The influence of rigid gas permeable lens wear on the concentrations of dinucleotides in tears and the effect on dry eye signs and symptoms in keratoconus

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ABSTRACT

Purpose
To evaluate the signs and symptoms of dry eye and dinucleotide secretion in tears of keratoconus patients (KC) and the potential effect of rigid gas permeable (RGP) contact lens wear.
Methods
Twenty-three KC patients and forty control subjects were enrolled in this study. Signs of dry eye including tear volume, tear stability and corneal staining along with symptoms were assessed using the McMonnies questionnaire. Tears were collected using Schirmer strips, and dinucleotide concentrations in collected tears measured using high pressure liquid chromatography. Values obtained in KC and controls were compared. The effect of contact lens wear in KC was also assessed.
Results
KC eyes showed a significantly lower tear volume compared to controls, shorter tear break up time (TBUT), higher corneal staining and higher McMonnies dry eye questionnaire scores ($p < 0.05$). When compared with non-wearers, KC contact lens wearers showed significantly higher symptoms, lower Schirmer and TBUT values ($p < 0.05$). Concentration of Ap_4A (0.695 ± 0.304 µM vs. 0.185 ± 0.178 µM) and Ap_5A (0.132 ± 0.128 µM vs. 0.045 ± 0.036 µM) were higher in KC compared to controls ($p < 0.001$) and only Ap_4A was statistically higher in RGP wearers compared to non-wearers (0.794 ± 0.478 µM vs. 0.417 ± 0.313 µM) ($p < 0.05$).
Conclusion
Signs and symptoms of dry eye as well as concentrations of Ap_4A and Ap_5A were markedly increased in KC patients compared to controls. Moreover, Ap_4A and symptoms of dry eye were statistically higher in RGP wearers compared to non-wearers. This seems to indicate that factors such as RGP contact lens wear might exacerbate the clinical condition of dry eye.

1. Introduction

Keratoconus (KC) is an ocular pathology causing a protrusion of the cornea. This pathology is bilateral, asymmetric and progressive [1,2] and affects about 2% of the population worldwide [2]. The cause of KC is not well understood, but it seems to be multifactorial. Factors such as an exposure to ultraviolet light, allergy and genetic mechanisms are the predominant reasons triggering the disease [3–5]. Other factors described in the literature as risk factors for the progression of KC include eye rubbing, with half of the patients reporting intense eye rubbing [6]. The need these patients feel to rub their eyes is due to ocular discomfort symptoms including itching, which might be closely related to eye dryness [7].

Only a very small number of studies have investigated the link between KC and dry eye [8–10]. In these studies, the results show a lower tear break time (BUT) in KC patients and a marked presence of corneal staining. In addition, patients with KC have a higher concen-tration of pro-inflammatory molecules such as interleukins and metalloproteinases compared to healthy subjects, this being even more elevated in patients using RGP contact lenses [11–13]. Inflammation is one of the most important signs described in the definition of dry eye given by the DEWS 2007 [14]. All these findings suggest a possible interaction between KC and dry eye.

In recent years, the presence of diadenosine polyphosphates in human tears and in aqueous humor has been shown [15,16]. These molecules can increase tear secretion when instilled topically [17]. Moreover, these substances can increase the production of lysozyme in tears, contributing to ocular surface defense [18]. Additionally, some of these molecules accelerate the rate of corneal wound healing (diadenosine tetraphosphate, Ap_4A) while others inhibit this process (diadenosine pentaphosphate, Ap_5A) [19]. These molecules have been proposed as objective biomarkers of dry eye, since their presence is significantly increased in patients with this disease [20,21]. In a recent study, it has been found that Ap_4A tear concentrations were higher in KC patients than healthy subjects. All patients with KC were RGP wearers [10]. A higher Ap_4A concentration in healthy RGP wearers has also been shown to be related [22].

The release of diadenosine polyphosphates to the tears is caused by shear stress. Corneal epithelial cells release these molecules as a

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consequence of the lid contact during the blinking process [23]. The CLEK study found an increase of about 25% of corneal scarring in KC patients wearing RGP contact lenses compared to non-users, possibly partly due to the abrasion caused by the contact lens to the corneal surface [24]. We hypothesized that patients with KC who wear RGP contact lenses have higher tear concentrations of diadenosine polyphosphates.

To the best of our knowledge, there is currently no study that evaluates the influence of RGP lenses on tears and ocular surface physiology in patients suffering from KC. Therefore, the aim of this study is to examine the signs, symptoms and adenosine polyphosphate tear concentrations in patients with and without KC as well as the influence of RGP contact lens wear on these parameters in KC.

2. Methods

2.1. Patients

Twenty-three patients (13 males and 10 females) with ages ranging from 20 to 59 (average 34.75 ± 6.12 years) with KC and forty healthy subjects (24 males and 16 females) with ages ranging from 20 to 61 (29.02 ± 6.92 years) were recruited at the Clinical & Experimental Optometry Research Lab (University of Minho, Braga, Portugal) and at the Optometric Clinic of the Faculty of Optic and Optometry (University of Complutense, Madrid, Spain). All patients with KC were diagnosed by an external ophthalmologist and recommended to the research. The study was conducted in compliance with good clinical practice guidelines, institutional review board regulations, informed consent regulations, and the tenets of the Declaration of Helsinki (WMA, 2008) [25]. The protocol of the study was approved by the institutional review board of the University Complutense of Madrid. All the subjects enrolled in the study were adults over 18 years of age, who were able to give informed consent. After the purpose of the study was explained and all doubts clarified to the participants, a consent form was presented and signed by both the patient and the researcher.

KC patients should have been wearing spectacles or aspheric RGP corneal design at least during the last year to be part of the study. Patients with soft contact lenses, hybrid or scleral or previous surgery were excluded. In order to study the influence of RGP lens wearing on the ocular surface integrity, the KC group was divided into users and non-users of RGP lenses. Control group subjects had all worn soft contact lenses on a monthly wear daily basis during the previous 2 years, except eight subjects who had never worn contact lenses at all. Additional demographic characteristics of the population are shown in Table 1.

Table 1
Demographic characteristics of participants in the study.

Parameter	Control	Keratoconus		
		Total	No RGP wear	RGP wear
Patients	40	23	11	12
Mean age (years) ± SD	29.02 ± 6.92	34.75 ± 6.12	35.96 ± 4.82	33.11 ± 3.16
Age range (years)	[20,61]	[18,59]	[23,59]	[18,44]
Gender (male/female)	[24,16]	[13,10]	[7,4]	[6,6]
Mean keratometry (D)				
Flat	43.87 ± 2.53	49.46 ± 6.32	46.97 ± 3.35	51.97 ± 5.62
Steep	45.31 ± 1.39	56.35 ± 8.76	53.25 ± 6.53	60.81 ± 6.79

2.2. Trials

Tests of tear volume, tear stability, corneal staining and recording of subjective symptoms including dryness and discomfort using the McMonnies questionnaire were carried out at the beginning of the day before contact lens wear [26,27].

Tear secretion was measured using the Schirmer I test (without anaesthesia). The tear collection was always performed following the Van Bijsterveld criteria [28]. The Schirmer strip (Tear Flo, HUB pharmaceuticals, USA) was placed on the temporal tarsal conjunctiva of the lower lid for 5 min with the eyes closed. The volume of tears, as millimetres of moistened strip, were recorded and the Schirmer strips were placed in Eppendorf tubes containing 500 µL of Ultrapure water, then the samples were frozen at $-80\,^{\circ}$C until the high pressure liquid chromatography (HPLC) analysis was performed [20].

To avoid contaminating the strip with fluorescein, fluorescein was applied five minutes after the Schirmer I test to evaluate tear break up time (TBUT) and corneal staining. In order to warrant repeatability of the staining procedure, a solution was prepared using a 10% concentration of sodium fluorescein diluted in saline (NaCl 0.9%). For each application, a micropipette with 5 µl of diluted fluorescein solution was applied in the inferior conjunctival sac and 20 s later TBUT was analyzed through a slit lamp at 16x, using a chronograph to record the time to break after the patient was asked to blink twice and then keep their eyes open. The cornea was divided into five areas to record the grade of staining and, as proposed by the Report of the National Eye Institute and Industry-Sponsored Dry Eye Workshop [29], Cornea and Contact Lens Research Unit (CCLRU) grading scales were used [30].

2.3. Tear preparation and HPLC analysis

After thawing, the samples were strongly vortexed for 5 min. The strips were carefully rinsed and the liquid in the Eppendorf tube was heated in a 100 °C bath for 20 min to precipitate proteins. In order to pellet the proteins, the tubes were centrifuged at 4000 rpm for 30 min. Diadenosine polyphosphates are not degraded by this treatment as previously demonstrated [31]. Supernatants were chromatographed through SEP-PAK Accell QMA cartridges [32]. Briefly, 250 µL of the supernatant was passed through the cartridges that were previously mixed with 3 mL of ultrapure water. The elution of the nucleotides and dinucleotides was performed by applying 1 mL of a solution containing 0.2 M KCl, 0.1 M HCl. Prior to injection into the HPLC, samples were neutralised with KOH. These eluents were injected at a volume of 10–100 µL.

Determination and quantification of diadenosine polyphosphates were performed by HPLC. The chromatographic system consisted of a Waters 1515 isocratic HPLC pump, a 2487 dual absorbance detector and a Reodyne injector, all managed by the Breeze software from Waters. The column was a Novapack C18 (15 cm length, 0.4 cm diameter) from Waters. The system was equilibrated overnight with the following mobile phase: 0.1 M KH_2PO_4, 2 mM tetrabutyl ammonium, 17% acetonitrile, pH 7.5 [19]. Detection was monitored at 260 nm wavelength. All the peaks identified as putative dinucleotides were taken for phosphodiesterase treatment. Phosphodiesterase from *Crotalus adamanteus*, from Sigma (St. Louis, Mo., USA; EC 3.1.4.1.) at a concentration of 1.0 U/mL was incubated at room temperature for 30 min with the corresponding putative dinucleotide. The digested products were analysed by HPLC. Peaks were transformed into concentrations by means of external standards of known concentrations of diadenosine polyphosphates.

2.4. Statistical analysis

Data were analysed by statistical package SPSS version 15.0 for Windows (SPSS, Inc., Chicago, IL). The values analyzed are the means ± SD of the experiments performed. For statistical analysis, one eye per subject was selected randomly. Normality of samples was analyzed using the Shapiro-Wilk test. Sample size calculations were performed with statistical software (Granmo 6.0; Institut Municipal d'Investigación Medica, Barcelona, Spain). With an accepted two-sided statistical significance threshold of 0.05 and a risk of 0.20, for a standard deviation of 0.16 units for the diadenosine tetraphosphate concentration and in order to detect a difference of 0.2 units or more, 13 subjects were needed to find statistically significant differences. Parametric tests were used to compare the studied groups. Differences between the control group and the KC group were estimated by the Student-t test for independent samples. Due to small sample sizes of KC subgroups, the Mann-Whitney U test was used to compare values between them. $p < 0.05$ was considered statistically significant.

3. Results

Of all the KC subjects enrolled in the study, 16 patients were of grade III, 5 patients of grade IV and 2 patients of grade V, according to the KC severity score grading scale (9 grade III, 1 grade IV and 1 grade V were included in non RGP wearers and 7 grade III, 4 grade IV and 1 grade V were included in RGP wearers) [33]. However, no data were recorded about when the patients were diagnosed of KC. Considering that no correlation between grades and any test performed was found, all the eyes were pooled together for further analysis. Moreover, no differences were found between soft contact lens wearers and non-lens wearers in the control group and therefore the control groups were not divided for additional analysis.

Results of the Schirmer test, TBUT, corneal staining, McMonnies score and concentrations of Ap4A and Ap5A are summarized in Table 2. When comparing the control group and the KC group, the Schirmer test indicated that the control group showed higher tear volume than the KC group ($p = 0.03$). TBUT was statistically shorter in the KC group than the control group ($p < 0.001$). Corneal staining scores were 3 fold higher in the KC patients than in healthy subjects ($p < 0.001$). Also, the McMonnies questionnaire showed that the KC subjects had statistically significant higher symptoms scores than the control group ($p < 0.001$). Among the symptoms more frequently reported by KC patients were: dryness (59%) and scratchiness (36%). Also, they more frequently reported to have uncomfortable eyes in smoke and/or air-conditioning conditions. Regarding Ap4A and Ap5A concentration, KC patients showed higher concentration than control group, this being statistically significant in both dinucleotides ($p < 0.001$).

When the KC group was divided into RGP wearers and non-RGP wearers, statistical differences were found in the Schirmer test, TBUT, the McMonnies test and Ap4A concentration ($p < 0.05$), but not in corneal staining scores and Ap5A concentration ($p > 0.05$, Table 3).

RGP wearers showed less tear volume than the KC non-RGP wearers. Moreover, the tear stability was shorter and symptoms were higher in RGP wearers than spectacle wearers. Ap4A concentration was higher in RGP wearers compared with non-wearers. Fig. 1 summarizes the main outcomes of this study.

Table 3
Comparison between wearers and non-wearers of GP lenses in keratoconus group. *p value < 0.05. Mann-Whitney U test. For details see Section 2.

Test	No RGP	RGP wearers	P value
Ap4A (μM) mean (SD)	0.417 ± 0.313	0.794 ± 0.478	0.041*
Ap5A (μM) mean (SD)	0.093 ± 0.086	0.217 ± 0.176	0.064
Schirmer (mm) mean (SD)	20.05 ± 7.45	13.45 ± 5.89	0.044*
TBUT (s.) mean (SD)	8.11 ± 1.85	5.83 ± 1.23	0.002*
Corneal staining mean (SD)	2.42 ± 0.46	2.59 ± 0.29	0.287
McMonnies score mean(SD)	7.93 ± 3.56	12.45 ± 3.32	0.005*

Table 2
Comparison between control group and keratoconus group. *p value < 0.05. Student-t test for independent samples. For details see Section 2.

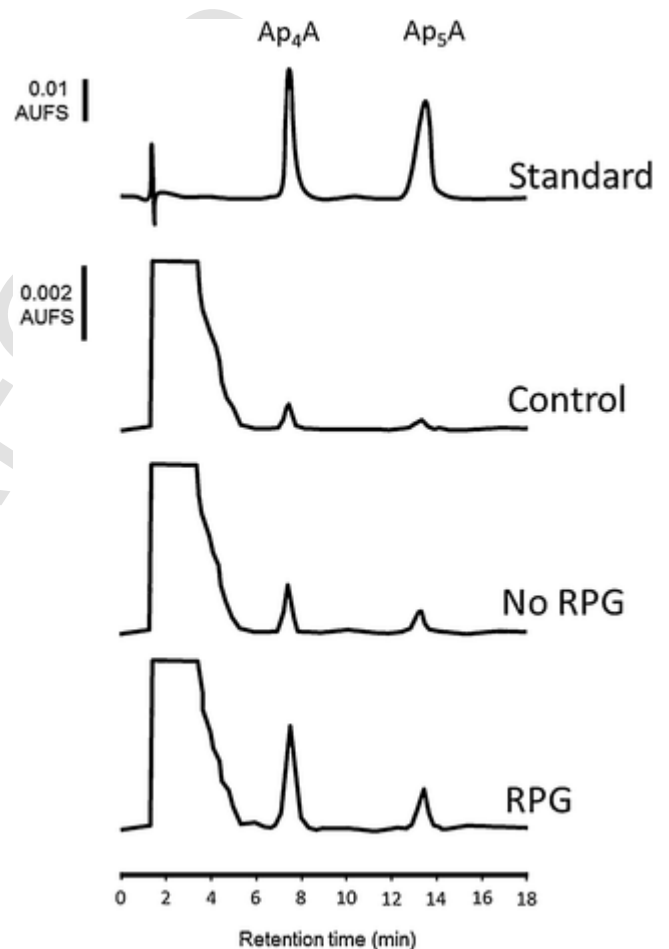| Test	Control	Keratoconus	P value
Ap4A (μM) mean (SD)	0.185 ± 0.178	0.695 ± 0.304	<0.001*
Ap5A (μM) mean (SD)	0.045 ± 0.036	0.132 ± 0.128	<0.001*
Schirmer (mm) mean (SD)	19.87 ± 5.71	16.75 ± 7.65	0.031*
TBUT (s.) mean (SD)	12.13 ± 1.85	6.56 ± 2.88	<0.001*
Corneal staining mean (SD)	0.82 ± 0.51	2.81 ± 0.76	<0.001*
McMonnies score mean(SD)	7.01 ± 3.15	10.87 ± 3.68	<0.001*

Fig. 1. Diadenosine polyphosphate detection in tears of KC patients. Chromatographic analysis of samples from KC patients showing the presence of two peaks identified as Ap4A and Ap5A when compared to a commercial standard (upper record). A control record (representing a healthy individual) can be compared with representative traces of KC patients grouped, according to methods, in RGP wearers and non-RGP wearers. Note that standards scale bar is different from the one for the samples. AUFS, Absorbance Units Full Scale.

4. Discussion

Currently, few scientific articles relate KC with dry eye syndrome [8]. Recent studies of the tears of KC patients have focused on the presence of pro-inflammatory molecules and their role on ectasia progression [11,34–36]. As we previously mentioned, the *Dry Eye Workshop*, published in 2007, includes the inflammation of ocular surface as one of the possible signs of dry eye syndrome [29]. This led us to consider that there could be a close relationship between dry eye syndrome and KC. This study evaluated and compared the signs of Ap_4A and Ap_5A concentrations and another signs and symptoms of dry eye in patients with KC and the influence of wearing GP contact lenses.

Previous studies examining dry eye signs and symptoms in KC patients who wore RGP lenses, had shown that the KC patients had increased concentrations of Ap_4A in their tears [10,37]. Current results match better with the most recent study published [37] than the older manuscript [10]. This is due to the low tear volume found in the KC patient of the study published in 2014, being almost 4 times lower than the other two studies (matched with Ap_4A ratio between studies).

With the aim to evaluate if KC disease causes changes in dinucleotide concentration, the KC group was divided into RGP wearers and RGP non-wearers. And the findings are very significant, because non-RGP wearing KC patients showed 2 times higher Ap_4A concentration than healthy subjects without contact lenses (0.417 ± 0.313 μM vs. 0.185 ± 0.178 μM) [20]. Therefore, KC may cause an increase in the concentration of Ap_4A. Also, this study corroborates the effect of RGP on the concentration of Ap_4A found in a previous study [22]. KC patients who wore RGP lenses had a 2 times higher Ap4A concentration than Kc patients who did not wear RGP lenses (0.794 ± 0.478 μM vs. 0.417 ± 0.313 μM).

The increase in Ap_4A in patients of KC may be due to a mechanical stimulation of the epithelial cells. It has been shown in both in vitro and in vivo studies, that the release of the nucleotides is driven by the shear stress produced on the corneal epithelial cells [23]. There are many studies that report a high prevalence of eye rubbing in patients with KC, with percentages between 46% and 80% of eye rubbing [3,6]. It is therefore likely that friction between lids and cornea is a mechanical stimulus for the release of dinucleotides to the tear. Furthermore, contact lenses exert mechanical pressure on the corneal epithelium in each blink, which may also stimulate the release of dinucleotides. In some studies, in which tear samples were taken before and after contact lens wear, significantly increased concentrations of Ap_4A and Ap_5A were found [22,38].

Nevertheless, KC patients who are not contact lens wearers also showed statistically higher concentrations of dinucleotides than the patients in the control group. The reasons for this observation are not clear, but we have several hypotheses. It might be possible that the increased curvature of the anterior surface of the cornea caused increased friction in each blink on the corneal surface which stimulates the release of the dinucleotides. No studies on the blinking frequency in KC patients has been performed but lower density of epithelial cells, primarily in the apical area have been reported, possibly due to the blinking stress [39,40]. Another possibility is the presence of dry eye associated with KC. There are some references in the literature that relate KC with dry eye but they describe the presence of cytokines such as interleukin-6 or Tumor necrosis factor (TNF-α) and metalloprotease 9 (MMP-9) in the tears of patients with KC [12,41]. These mediators are very important in the pathogenesis of dry eye [42,43]. In another study published in 2003, the authors reported a decrease in tear volume and TBUT in patients with KC [8].

Besides the concentration of dinucleotides, other signs and symptoms of dry eye in patients with KC were evaluated. When compared with the control group, we have found that patients with KC have less tear volume, reduced tear stability, and more corneal staining, all of which are statistically significant. These findings strongly suggest that the increased concentrations of dinucleotides in KC are mainly caused by the associated dry eye.

Regarding the tear volume and stability, KC patients who use contact lenses had significantly lower values than those who are non-users. A possible reason could be that GP contact lenses disrupt normal tear physiology by thinning and breakup of the tear film lipid layer favoring the consequent increase in tear film evaporation [44]. Patients using contact lenses show a significant increase in the Mc-Monnies test. These scores are considered marginal dry eye [45]. It seems possible that the use of rigid gas permeable contact lenses is related to dry eye symptoms in these KC patients, though it would be interesting to know how long each subjects had the disease as this may be an influential factor.

Dogru et al. [8] also indicated that 81.5% of their patients reported discomfort, irritation and foreign body sensation, although that study does not differentiate between contact lens wearers and non-wearers. Although, our KC patients are older, the percentage of patients diagnosed or treated of dry eye in the age range between 25 to 29 years old and 30–34 years old was similar [46] and moreover, signs and symptoms of dry eye worsen with age, but it is accepted that the age of onset is 45–50 years old, which could be potentially linked to hormonal factors [47]. However, KC patients did not show a relationship between age and the parameters evaluated, which could be related to the narrow age range and low subject numbers in our study. A future study with a larger population may be necessary to evaluate the symptomatology of dry eye in KC patients as a function of age including ages up to 60–70 years old. Otherwise, the same number of patients was recruited from both places for both groups, control and KC group. We found no differences between places for any parameter evaluated, but the KC subgroups division in function of the centre gives us very small samples to make any conclusion. Therefore, more studies about environmental conditions and dry eye signs and symptoms in KC patients should be performed.

On the one hand, our patients using RGP lenses have no more staining than non-wearers. This would indicate that symptoms in these patients are not due to corneal damage but due to the contact lens wear. In the case of contact lens wearers, several types of contact lenses are currently fitted in KC patients, from soft contact lenses or GP corneal contact lenses to gas permeable scleral contact lenses or hybrid contact lenses [48,49]. On the other hand some studies have found that gas permeable contact lens wear increases the presence of pro-inflammatory molecules in tears of patients with KC [13]. Furthermore, several studies have demonstrated that soft contact lens wear provokes symptoms of dryness, especially at the end of the day [50], but no studies with scleral or hybrid contact lenses have been performed.

Finally, limitations of this study are the lack of patients with different severity of KC. It might be interesting to investigate the influence of KC severity to confirm our lack of correlation between severity, dry eye signs and symptoms. Eye rubbing was reported by 13 keratoconus patients, 6 non-RPG lens wearers and 7 RGP wearers. The patients who related eye rubbing showed a higher concentration of dinucleotides and Mcmonnies score than non-rubbing eyes in both KC subgroups. However, the sample size for this parameter is too small for statistical analysis and more patients would be necessary to confirm this trend.

In conclusion, the parameters evaluated in this study indicate that patients with KC have more severe signs and symptoms of dry eye than subjects without KC, which could be exacerbated when the patient wears gas permeable contact lenses.

Disclosure

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