Is oxidative stress involved in Vernal keratocongiuntivitis? Results from a pilot study in children

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Abstract

Background: Vernal keratoconjunctivitis (VKC) is a rare chronic conjunctivitis characterized by a predominantly eosinophil-mediated inflammatory disorder that could develop critical complications such as blindness. Oxidative stress plays a pivotal role in the pathogenesis of several allergic diseases. The role of oxidative stress has been hypothesized in VKC, but no study explored this issue.Furthermore, cyclosporine A (CsA) exerts an anti-inflammatory and antioxidant action on the conjunctiva. This study aims to assess oxidative stress in VKC patients and controls and to study the effect ofCsA on oxidative stress in these subjects. Methods:Thirty-six consecutive children, including 12 VKC(9 males, 75%; mean age 10,17; SD ± 2.48) patients without treatment,12 VKC treated with CsA(9 males, 75%; mean age 9,08; SD± 2.75) and 12 controls (CT) (7males,58%; mean age8,58; SD ±1,78) were recruited. A cross-sectional study was performed to compare H2O2 in the serum and the tears ofthese children. Results: Compared with CT and VKC children treated with CsA, VKCuntreated children had significantly higher values ofHydrogen peroxide (H2O2) in theserum and the tears.No significant differences were observed between CT and VKC treated with CsA. A significant correlation was found at the linear regression analysis between serum and tear H2O2 levels. Conclusion: This study provides the first report attesting that patients with VKC have high oxidative stress; furthermore, it suggests that CsA could have an anti-inflammatory and antioxidant action that could be useful to prevent the poor VKC outcome.

Keywords

Vernal keratoconjunctivitis, oxidative stress, children, cyclosporine A, Hydrogen peroxide

INTRODUCTION

Vernal keratoconjunctivitis (VKC) is a rare (<1: 10,000) and chronic conjunctivitis that arose in the pediatric population and is often self-limiting during puberty, underlying a possible hormonal correlation [1]. Males are affected more than females with an approximate ratio from 4:1 to 2:1.[2]

Typical phenotypes of VKC disease are: the tarsal one with papillae on the tarsal conjunctiva, the limbal one with limbal papillae covered with Horner Trantas points and the mixed phenotype with characteristics of both the ones above.[1]

Even if the typical symptoms are in common with allergic conjunctivitis, such as intense photophobia, hyperemia, itching, and tearing and not improved by common therapeutic drugs[1], VKC deserves more attention because of its severe complications that can cause permanent eye damage including blindness.

Although many factors have been considered in VKC etiology, the real mechanism supporting chronic ocular inflammation remains a debated topic[3,4] VKC was initially defined as an IgE-mediated disease but the presence of atopy, positive SPT, and high serum IgE levels, is reported only in 50% cases.[5] Moreover a high percentage of ANA positivity and a family history of autoimmunity is common in VKC.[5]

Ocular conjunctiva is characterized by a predominantly eosinophil-mediated inflammation where histamine, released from mast cell degranulation in response to environmental allergens, has a key role in keeping active the inflammatory pathways. The local funding of eosinophil cationic protein (ECP) is a marker of this eosinophilic inflammation.[6] Besides a Th2 response, many studies have outlined in VKC ocular inflammation several cytokines, such as IL1, 4, 5, 6, 8, 13, and the transforming growth factor-beta 1 (TGFß 1). Also, systemic pro-inflammatory markers, like High-mobility group box 1 (HMGB1) and its receptor for advanced glycation end-product (sRAGE), were found in VKC, underlying a chronic inflammation of the conjunctiva with systemic involvement .[3,5,7,8,6]

Periostin, produced by fibroblasts and endothelial cells in response to IL-4 or IL-13, resulted in VKC chronic inflammation too.[6]

Given this crosstalk between allergic Th2-mediated response and inflammatory Th1-mediated one, it is understandable that the lack of efficacy in VKC disease of standard allergic treatments is mostly based on antihistamines.[5,7,9,10]

The timely use of topical immunosuppressant therapy with cyclosporine A 1% eye drops (CsA) is considered the best treatment for the complete control of ocular symptoms. Furthermore, CsA aims to reduce any future complications, reinforcing the hypothesis of local/systemic inflammation. [11,12,13]

Oxidative stress is a well-known mediator that has been identified as a key role in the pathogenesis of many diseases, such as conjunctivitis. In particular, oxidative stress is considered as the pathogenic result of generalized allergy-related inflammation in patients with seasonal allergic conjunctivitis.[14]

Thus oxidative stress contributes to the onset and persistence of conjunctival damage. A typical example of this condition is common in patients with allergic conjunctivitis, where NADPH oxidase generates superoxide anions that are converted by superoxide dismutase in Hydrogen peroxide (H2O2). This latter oxidative molecule is increased in subjects with allergic conjunctivitis and can contribute to maintaining ocular damage.[15]

To the best of our knowledge, no study has analyzed oxidative stress in VKC, besides the already well investigated inflammatory cascade.

Therefore, the first aim of this pilot study was to assess if oxidative stress is increased in VKC patients.

Furthermore, previous studies showed that CsA inhibits ROS production[16]. Thus the second aim of this study was to analyze the effect of CsA on oxidative stress in VKC children.

METHODS

From April 2019 to June 2019, children affected by VKC, between 6 and 14 years of age, were enrolled at the Department of Pediatrics, Division of Allergy and Immunology, 'Sapienza' University of Rome.

Healthy children, cross-matched to the enrolled VKC children for gender and age, were recruited as controls.

Exclusion criteria were the diagnosis of ocular pathologies and the use of antihistamines and/or corticosteroids in the four weeks before the enrollment.

Diagnosis of VKC was performed by an ophthalmologist investigating ocular signs (conjunctival hyperemia, tarsal and/or limbal papillae, giant papillae) and subjective ocular symptoms (itching, photophobia, tearing, foreign body sensation, and burning sensation), according to the two disease severity scales, graded as follows: 0 = absent; 1 = mild; 2 = moderate; 3 =severe.

Children were classified as having severe VKC if the score was >3 points for one eye for each scale.[17]

Half of them were treated with CsA 1% eye drops suspension (Sandimmun galenical collyrium Pharmacy Umberto I hospital, Rome, Italy) 1 drop/eye twice daily, prepared by the Chemistry Service Institute at the University of Rome, following a formulation which included one part of commercially available CsA solution (Sandimmun Novartis Farma S.p.A.S.S., Varese, Italy), diluted in an aqueous vehicle (Vismed Light_TRB Chemedica, Haar/ Munich, Germany).

Parents were asked to keep the vial with the eye drops shielded from light and to use it within 15 days from the opening. CsA treatment was started for at least four weeks; antihistamines and/or corticosteroids had been suspended at least eight weeks before the beginning of CsA treatment.

The remaining VCK children were enrolled at the onset of disease and tested before starting CsA therapy. Atopic status was assessed by skin prick tests (SPTs) to aeroallergens and food allergens (Lofarma, Milan, Italy) and /or elevated specific (>0.35 kU/l) and total IgE (>100kU/l). SPT panels included: Dermatophagoides pteronissinus (DPT), Dermatophagoides farinae, dog/cat dander, Olea Europea, Lolium perenne, Alternaria tenuis, Parietaria officinalis, lactalbumin, ss-lactoglobulin, casein, egg white and yolk, soy, codfish. Histamine dihydrochloride 10 mg/mL and glycerol saline solution were used as positive and negative controls, respectively. Wheal reactions, read after 15 minutes, [?]3 mm was regarded as positive.[18]

Serum was obtained from peripheral blood samples to evaluate routine blood count, total, and specific IgE levels (sIgE) and oxidative stress. Serum total IgE and sIgE levels were detected for the same allergens tested in the SPTs, using a fluorescence enzyme immunoassay (FEIA) with capsulated cellulose polymer solid- phase (Immuno CAP(r)) coupled allergens (Thermo Fisher Scientific Inc, Phadia AB, Uppsala, Sweden). Results were expressed in kU/L: a cutoff point of 0.35 kU/L has been used as positivity for the specific IgE and > 100 kU/L for the total IgE.

Tear samples were obtained as follows: 20–50 μl of open eye tears were gently collected from the external canthus of the most affected eye using a microcapillary tube and avoiding the tear reflex as much as possible. The samples were placed in Eppendorf Tubes, centrifuged at 160 ×g for 8 min, and stored at -80degC.[19]

The concentration of H2O2 was analyzed in serum and tear samples using a commercial colorimetric assay (Sigma, Saint Louis, Missouri).

The sensitivity, determined by subtracting two standard deviations from the mean absorbance value of sixteen zero standard replicates and calculating the corresponding concentration, was around 50ng/ml.

The inter-assay Precision coefficient was 1,7 %, and the inter-assay Precision coefficient was 3,0%

Values are expressed in terms of micro molarity, where 1,0 m corresponds to 8,46ng/ml. This study was approved by the International Review Board of 'Sapienza' University of Rome and performed with the written informed consent of the parents of all children.

Statistical analysis

Statistical analysis was performed with SPSS 18.0 software for Windows (SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. Normally distributed data are described as means+-standard deviations (SDs). Group differences were analyzed by Mann-Whitney tests (for non-normally distributed data) or analysis of variance (ANOVA). Differences between categorical variables were assessed by the $\chi 2$ test. Simple linear regression analysis was performed by Spearman's rank correlation test. A p-value < 0.05 was considered as statistically significant.

Sample size determination

The minimum sample size was computed with respect to a two-tailed, one-sample Student t-test considering, on the basis of data from a previous pilot study (data not shown): a difference of 8 pg/ml for H2O2 levels between children affected by VKC and controls, 5.5 as SD, 0.05 (α) as type I error probability and 0.95 as power 1 - β. The sample size was n = 10 patients/group.

RESULTS

The clinical characteristics of each group are described in Table 1. Twenty-four children affected by VKC (18 males, 75%) between 6 and 14 years of age (mean 9,63; SD±2,62) were enrolled. Of them, 12 VKC (9 males, 75%; mean age 9,08; SD± 2.75) were treated with CsA for at least four weeks and 12 VKC children (9 males, 75%; mean age 10,17; SD ± 2.48) at the onset in the active phase of the disease.

The control group (CT) composed of 12 children (7 males, 58%; mean age of 8,58; SD±1.78) well matched for gender and age with disease groups. No differences were reported for age, gender distribution, and body mass index among all children.

Positive SPTs were found in 7 (58%) VKC treated children: 6 (50%) to DPT, 5 (42%) to Lolium perenne, 4 (33%) to Olea europea, 3 (25%) to Alternaria, 1 (8%) to cat dander.

Among VKC untreated children, 6 (50%) had a positive SPT: 5 (42%) to DPT, 5 (42%) to Lolium perenne, 2 (17%) to Olea Europea, 1 (8%) to Parietaria officinalis**.** SPT positivity was confirmed by sIgE values in the blood, which reported overlapping serum values.

Among VKC treated children, the tarsal form was diagnosed in 7 (58%) children, the limbal form in 2 (17%), while the mixed phenotype in 3 (25%) children. Instead, among untreated VKC children, 9 (75%) children presented the tarsal form, 1 (8%) the limbal one, and 2 (17 %) the mixed phenotype. No corneal involvement was reported.

VKC score was <3 points for one eye for each scale for all children.

H2O2 was detected to assess systemic oxidative stress. Compared to VKC untreated children at the onset of disease, children undergoing CsA and controls had statistically significant lower values of H2O2 (Figure 1, Panel A). No significant differences were reported between controls, and VKC treated children concerning H2O2 blood values. (Figure 1, Panel A). Finally, we analyzed H2O2 in the tears of all the enrolled children. Compared to VKC untreated children, those undergoing CsA and controls had statistically significant lower H2O2 levels, also in the tears samples (Figure 1, Panel B). No statistical differences were found between controls, and VKC treated children regarding H2O2 tears values. (Figure 1, Panel B). Interestingly among all VKC children, H2O2 serum values were linearly and significantly correlated with levels of H2O2 in tears (Rs=0.629, p<0.001**)** (Figure1, Panel C)

DISCUSSION

VKC is characterized by a severe inflammatory pattern, generally resistant to standard anti-inflammatory therapies and sensitive to topical steroids or immunosuppressants.[1,3]

Several cytokines and chemokines play a pivotal role in keeping the inflammatory cascade active, as it results from previous studies[3,6,8]

The role of oxidative stress has been studied with increasing interest in several allergic or immune-mediated inflammatory diseases[20], except for VKC.

Ocular oxidative stress has been investigated both in humans and in animal models in many eye diseases such as uveitis[21], corneal inflammation[22], and allergic keratoconjunctivitis[23]. To our knowledge in only one study, Tais H. Wakamatsu et al. [24] evaluated tears and brush cytology samples in 14 adolescent-young adults with atopic dermatitis and allergic conjunctivitis, to assess if there were oxidative-stress related changes in these patients in comparison to healthy children. The authors[24] found higher percentages of cells positively stained for the early phase lipid peroxidation marker and the late phase lipid peroxidation marker, in palpebral conjunctiva and tears, suggesting an increased lipid peroxidation status in atopic keratoconjunctivitis, leading to endothelial damage.

Dadaci Z et al., evaluated oxidative stress parameters in 35 patients with seasonal allergic conjunctivitis (SAC) during the pollen season vs. 38 healthy subjects, demonstrating higher levels of oxidative stress

parameters in SAC compared to controls, outlining a possible role of oxidative stress in the pathogenesis of this disease.[2]

This study reports new and intriguing results, never investigated in VKC, providing the first evidence that oxidative stress characterizes VKC disease. We found significantly higher levels of hydrogen peroxide in the serum and in the tears of children in the active phase of disease compared to VKC treated children and CT. No significant differences were observed between CT and VKC undergoing CsA. Moreover, a linear regression analysis reported a significant correlation between serum and tear H2O2 levels.

The reduction of H2O2 values in tears and the serum of VKC treated children, could be considered a parameter for the efficacy of CsA treatment, confirming that this therapy effectively controls both systemic and local inflammation.

CsA reduces H2O2-induced intracellular generation of reactive oxygen species, protecting against H2O2-induced cell injury.[25]

To confirm the importance of the timely use of CsA, Leonardi A et al. have conducted a trial where 169 children were randomized to receive CsA 0.1% (1 mg/ml) eye drops with higher dosages compared to lower ones, reporting a better efficacy of high-dose CsA to improve keratitis, symptoms and also the quality of life of VKC children.[26]

Moreover, CsA can interrupt both the helper T-lymphocyte proliferation and the IL-2 production, which inhibit the release of histamine from basophils and mast cells, reducing IL-5 levels.[27]

This finding leads us to speculate that local immunosuppressive therapy with CsA may control the ocular immunological response and the systemic one.[5,7]

Although eyes are defined as immunological sanctuaries, the finding of high local H2O2 values lets us speculate that H2O2 could play a key role in ocular dysfunction and inflammation, which is responsible for the serious complications typical of VKC. This reinforces the importance of a prompt start of target therapy with CsA in VKC patients.

Our results must be interpreted, considering some limitations.

First of all, we have studied a small, single-center sample that needs to be implemented into a larger cohort. The presence of allergic diseases in 54% of VKC children may interfere with a realistic estimate of the systemic oxidative stress caused by VKC disease alone, rather than by other allergic coexisting conditions. However, the randomized distribution of these patients exceeds a possible risk of bias. Summing up, this is a pilot study with a cross-sectional design that may reports only associations between H2O2 mediated oxidative stress and inflammation in VKC children.

So further studies are warranted to confirm these results in a long-lasting follow-up.

Several questions remain unsolved, such as the correlation between oxidative stress and the degree of severity of VKC disease.

Conclusion

Our study shows that oxidative stress potentially plays a pivotal role in VCK children. It may be responsible for VKC ocular inflammation, triggering an increase of systemic, as well as local, H2O2 levels that lead to serious complications. Hence the importance of prompt therapy with CsA, able to reduce H2O2 levels.

However, more studies should be carried out to clarify the H2O2 role in the immunopathogenesis of VKC diseases and the connection with its severity.

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