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Maximal tear secretion evoked by controlled stimulation of corneal sensory nerves in healthy individuals and dry eye subjects

María L. Merino [a,b], Javier Belmonte [c], José Rosas [d], M. Carmen Acosta [a], Juana Gallar [a,e], Carlos Belmonte [a,*]

[a] Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, San Juan de Alicante, Spain
[b] Oftalmología, Hospital de la Marina Baixa, Villajoyosa, Spain
[c] Oftalmología, Hospital General Universitario de Alicante, Alicante, Spain
[d] Reumatología, Hospital de la Marina Baixa, Villajoyosa, Spain
[e] Instituto de Investigación Biomédica y Sanitaria de Alicante, San Juan de Alicante, Spain

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ABSTRACT

Purpose: To measure, the tear flow changes evoked in healthy subjects and dry eye disease (DED) patients by controlled sensory stimulation of the eye surface with i-Onion™, a new stimulation device.
Methods: Sensory corneal nerves were stimulated with an instrument (i-Onion™) that ejects puffs of CO_2 gas (99.9%) at 200 ml·min^{-1} for 3s, delivered 5 mm from the cornea. Using Schirmer test strips, tear volumes were measured over 3 min in the cornea of one eye before (basal tear volume -BTV) and in the other eye after stimulation of the sensory nerves with CO_2 (stimulated tear volume -STV). These measurements were obtained from a control group of adults of either sex (17 students aged 20–30 and 29 subjects without signs of dry eye aged 25–61), a cohort of DED patients (aged 34–75) that included 12 asymptomatic, suspected DED subjects (Schirmer <7 mm and/or TBUT <10s), and 30 Sjögren's syndrome (SS) patients.
Results: CO_2 stimulation significantly increased the tear volume (BTV = 14.6 ± 1.0 mm, STV = 19.0 ± 1.1 mm: n = 46) in 78% of control subjects, reflecting a mean tear reserve volume (TRV = STV-BTV) of 4.4 ± 0.8 mm. Individual differences were wide, and while no increase in reflex tearing was evoked in 30% of subjects with a BTV >10 mm, the remaining 70% responded vigorously to stimulation, even those with a BTV >18 mm. Asymptomatic DED subjects displayed weaker responses to CO_2 stimulation, with lower STVs. Both the BTV and STV of SS patients were low, significantly below those of the healthy controls.
Conclusions: Measuring the rise in reflex tearing volume evoked by controlled corneal stimulation provides objective information about the tear glands' secretory capacity in health and disease.

1. Introduction

Under resting conditions, including when sleeping, a moist and lubricated eye surface is maintained through the continuous basal secretion of tears by the principal and accessory lacrimal glands, supplemented by goblet cell mucins. In the waking state, reflex adjustments of tear flow compensate for changes in evaporation brought about by varying environmental conditions [1–3], reflexes controlled by a system referred to as the lacrimal functional unit [4]. In addition, enhanced acute, defensive reflex tearing may be evoked by noxious stimulation of the cornea, conjunctiva or other orofacial mucosae, such as those lining the nose, oropharynx or upper respiratory pathways [5,6].

Peripheral neural information that modulates basal and reflex tear flow is provided by afferent sensory nerve terminals that belong to functionally distinct classes of trigeminal ganglion neurons [7,8]. Indeed, these neurons project to higher order neuronal clusters in the brain that are located at various levels of the trigeminal brainstem nuclear complex [9–11]. These neurons process sensory information and they regulate lacrimation through their connections in the superior salivatory nucleus [11]. In turn, they activate peripheral parasympathetic and some sympathetic ganglion neurons of the pterygopalatine and superior cervical ganglia, respectively, stimulating tear secretion by the main and accessory lacrimal glands [12,13].

Dry eye disease (DED) is a multifactorial disease of the ocular surface

* Corresponding author. Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, Avda Santiago Ramón y Cajal 2, 03550, San Juan de Alicante, Spain.
E-mail address: carlos.belmonte@umh.es (C. Belmonte).

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characterized by a loss of tear film homeostasis and other symptoms [14]. DED affects millions of people worldwide and it has been classified into two main subcategories: aqueous tear deficient (ADDE) and evaporative (EDE) [15]. The reduced aqueous tear flow in ADDE is also a frequent clinical sign of systemic autoimmune disorders that cause lacrimal gland damage, such as primary Sjögren's syndrome (SS), SS secondary to rheumatoid arthritis, or systemic lupus erythematosus [14,16]. Disruption of sensory innervation at the eye surface as a consequence of trigeminal nerve injury, refractive surgery or chronic contact lens use are other potential causes of ADDE [17]. In recent years, a borderline type of DED has been described, referred to as short tear break-up time DED (Short TBUT DED), which is characterized by marked tear film instability, subjective symptoms of pain and similar dryness to ADDE, yet with normal basal tear secretion and little or no corneal damage. This Short TBUT DED has been associated with the widespread use of visual display terminals [18,19].

DED is initially diagnosed through tests involving tear volume measurement, meibography and lipid interferometry [20]. Quantifying tear flow is an essential step in DED diagnosis and while it has traditionally been performed with the Schirmer test, more recently it has also been measured by meniscometry or fluorometry [20,21]. The Schirmer test calculates aqueous tear production by placing a strip of filter paper at the inferior-temporal aspect of the conjunctival sac and measuring the extent to which it is wetted length (in mm), usually after 5 min. When performed under conditions of topical anesthesia the sensory input from the eye surface is suppressed, providing information about the basal tear flow rate even though reflex input from other mucosae of the cephalic area is not eliminated [22,232]. When the Schirmer test is performed without anesthesia, the tear volume measured is the result of the autonomous, basal gland secretion plus a variable neural component, which includes the tonic reflex stimulation evoked by cold thermoreceptors [24]. In addition, the tear volume may also be influenced by an irritative reflex effect caused by the filter paper strip exciting conjunctival nociceptors, which may influence the reproducibility and specificity of the data [22].

When the eye remains open, environmental factors strongly influence tear flow and this varies markedly in healthy individuals. Indeed, there is continuous modification of the peripheral sensory inflow from the eye surface, which also reduces dramatically upon eye closure [25]. Thus, basal tear flow in rested subjects should be determined under conditions of bilateral eye closure or when evaporative loss is prevented with the eyes open under ambient conditions of 100% humidity [25–27]. Likewise, the maximal capacity of tear secretion has been determined by evoking tearing through stimulation of the nasal mucosa and measuring this with a Schirmer test with anesthesia [28]. Nonetheless, differences between basal tear flow and the maximal reflex lacrimal secretory response in healthy or DED subjects has received little attention in general [5,21,29].

Controlled supramaximal stimulation of nociceptive corneo-conjunctival nerves can be obtained by directing a jet of high-concentration CO_2 gas at the surface of the eye [30–33]. Local formation of carbonic acid immediately decreases the pH of the precorneal tear film, which in turn stimulates polymodal nociceptors and evokes sensations of discomfort, as well as irritative tearing [5,30–34]. Here we used a new instrument based on this principle to obtain transient, and presumably complete activation of polymodal nociceptor nerve terminals in the stimulated corneo-conjunctival area, to produce well-controlled and reproducible reflex tear secretion in humans. Our aim was to determine whether this technique may permit the simple and objective clinical assessment of an individual's maximal secretory capacity, helping to quantitatively evaluate the functional reserve of the lacrimal glands.

2. Methods

2.1. i-Onion™

Gas stimulation was performed with a tear flow stimulation apparatus (i-Onion™), a portable device designed in our laboratory and built by Tearful SL (Alicante, Spain). The i-Onion™ applies a puff of human-grade, 99.9% CO_2 gas onto the cornea through an outlet nozzle at a flow rate of 200 ml·min^{-1}. When in use, the nozzle is placed 5 mm from the center of the cornea aided by a spacer attached to the front of the instrument, an element that rests on and is supported by the bony margins of the orbit. The latest version of the i-Onion™ instrument is shown in use in Fig. 1.

2.2. Experimental protocol

Preliminary testing of a prototype tear flow stimulator was performed on healthy volunteers at the Human Esthesiometry Laboratory of the Instituto de Neurociencias (UMH-CSIC, San Juan de Alicante, Spain). Previous animal and human studies with the Belmonte gas esthesiometer [30,34] had shown that CO_2 concentrations >80%, with a gas flow >150 ml·min^{-1} evoked maximal activation of polymodal nociceptors in cats, and the highest discomfort score in humans [30]. Accordingly, 3s pulses at a flow rate of 200 ml·min^{-1} were the parameters chosen to stimulate maximal reflex tearing with the i-Onion apparatus without causing discomfort/corneal injury. The effect of this protocol was first tested with the first iOnion™ prototype on young healthy students (Control group A). The remaining subjects (Control

Fig. 1. A. The i-Onion™ instrument. **B.** Application of the instrument to the orbital margins of a subject's right eye. In this example, the Schirmer test strip has been place to illustrate how tear flow is measured.

group B: see below) were selected from patients at the Hospital de la Marina Baixa in Villajoyosa (Spain). After explaining the goals of the study and obtaining a short clinical history, the patients received a written information package detailing the objectives of the study and the protocol to be followed, and an Informed Consent sheet to be signed in the case of acceptance. All the participants were unpaid and were free to withdraw from the study at any time. On the second visit, the patient's clinical history was completed and their tear meniscus height was measured in both eyes while comfortably seated at the OCT table [21]. Thereafter, the Schirmer test was performed, placing a strip wetted with 10 μL lidocaine behind the lower lid of one of the eyes (chosen arbitrarily, unstimulated control eye) for 3 min to measure basal tear volume (BTV). The i-Onion™ device was then placed on the bony orbital margin of the contralateral eye (stimulated, test eye); the patient was instructed to look at the stimulator's nozzle and for 3s a CO_2 puff was directed towards the center of the cornea. Immediately afterwards, a Schirmer test strip was introduced into the palpebral border of the stimulated eye, maintaining the eye closed for 3 min to measure the stimulus-evoked tear secretion volume (STV). Subsequently, the patient's were asked to respond to the McMonnies Questionnaire in Spanish [35]. Finally, a fluorescein strip was applied to both eyes to measure the TBUT and visible damage of the corneo-conjunctival surface was evaluated by slit lamp examination, quantified according to the Oxford Scale [36].

2.3. Experimental subjects

A total of 88 adult volunteers of both sexes participated in the study and these subjects were divided into two different groups.

2.3.1. Control group

Control group "A" - Students of both sexes from the Medical School at the University Miguel Hernandez (San Juan de Alicante, Spain), and between 20 and 30 years of age (n = 17), were initially recruited as volunteers to test the first i-Onion prototype in healthy subjects in order to define the optimal stimulation parameters (CO_2 concentration, gas flow rate, and distance between the nozzle tip and the corneal surface). Recruitment was based on the absence of ophthalmic pathologies or other medical conditions, a healthy eye surface confirmed by slit lamp examination and basal Schirmer test values ≥7 mm when assessed by a registered ophthalmologist. Control group "B" - An additional 29 healthy volunteers (20 female/9 male) aged from 25 to 61 years-old were recruited from among the personnel at the Hospital de la Marina Baixa after a general ophthalmic examination was performed. These individuals had no previous DED symptomatology, McMonnies questionnaire [35,37] values below 10 points, Schirmer test values ≥ 7 mm and/or TBUT ≥10s, and a score of "0" on the Oxford Scale of ocular surface staining [36].

2.3.2. DED group

This DED group was composed of asymptomatic DED subjects (n = 12, 8 females/4 males) aged from 46 to 75 years-old, identified from the Hospital personnel that when assessed with a standard routine ocular exploration for recruitment had a Schirmer test value < 7 mm and/or TBUT <10s. In addition, it included 30 SS DED patients (SS-DED, 27 female/3 male) aged from 34 to 67 years-old, previously diagnosed at the hospital's Rheumatology Service (based on the criteria of the American-European Consensus Group -AECG) [16] and with a mean disease evolution time of 12 (±8) years. According to the AECG criteria, these patients were classified as primary (n = 9) or secondary SS patients (n = 21: 10 with systemic lupus erythematosus; 9 with rheumatoid arthritis; and 2 with ankylosing spondylitis).

All the participants in the control and DED groups recruited at the hospital completed a McMonnies questionnaire in Spanish [35]. They were then assessed at the hospital eye clinic by a registered ophthalmologist (MM or JB) for visual acuity, eye fundus exploration, eye surface slit lamp examination, intraocular pressure (IOP) measurement, TBUT and our modified Schirmer test with limited anesthesia (see below) to detect potential incompatibilities prior to their final inclusion on the study. Subjects that wear contact lenses, those who had undergone prior eye surgery, individual's with any meibomian gland pathology and pregnant women were all excluded from the study. The treatment of patients with systemic diseases was recorded to evaluate any possible influence on DED signs.

This study was carried out in accordance with the guidelines of the Helsinki Declaration and it was approved by the Ethic Committees of the University Miguel Hernandez, the Clinical Trials Committee of the Hospital de la Marina Baixa and the Consejería de Sanidad y Consumo. The experiments were performed in a room maintained at a controlled temperature and humidity (23.1 ± 0.7 °C; 43.9 ± 7.3%). The demographic and ocular surface data of the subjects recruited at the hospital are summarized in Table 1.

2.4. Tear measurement

Tear volume was measured using Schirmer test strips (35 × 5 mm: Haag-Streit®, Entod Research Cell, London, UK). The 5 mm tab at the end of the strip was folded and 10 μL of 2% lidocaine was applied to the tab with a micropipette. The folded end was then gently introduced between the bulbar and the palpebral conjunctiva, near the external canthus, and the eye was then maintained closed for 3 min until the strip was removed. Due to the small volume of anesthetic it remained restricted to the tab, presumably limiting its anesthetic action to the bulbar and tarsal conjunctival surfaces in direct contact with the tab. The length of the strip wetted by the tears was measured in half millimeters and the tear volume collected was expressed as mm of wetted Schirmer strip.

The BTV was defined by the length of strip wetted over 3 min [38] when measured in one eye closed and at rest, and with no stimulation (as described above). The STV represented the length of the Schirmer strip wetted in the contralateral eye immediately after stimulation with i-Onion™, measured as indicated above. The Tear Reserve Volume (TRV, in mm) corresponds to the difference between STV and BTV and was calculated at the end of the stimulation protocol.

2.5. TBUT, corneal staining and tear meniscus height

A fluorescein strip (Haag-Streit®, Entod Research Cell, London, UK) was applied to the bulbar conjunctiva and a slit-lamp equipped with a blue cobalt filter was used to detect when the first dark spot appeared, registering the time lapsed. TBUT measurements longer than 7 s were

Table 1
Demographic and ocular data from healthy and DED subjects.

	Control group "B"	Asymptomatic DED	SS DED
Number of subjects	29	12	30
Gender (%; F/M)	69/31	67/33	90/10
Age (years)	47 ± 2	56 ± 2	51 ± 2
BTV (mm)	14.0 ± 1.2	7.1 ± 0.7*	6.6 ± 0.6*
STV CO_2 (mm)	18.3 ± 1.5	9.7 ± 1.1*	8.5 ± 0.6*
TBUT (s)	9.4 ± 0.3	6.4 ± 1.1	3.7 ± 0.5*
Corneal epitheliopathy	0	0.5 ± 0.2	1.8 ± 1.2*
Meniscus height (μm)	218.4 ± 13.2	174.5 ± 21.9	161.9 ± 14.5*
McMonnies score	3.6 ± 0.4	4.4 ± 0.6	13.3 ± 0.6*

Abbreviations: SS, Sjögren's syndrome; BTV, basal tearing volume; STV CO_2, CO_2 stimulated tearing volume; Corneal epitheliopathy, as scored according to the Oxford scale. The data are expressed as the mean ± sem: *p < 0.05, differences relative to the Control group B, One Way ANOVA with post hoc Holm-Sidak or Kruskal-Wallis with Dunn's method, as appropriate. Control Group A was not included because some ophthalmic explorations were not performed on these participants.

considered normal. Corneal and conjunctival epithelial damage were assessed on the Oxford scale (0–5) [36], also using a fluorescein strip and a slit-lamp with a blue cobalt filter. The lower tear meniscus height was measured in both the subject's eyes using optic coherence tomography (OCT 2000: Topcon, Tokyo, Japan), in the center of the palpebral border and at a working distance of 63.7 mm.

2.6. Data analysis

Pseudo-anonymized data were collected and processed for statistical analysis using SigmaPlot (v.11; Systat Software, San Jose, CA, USA), and they were expressed as the mean ± SEM. Differences in the clinical features were compared using parametric and non-parametric tests according to the normality of data. Details on the specific statistical tests applied are provided throughout the text, and in the figures and tables.

3. Results

3.1. Acidic stimulation of the corneal surface with CO_2 increases the secreted tear volume in healthy subjects

To exclude any possible differences in the responsiveness of the participants in the control subgroups A and B, the BTV and STV values of these two subgroups were compared. No differences were observed in the incidence of positive responses, in the mean BTV and STV, or in the magnitude of the tearing rise between these two subgroups (data not shown) and hence, the data obtained from them was pooled into a single Control group to define the characteristics of the stimulus-evoked tearing response in healthy subjects (see Fig. 2 for the BTVs and STVs from each member of the Control group). CO_2 stimulation increased tear flow ≥1 mm over the BTV in the contralateral eye of 36 of the 46 subjects included in this group (78.3%), although the mean tearing rates differed significantly both before and after stimulation (Table 2).

The individual BTVs and STVs of all control subjects (n = 46) were ordered sequentially according to their BTV (Fig. 3: control subgroup A, grey circles; control subgroup B, black circles), reflecting the high incidence of positive responses to the stimulus (red arrows: STV > BTV, n = 36), although in a few subjects the STVs were similar or less than the contralateral BTV (blue arrows: STV=BTV, n = 2; STV < BTV, n = 8). Notably, the absolute increase in tear secretion (STV-BTV) of the positive responders varied considerably among the healthy participants, ranging from +1 mm to the highest measurable peak of +13 mm (see Fig. 3). Hence, we also calculated the difference in the BTV and STV for each individual to obtain the TRV (graphically represented by the red arrows in Fig. 3). Null or negative responses to the stimulus were not observed in any control subject with a moderate pre-stimulus BTV

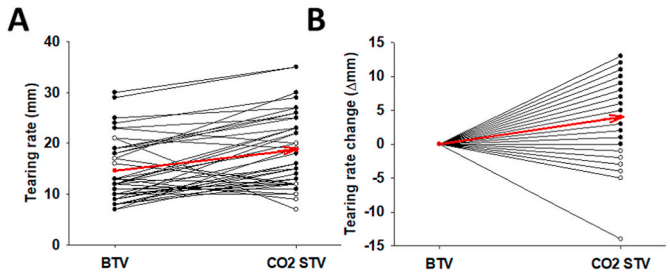

Fig. 2. Tear rate changes evoked by CO_2 stimulation in all control subjects (n = 46). Basal tear volume (BTV) and stimulated tear volume (CO_2 STV) of all the subjects is represented individually and connected by a line. The tear volume variations are presented as (**A**) absolute Schirmer test values in mm and (**B**), as the stimulus-evoked deviation of the stimulated tear volume from its basal value in mm. White circles correspond to subjects where a negative tearing response to stimulation was obtained. Red arrows show the average value of all the subjects and their ascending direction reflects the increase in tearing after stimulation.

Table 2
Basal tear volume (BTV), stimulated tear volume (STV) and tear reserve volume (TRV) of control subjects.

	ALL CONTROL SUBJECTS		
	Pooled	Moderate tearing	High tearing
BTV range	7–35 mm	7–10 mm	10.5–35 mm
BTV (mm)	14.6 ± 1.0	8.8 ± 0.3	17.7 ± 1.1
STV (mm)	19.0 ± 1.1**	15.2 ± 1.0**	21.1 ± 1.4†
TRV (mm)	4.4 ± 0.8	6.4 ± 1.0	3.4 ± 1.1
N	46	16	30
	POSITIVELY-RESPONDING (TRV > 1 mm) CONTROL SUBJECTS		
	Pooled	Moderate tearing	High tearing
BTV range	7–35 mm	7–10 mm	10.5–35 mm
BTV (mm)	14.2 ± 1.1	8.7 ± 0.3	18.1 ± 1.4
STV (mm)	20.8 ± 1.2**	15.5 ± 1.0**	24.6 ± 1.4**
TRV (mm)	6.6 ± 0.5	6.9 ± 1.0	6.5 ± 0.7
N	36	15	21
%	78.3	93.8	70

In the table the data from the 46 control group subjects are shown in the "Pooled" column and then classified according to their basal tear volume: "Moderate tearing" - BTV values ≤ 10 mm (n = 16); or "High tearing" - BTV >10 mm (n = 30). In addition, the mean data of the control subjects that responded positively to CO_2 stimulation (TRV >1 mm; 36 out of 46) are shown in the lower part of the table. Note that almost all the control subjects with moderate basal tearing (93.8%, 15 out of 16) responded positively to stimulation. The data are expressed as the mean ± sem of the differences between the BTV and STV in each group: **p < 0.001, †p < 0.005, paired *t*-test or Wilcoxon Signed Rank test. BTV, Basal tear volume; STV, stimulated tear volume; and TRV, tear reserve volume in all control subjects (Control groups A + B).

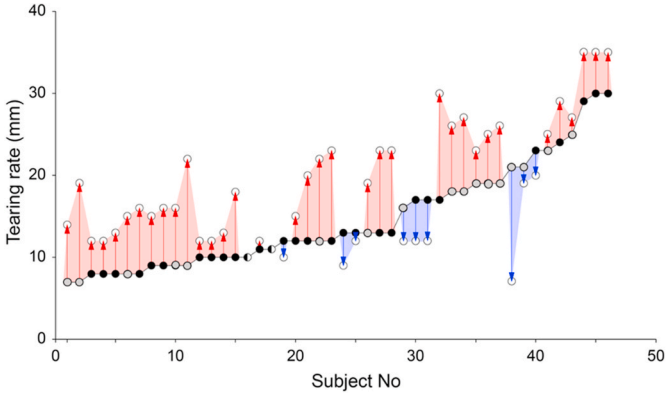

Fig. 3. The effect of CO_2 stimulation with the i-Onion™ on the tear rate of control subjects. Individual BTV (Control group A, grey circles; Control group B, black circles) and STV values (white circles) from each subject are connected by an arrow and ordered sequentially by their BTV magnitude. To emphasize the higher incidence of negative TRVs among subjects with higher BTVs, positive and negative values of the TRV are shaded in red and blue, respectively.

(between 7 and 10 mm), whereas null or negative tearing volumes evoked by CO_2 were more frequent, and with larger negative values among subjects showing higher pre-stimulus BTVs (Fig. 3, blue arrows). These observations were consistent with the mean tear secretion parameters in the control subjects, either pooled or selecting only the values from those subjects with a positive STV response (Table 2). The participants were also segregated accordingly to their initial BTV, moderate (≤10 mm) or high (>10 mm). Practically all the subjects with a BTV ≤10 mm responded positively to stimulation, while the tear rate in 30% of those exhibiting BTV >10 mm did not increase further with CO_2 application (Table 2). Note, that the mean absolute TRV was very similar in both these groups, indicating that a fraction of the individuals with a high basal tear flow still maintained a remarkable capacity to respond to CO_2 stimulation with an increase in their tear volume.

Indeed, the mean STVs of the subjects with a high BTV were significantly larger than those that had a moderate BTV.

The STV values of all the control subjects were grouped according to their BTV (Fig. 4) and all but one (with a TRV = 0) of the 16 subjects with BTV ≤10 mm responded positively to CO_2 stimulation (Table 2 and Fig. 4, pink box). The subjects with higher BTV values (BTV >10 mm, n = 30) could be segregated into a subgroup that were unable to further increase their tearing rate upon stimulus or that even showed a lower tearing than the BTV (n = 9: Fig. 4, blue box), and another subgroup of subjects that maintained the capacity to increase tearing under stimulation (n = 21: Fig. 4, red box).

Together, these results reflected the large individual differences in total tear secretion capacity among the healthy subjects in the Control group. Moreover, some participants with a modest BTV appear to already be close to their maximal tearing volume given their limited capacity to surpass the BTV when exposed to reflex stimulation. No significant correlation was found between age and the BTV (p = 0.742) or STV (p = 0.33, Pearson correlation) when explored in the control group B of healthy subjects, whose ages ranged from 25 to 61 years. Likewise, no significant differences in BTV and STV values were found between male (p = 0.537) and female subjects (p = 0.341, Mann-Whitney test). However, it noteworthy that participants below 30 years of age represented only 31.2% of those with a BTV <10 mm, whereas a larger proportion of subjects (58.3%) with a BTV ≥18 mm that still responded positively to stimulation were below 30 years of age.

3.2. Asymptomatic DED subjects have lower BTVs and STVs

During the routine screening of the hospital personnel recruited for the control group, we detected some individuals with a BTV <7 mm and/or a TBUT <10 s, and also with a low tear meniscus height and a mild degree of corneo-conjunctival epitheliopathy (i.e.: clinically suggestive of moderate DED but with no disease symptoms according to the McMonnies questionnaire - Table 1). We assessed these patients as a separate cohort of asymptomatic DED patients and studied their response to CO_2 stimulation.

The individual tear volumes of the 12 asymptomatic DED subjects were recorded before and after CO_2 stimulation (Fig. 5A and B), and the curve of their individual responses to CO_2 was established based on their BTV to compare their behavior with that of the healthy control subjects (see Figs. 5C and 3). The number of asymptomatic DED subjects with a negative or null response to CO_2 stimulation was almost twice that of the

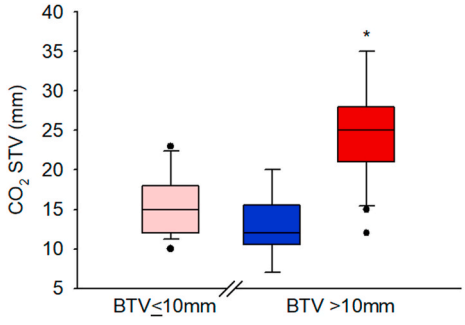

Fig. 4. Box plot showing the differences in CO_2-stimulated tear values (STVs) within the population of control, healthy subjects. Healthy subjects with a basal tear volume (BTV) ≤10 mm maintain a positive reflex response to maximal ocular surface stimulation (pink box). Healthy subjects with BTVs >10 mm segregate in two distinct groups, those in which stimulation does not augment or that decreases tearing (blue box), and those in which tearing augments after stimulation (red box). Although the data is represented as box plots (Q1-Q3 interquartile range and the median), they are normally distributed. STVs of subjects with BTV >10 mm and that retain a large tearing reserve (red box) differed significantly from the other groups: *p < 0.05, One Way ANOVA, Holm-Sidak method.

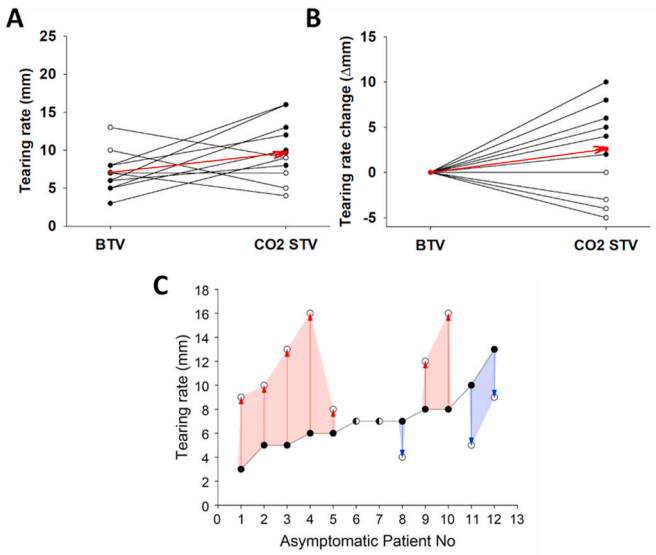

Fig. 5. Effects of CO_2 stimulation with the i-Onion™ on the tear rate of asymptomatic DED subjects. **A,B.** The change in tear rate evoked by CO_2 stimulation expressed as absolute values (**A**) and as the relative change from the basal values (**B**): white circles, asymptomatic DED subjects with a negative tearing response to stimulation; red arrows, average value of all the subjects. Although small, the ascending direction of these arrows reflects the average increase in tearing after stimulation. **C.** Individual BTVs (black circles) and STVs (white circles) of each subject are connected by an arrow, and ordered sequentially according to their BTV magnitude. Positive and negative TRVs are shaded in red and blue, respectively.

controls, apparent even in subjects with low BTV values. More than half of the asymptomatic DED subjects had a STV of 10 mm and only two surpassed a STV of 16 mm. BTV values > 7 mm were detected in almost half of the asymptomatic individuals but this did not predict their incapacity to increase secretion under CO_2 stimulation. In fact, the mean TRV of the positive asymptomatic responders was 5.9 ± 0.7 mm (n = 7), only slightly lower than the equivalent TRV of healthy control subjects (compare Figs. 3 and 5). There was no correlation between BTV and STV values in subclinical DED subjects (p = 0.385, Pearson correlation coefficient = −0.276), while a significant negative correlation was found between their BTV and TRV values (p = 0.011, Pearson correlation coefficient = 0.7). No significant differences in the BTV (p = 0.668) and STV (p = 0.979, *t*-test) were found between male and female asymptomatic DED subjects. Similarly, the BTV (p = 0.917) and STV (p = 0.689, Pearson correlation) did not appear to be associated with the age of these patients.

3.3. BTV and STV values were markedly reduced in SS-DED patients

The group of SS-DED patients was composed of 30 patients derived to the Ophthalmology department by the Rheumatology Service and diagnosed with primary (n = 9) or secondary SS (n = 21: Table 1). As for the control and asymptomatic DED subjects, the individual responses to CO_2 of all the members of the SS-DED subjects were recorded (Fig. 6A and B) and organized according to their BTV (Fig. 6C). For BTV values up to 7 mm, the majority of SS-DED patients (17/21) exhibited positive but modest STVs in response to CO_2 stimulation, as reflected in a mean TRV of 3.1 ± 0.6 mm (n = 17). Among those with a BTV >7 mm (n = 9) only three had STV values 1–3 mm higher following CO_2 stimulation, while no positive STV responses were observed in the remaining 6 patients of which, one recorded a negative response (Fig. 6). As expected, the mean TRV of all SS-DED patients was generally very low (1.9 ± 0.5 mm, n = 30), suggesting that in about 50% of these patients tear secretion under basal conditions was already at or close to the maximal

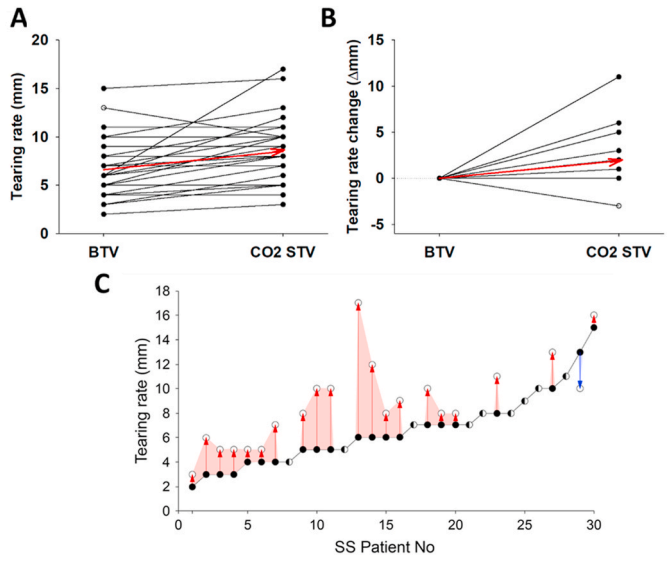

Fig. 6. Effects of CO_2 stimulation with i-Onion™ on the tear rate of SS-DED patients. **A, B.** Change in the tear rate in SS-DED patients evoked by CO_2 stimulation, expressed as the absolute (**A**) and relative values (**B**). The white circle represents the only SS-DED patient with a negative tearing response to stimulation, and the red arrows are the average values of all the patients. The slightly ascending direction of the arrows reflects the small increase in tearing evoked by stimulation. **C.** Individual BTVs (black circles) and STVs (white circles) from each SS-DED patient are connected by an arrow and ordered sequentially according to the BTV magnitude. Positive and negative TRV values are shaded in red and blue, respectively.

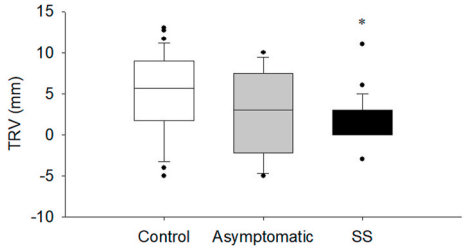

Fig. 7. Box plots showing the tear reserve volume (TRV) of control, healthy subjects (Control, n = 46), asymptomatic DED subjects (Asymptomatic, n = 12) and SS-DED patients (SS, n = 30). The data represents the Q1-Q3 interquartile range and the median: *p < 0.05 relative to the Controls, Kruskal-Wallis test with a post hoc Dunn's method.

secretion capacity. There was a strong correlation (Pearson correlation coefficient = 0.675, p < 0.001) between the BTV and STV in SS-DED patients, although no significant correlation was found between the BTV and TRV (Pearson correlation coefficient = −0.317, p = 0.087). As there were only three men in this group it was not possibly to study the differences between male and female patients but as in the other experimental groups, there was no correlation of BTV or STV with age (see Fig. 7).

3.4. TRV values reflect the functional capacity of lacrimal glands

The mean values of the TRV obtained were evaluated in healthy and asymptomatic DED subjects, and in SS-DED patients, confirming that SS-DED patients have a significantly lower capacity to increase their reflex tear flow under maximal sensory stimulation. However, the differences in the mean TRV between asymptomatic DED subjects and the healthy controls was not significant.

4. Discussion

We report here an increase in the volume of tear secretion provoked by CO_2 application with iOnion™, a new ophthalmic instrument that produces controlled stimulation of sensory nerve terminals at the corneal surface, resulting in a strong reflex activation of aqueous secretion by the tear glands. Comparing the results obtained with this procedure in healthy subjects and in subjects suspected to have or already diagnosed with DED, indicates that the elevation in tear flow volume above the basal levels evoked by CO_2 stimulation was small or absent in SS patients that display well-defined ocular signs and symptoms of DED. A sluggish response was also observed in patients with only mild signs of DED, such as abnormal tear film stability and volume, and discrete corneal epithelium damage, yet in the absence of subjective symptoms of DED. Thus, stimulation of acute and reproducible reflex tear gland secretion using i-Onion™ appears to be a promising and simple procedure to obtain reliable information about the functional status of a patient's tear glands, helping to predict the onset and evolution of DED.

The Schirmer test is currently the conventional, first-line method used in routine clinical exploration to assess the functional status of the lacrimal gland. The practical limitations of this test have been discussed extensively and they are related to the uncertainty of the BTV measurements [23]. Eye surface anesthesia has been estimated to reduce the mean test values and tear meniscus height in normal eyes by 40% [27]. This effect has been attributed to the blockade of the ongoing activity of cold thermoreceptors [24], and to a suppression of the irritant afferent sensory input evoked by contact of the filter paper strip with the richly innervated mucosa of bulbar and tarsal conjunctiva [22,25,27]. In the experiments performed, this uncontrolled stimulation was limited by wetting the tab of the Schirmer strip touching the conjunctiva with a local anesthetic solution when measuring the tear volumes in both the unstimulated and stimulated eye of each patient. On average, the BTV of control healthy subjects did not differ from those obtained previously in healthy subjects using the conventional Schirmer test with anesthesia. Hence, wetting the Schirmer strip with anesthesia where it contacts the conjunctiva reduces irritation and allows reasonable steady-state BTVs to be obtained, without compromising the reflex tearing excitation evoked by CO_2 stimulation of corneal sensory terminals.

Measuring the secretory capacity of the lacrimal glands in patients with eye surface disorders provides important information to assess the severity of glandular damage in these ocular pathologies where tear dynamics are compromised, of which SS is a prominent example [22,39,40]. Different stimulation procedures have been tested to induce a near-maximal reflex secretion by the tear glands, including mechanical stimulation of the nose mucosa with a cotton-tip applicator [6,22,41], or exposure of the eye surface to chemical irritants like citric acid [42] or onion lachrymatory factor [43]. A common limitation of these methods is that they cause significant irritation and discomfort to the patient, and the intensity and duration of the stimulus applied is not controlled. Moreover, it is not known whether the neural pathways recruited by the stimulation of non-ocular trigeminal territories fully overlap with those specifically involved in the continuous adjustment of the tearing rate to maintain wetness and to prevent damage of the eye surface [7,8,44,45].

Application of a CO_2 gas jet to the cornea with i-Onion™ evokes an immediate, transient activation of the corneo-conjunctival nerve endings that innervate the exposed eye surface. These terminals correspond to the population of nociceptive ocular TG neurons directly involved in the detection of stimuli threatening the integrity of the eye surface. CO_2 dissolves rapidly in the aqueous tear film layer, combining with water molecules to produce carbonic acid. This compound dissociates immediately, releasing protons and lowering the tear film pH [30]. A reduction in the corneal pH by applying acidic solutions or CO_2 represents an effective stimulus for polymodal nociceptor sensory endings of the cornea in experimental animals [30,34]. Moreover, applying different CO_2 gas concentrations to the surface of cat and human eyes

demonstrated that gas jets at a concentration above 70%, and at a flow rate over 150 ml·min^{-1} cause vigorous polymodal nociceptor corneal nerve fiber discharges in anesthetized cats [34], and a distinct, stinging unpleasant sensation accompanied by augmented tearing in humans [5,34,46–48]. In addition, high CO_2 flow rates also recruit a fraction of corneal mechano-nociceptor nerve fibers, possibly due to the pressure exerted by the CO_2 gas jet on the cornea, as well as low-threshold corneal cold thermoreceptor fibers that are activated by the evaporative and direct cooling effects of the CO_2 gas flow [30,34].

Polymodal nociceptors represent around 70% of the total sensory innervation to the cornea [7] and they appear to be the main source of peripheral nerve impulses encoding ocular surface pain [7,8,34,47,48], as well as the dominant afferent branch of reflex irritative tearing responses [2]. The high-flow CO_2 jet produced by i-Onion™ is directed towards the center of the cornea located 5 mm away, although it diffuses along its trajectory, adopting the form of an inverted cone the base of which hits the cornea and surrounding conjunctiva richly innervated by nociceptor terminals. The largescale recruitment of the different populations of corneal sensory nerve fibers evoked by the gas stream is apparently sufficient to activate the reflex secretory capacity of the lacrimal glands to near-maximal levels in humans. Direct supramaximal electrical stimulation of parasympathetic nerve fibers innervating the tear glands or indirect electrical excitation of corneal surface sensory nerve terminals, provoke rises in basal tear flow to around 100% in rabbits and rats [49,50], an increase roughly equivalent to that obtained here in healthy humans. Thus, the i-Onion™ instrument would seem to selectively recruit most ocular surface sensory nerve fibers in charge of the physiological regulation of tear flow. This recruitment leads to reflex activation of the efferent parasympathetic neurons that innervate the lacrimal glands, possibly mimicking their maximal reflex excitation under physiological conditions [12].

The BTV varied widely in the control group of healthy subjects, and this value was not significantly correlated with age or gender, as seen previously [51]. This is somewhat surprising considering the well-documented and gradual appearance of histopathological degenerative abnormalities, and functional decline, of the tear gland with age [52,53]. Nonetheless, such aging related events commence relatively late and are slow, resulting in an estimated mean decrease in tear meniscus volume of 1.0% per year [54]. Hence, it is conceivable that the reduction in tearing capacity associated with age or gender is too small to be detected in our relatively limited control group (of both sexes and between 25 and 61 years of age). Nonetheless, the proportion of control subjects under 30 years of age that exhibited a high BTV and strong responses to CO_2 stimulation was higher than among the older subjects. Hence, it might be speculated that this difference is an early sign of the onset of lacrimal gland degeneration at ages above 30 years-old.

A number of control subjects with BTV values over 15 mm are unable to secrete more tears under stimulation, although around 25% of the control subjects with a high basal tear rate still further increase their tear secretion in response to CO_2. Notably, the mean tear volume in CO_2-responders adds close to 6–7 mm to their BTV, as reflected by the TRV value. The differences in basal secretory capacity among healthy individuals may depend on the volume of the working secretory tissue. Acinar and ductal epithelium cells are responsible for electrolyte and water secretion associated with basal tearing [40], although it is possible that basal and reflex tear gland secretion do not depend on the same cellular mechanisms.

The identification of a group of asymptomatic DED patients using i-Onion™ stimulation is evidence of the potential diagnostic interest of measuring STV values. This value may serve to predict and follow the evolution towards ADDE in subjects with a borderline BTV and eventually, other signs of reduced eye surface wetness in the absence of clear symptoms of the disease. Almost all asymptomatic DED subjects analyzed in the present study were unable to increase their basal tearing rate under CO_2 stimulation, and the STV of those responding positively never surpassed the mean BTV exhibited by healthy subjects. The general response curve of the asymptomatic DED patients closely resembles that of the SS-DED patients, reinforcing the hypothesis that they were probably borderline cases of DED at an early stage of lacrimal gland insufficiency.

Lacrimal gland inflammation is the most relevant pathogenic mechanism of reduced tearing in SS patients [55]. The disease produces periductal and focal inflammatory aggregates, atrophy of acini with fibrosis and a disorganization of the lacrimal ducts [40]. Atrophy compromises function and in combination with periductal fibrosis, it interferes with normal tear secretion. The degree of lacrimal gland tissue damage in SS patients is variable and difficult to evaluate directly, despite the efforts to identify cellular and molecular markers, or the use of lacrimal gland imaging *in vivo* [56,57]. Accordingly, low BTV values are commonly used in the clinic as a functional sign of SS, reflecting the magnitude of lacrimal gland damage produced by the autoimmune disorder [3,20,57]. Our data indicate that in addition to low BTVs and in contrast to healthy subjects, most SS-DED patients have a maximal secretory capacity that rarely surpasses a STV of 12 mm, with TRVs significantly lower than those of healthy subjects. The impaired secretion in SS patients, and of the drainage of water and electrolytes through the tear fluid, has been associated with the integrity of acini and ducts. Measuring the i-Onion stimulus-evoked maximal tear secretion may serve as an indirect, quantitative index of the functional status of the tear gland. The TRV probably reflects the remaining tearing capacity of injured gland tissues in very demanding conditions and this value may therefore help to realistically define the therapeutic alternatives that can be employed in these patients to restore their eye surface wetness.

The present study has some limitations that should be considered when comparing the results with those reported elsewhere. The time lapse to measure tear flow with the Schirmer test was shortened to 3 min rather than the conventional 5 min used generally in the clinic and in many publications. Also, the study's sample size was relatively small, particularly in the case of the asymptomatic patients, reducing the statistical power of the comparisons. Wetting of the Schirmer strip tab with a small volume of anesthesia to avoid direct irritation of the tarsal conjunctiva by the strip assumes but does not ensure that all other sensory nerve fibers contribute to the basal tear flow under resting conditions. Finally, the BTV was obtained from one eye in each subject and the STV was assessed immediately afterwards in the contralateral eye. However, the degree of correspondence between BTV values in both eyes was not established and the possibility of a consensual influence from the manipulation of one eye to the contralateral one cannot be excluded. A contralateral effect might explain why the STV value obtained was lower on occasions than the control BVT. Hence, the data obtained here needs to be extended to a larger number of patients to confirm some of the observations. However, it is worth noting that the primary goal of this study was to assess the efficacy of i-Onion as a simple and easy-to-use instrument to reliably evoke maximal reflex tear secretion without producing discomfort and/or eye surface irritation. In addition, the study aimed to confirm that reduced maximal tearing can be observed in disorders affecting tear gland function. Together, the data obtained supports the possibility that STV and TRV values might serve as clinical markers of the tear secretion capacity in humans. These parameters, obtained with the i-Onion™, can be particularly useful to identify the asymptomatic tear-deficient dry eye patients. In addition, this device may also be used in animals to study and diagnose DED and to potentially evaluate, in animal models, the benefits of novel therapies. To achieve these goals, more studies using the i-Onion™ in larger populations of patients will be required to define the potential heterogeneity generated by the stimulus, as well as any characteristic age, male-female or racial differences in healthy subjects, and in patients with suspected tear secretion disorders of different etiology.

5. Conclusions

The maximal tearing capacity of an individual can be assessed

through controlled and reproducible stimulation of ocular surface innervation with a CO_2 puff delivered with a new instrument named i-Onion™. This procedure may be useful for the diagnosis and follow-up of DED patients. Moreover, it may help evaluate and prevent the appearance of eye dryness in patients with a critically low TRV before they are exposed to therapeutic procedures that may potentially reduce the corneal sensory nerve input that maintains reflex tear secretion.

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Disclosure/conflicts of interest

CB, JG, MM and MCA are co-inventors in the patent of i-Onion™ held by the Universidad Miguel Hernandez (San Juan de Alicante, Spain); CB and JG own stock of Tearful SL (Alicante, Spain). CB receives consulting income from TwentyTwenty (San Francisco, USA).

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