the conjunctiva.

Table 3: The ophthalmic parameters were evaluated 1 day after surgery.

We also showed that ALTP-based surgery could improve the OSDI score in CCh patients, e.g. ameliorating the CCh severity from the moderate-to-severe to normal ranges. Interestingly, we showed that the CCh severity was substantially decreased after ALTP-based surgery, e.g. from grade 4 to 2. Further following ups verified that this decrease constantly remained for up to 6 months after the surgery. In order to show whether intraocular pressure can change because of ALTP-based surgery or not, an Icare tonometer was performed. Expectedly, we detected no abnormality by this method (Figure 2).

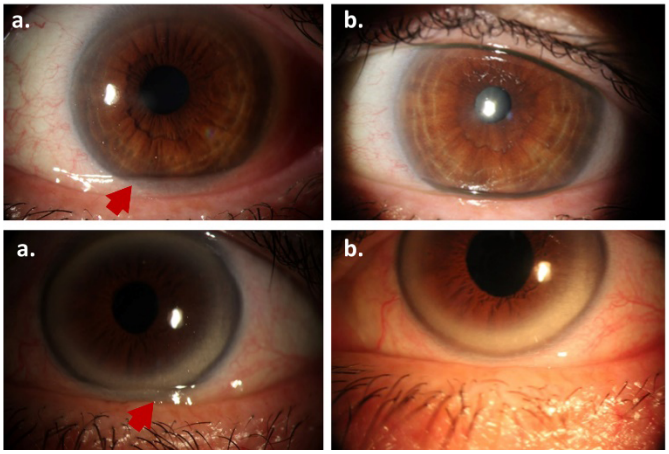

Figure 2: PANIS method was employed to treat Conjunctivochalasis. In two cases, a and c represents the disease before treatment, while b and d show post-treatment periods. Examinations showed that after surgery, the severity of the CCh was decreased to the lower grades, videlicet grades 3/4 to grades 1/2. The red arrows show the abnormal structures caused by CCh.

Cytokine concentration in Tear and Serum Samples

Each candidate cytokine was in a detectable range in tear and serum samples. The concentrations of the pro-inflammatory (IFN-γ, IL-2, IL-6, IL-17A, TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokines in basal tear and serum samples from CCh patients are presented in Figure 3a-n. The concentrations were reported according to pg/mL. No significant differences were detected in all groups considering the concentration of candidate cytokines in patients' tear and serum samples.

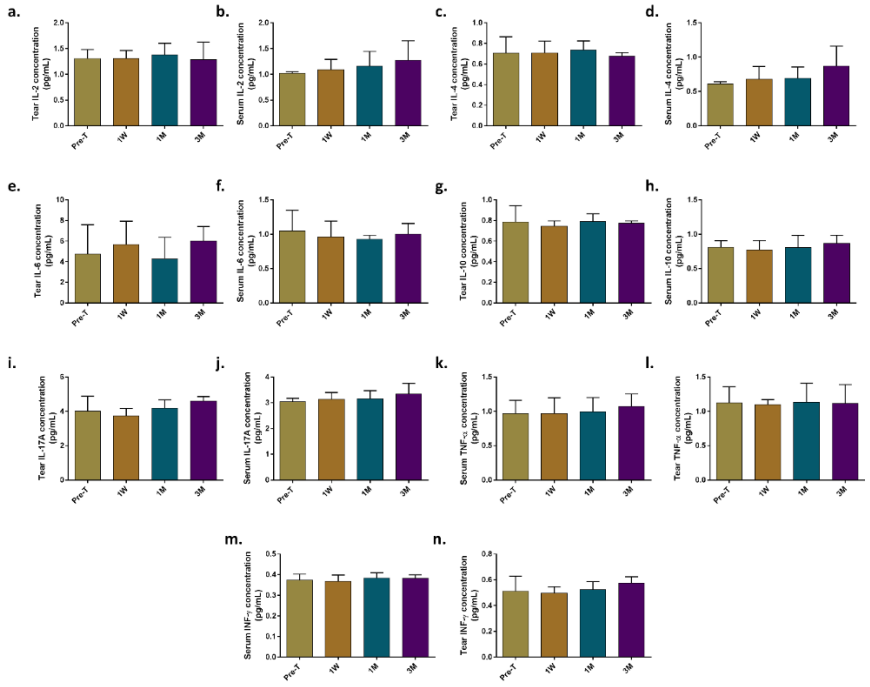

Figure 3: Flow cytometry was used to measure the concentration of candidate cytokines in tear and serum samples. a and b) the concentration of Interleukin-2 (IL-2) was assessed respectively in tear and serum samples. c and d) The concentration of IL-4 was unchanged before and after surgery. e and f) flow-cytometry analyses showed no significant differences in the concentration of IL-6 in patients' tear and serum respectively, before and after doing ALTP-based surgery. g and h) the concentration of IL-10 was unchanged in pre- and post-surgery groups. i and j) the findings showed that ALTP-based surgery did not affect the concentration of IL-17A in CCh patients' tear and serum samples. k and l) Data demonstrated that the concentration of TNF-α cannot change owing to doing the ALTP-based surgery, in the patients' tear and serum, respectively. m and n) the concentration of INF-ϒ was assessed in the patients' tear and serum samples, while no differences were identified before and after doing the surgery. All data represent the mean ± SD.

Discussion

A bunch of factors such as age, ocular inflammation, mechanical friction, tear film instability, and delayed tear clearance have been documented to contribute to CCh pathogenesis [7,24]; however, the exact etiology is still unclear. Symptomatic CCh can be very imposing and has substantial impacts on patients' quality of life. CCh is considered an age-dependent disease that is usually manifested by different symptoms to touch upon ocular discomfort, unstable tear film, and exacerbated vision-related quality of life [7,25]. Although in clinical practice, CCh is a common ailment, there is little to no definite treatment for this disease [7]. These patients usually take artificial tears to cut down on dry eye symptoms [26], but this procedure is not satisfactory. In fact, for asymptomatic CCh, no treatment is required, while for symptomatic ones, different medical and surgical interventions are available. For the patients who yet respond to the medicine, taking lubricants and/or anti-inflammatory eye drops substantially reduces various tear cytokines and ameliorates symptoms and signs [17,27], so the immune system can be considered as the first line promoting/exacerbating CCh symptoms. Regarding the cases with the most severe form of CCh, such treatments should be put behind and replaced with surgical ones.

Despite the fact that a number of surgical methods have been developed so far, most surgeons often use conjunctival cauterization or surgical excision with/without tissue graft [28]. This technique is disadvantageous because it needs to be repeated in some cases, hence, can per se increase the recurrence rate of CCh and also put the patients on inconveniences [28]. In symptomatic CCh cases, the redundant conjunctiva can be surgically removed, however, it is somehow demanding to determine how much of conjunctiva tissue is supposed to be excised [8, 29]. Additionally, conjunctival resection with Tenon's capsule excision can also be used as a common method to remove keratinized bulbar conjunctiva and make firm adhesion of bulbar conjunctiva to sclera [7, 30]. In sum, due to the minimized mechanical friction force, this method reduces the inflammatory reaction over the

ocular surface, confirmed by the unchanged concentration of cytokines in patients' tears after surgery. However, the anatomy of the Tenon's capsule can pose some potential disadvantages [31]. One of the major drawbacks of surgery-based techniques is over-resection that may result in a compromised inferior fornix, cicatricial entropion of the lower lid, or limited ocular movement. Under-resection, on the other side, may lead to the persistence of symptoms [7,17]. Thus, the risk of recurrence and also unwanted tissue damages are increased. Furthermore, the surgery-based techniques usually need sutures that in turn prolong the operating time, to say nothing of delaying the healing process [19]. On top of that, suture-related complications -e.g. foreign body sensation, pyogenic granuloma formation, giant papillary conjunctivitis, and extended inflammation -have been reported to occur in some cases [7,9,19]. To avoid such complications, fibrin glue has recently been developed to use in CCh excision [12,32], but it is not free from side effects though.

According to Wang et al., Inflammation may be a consequence or a cause of CCh [33]. Pro-inflammatory cytokines, e.g. TNF-α and IL-1β, can promote the overexpression of Matrix metalloproteinases in CCh. These events demonstrate the self-sustaining role of ocular surface inflammation in CCh pathogenesis. Supportedly, tears from CCh patients contain substantially higher levels of proinflammatory cytokines [34]. Indeed, a delay in tear clearance can aggravate pre-existing ocular surface inflammation caused by mechanical friction or oxidative stress [7]; interestingly, ocular surface inflammation has been hypothesized to cause delayed tear clearance and tear film instability in CCh patients [8, 35]. The role of inflammatory cells in CCh samples is somehow debatable; while many investigations have not shown any significant infiltration of inflammatory cells in the conjunctiva of eyes with CCh [36], Zhang et al. reported lymphocyte and plasmocyte infiltration in CCh patients [37]. However, it is unclear that whether such findings are pertinent to CCh itself or the coexisting nasolacrimal duct obstruction. Consistently, we evaluated the important inflammatory mediators, i.e. pro- and anti-inflammatory cytokines, in the patients' serum and tear to show whether this method is safe immunologically or not. None of each cytokine was increased in the putative samples; this was in line with our previous study showed that the ALTP-treatment has minimal effects on the inflammatory responses in animal models, e.g. rats and rabbits [20, 38]. These confirm that ALTP-based surgery is unlikely to promote the secretion/expression of pro- and anti-inflammatory cytokines [4,20,38].

In sum, according to the literature review, in near all surgical-based procedures, the patients should be hospitalized, although according to our previous studies, using the PANIS method (an ALTP-based surgery), the patient will experience minimal surgery time and complications without any needs for hospitalization. In the present study, the PANIS method as a new plasma-based method was used to treat CCh. This technique uses the sublimation process, so minimizing the side effects and pertinent complications. In line with the previous study [4], herein, we extended the sample size to 20 individuals; we underscored that after surgery, no clinically intraoperative and postoperative complications were detected in each patient, confirmed by clinically 6 months following ups. The further postoperatively follow-ups also showed that CCh scares were completely healed and no sign of recurrence was documented. We should point out that this approach is advantageous because, in case of recurrence, the patients can be easily subjected to do surgery again using this office-based method.

In the present study, we used two major groups of candidate cytokines, as the most important molecules triggering inflammatory responses after eye surgeries. To eliminate such locally inflammatory responses, some immunosuppressive drugs are prescribed. Ocular surface cells themselves also express cytokines e.g. IL-1α, IL-2, and TNF-α that serve as an important defending line against pathogens [39]. According to Lam et al., the severity of the ocular surface epithelial disease is related to various cytokines and chemokines, including IFN-γ, IL-1α, IL-1β, and IL-6 [40]. Tear IL-6 and IL-8 levels have been identified to increase in parallel with the severity and stages of the diseases in CCh patients [41]. Interestingly, it has been recently demonstrated that tear IL-1β, IL-17A, and TNF-α were correlated with the severity of dry eye diseases, so these kinds of inflammatory cytokines were defined as potential biomarkers for this disease [42]. In the present study, we nominated 7 cytokines to show how ALTP-based surgery can change the inflammation responses.

Interestingly, no differences before and after doing the surgery was documented; this per se shows if PANIS method is performed in a combination of taking medicines (e.g. ciprofloxacin and betamethasone), it can be considered as a handy, safe, office-based method to treat CCh. One of the major limitations of our study is the limited sample size; in fact, further studies are needed to replicate the data in CCh patients at different stages. Another drawback is the limited number of candidate cytokines. In this study, we focused on the important candidate pro- and anti-inflammatory cytokines, but future investigations are recommended to unveil the throughout immune profile embracing all critical cytokines, chemokines, immune cells after ALTP-based surgery. Last but not least, we could not determine that the immunologically unchanged profile was absolutely due to our used method or was chalked up to the taken immunosuppressive medicines, e.g. ciprofloxacin and betamethasone eye drops. Although, our previous studies using the rat models have answered these to some extent [38].

Conclusion

In this study, we showed that ALTP-based surgery is a safe, effective technique to treat symptomatic CCh. This method can provide

greater symptom relief after surgery and also doesn't have any undesirable systemic effects. Besides, no clinically complications were detected after surgery in the candidate CCh patients. We also showed that the PANIS application did not cause any inflammatory responses (followed up for 3 months after surgery), confirmed by measuring the concentration of 7 candidate cytokines in patients' tear and serum samples.

Conflicts of interest/Competing interests: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author Contributions: Farhad Nejat: Conceptualization, Data curation, Supervision, Funding acquisition, Investigation, Visualization, Roles/Writing - original draft. Khosrow Jadidi: Conceptualization, Data curation. Shima Eghtedari: Conceptualization, Data curation, Formal analysis, Project administration, Methodology, Writing - review & editing. Hosein Aghamollaei: Conceptualization, Data curation, review & editing.

Ethics approval: This research fulfilled a procedure that was accepted by ethics committee of Semnan University of Medical Science, Semnan, Iran, and was also in accordance with the tenets of the Declaration of Helsinki.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent for publication: Each participant provided signed written consent to publish all personal and medical details included in this study.

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