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Using Immunofluorescence to Investigate an Associated Specific Humoral Immune Response Role in Climatic Droplet Keratopathy

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Abstract

Purpose: To find out any associated specific immune role in Climatic Droplet Keratopathy (CDK) through immune fluorescent study and electron microscopy that can provide a new therapeutic strategy in the prevention and in the progression of the disease.

Methods: A total of 22 case of CDK who underwent keratectomy or scraping were incorporated in this study. Twenty of them were subjected to immuno-fluorescent study using IgG, IgM, IgA and complement 3 and the remaining two cases were subjected to electron microscopic study.

Results: Although, the electron microscopic study showed extracellular electron dense like globules in the superficial corneal stroma just beneath the epithelium that may stimulate the immune complex humps of immune-mediated glomerulonephritis, the immuno fluorescent examination for all the studied cases revealed total negative stain for all studied markers (IgG, IgM, IgA and C3).

Conclusion: This work suggests the absence of specific immune response in climatic droplet keratopathy.

Keywords: Climatic droplet keratopathy, immune complex, immuno fluorescent.

Abbreviations

CDK: Climatic Droplet Keratopathy; IgA: Immuno Globulin Alpha; IgG: Immuno Globulin Gamma; IgM: Immuno Globulin Macroglobulin; C3: component 3

Introduction

Climatic Droplet Keratopathy (CDK) (Figure 1) has been given a variety of names, including Lapradore keratopathy, spheroidal degeneration, chronic actinic keratopathy, oil droplet degeneration, keratinous corneal degeneration, elastic degeneration, hyaline degeneration; fisher mans keratopathy, Name keratopathy and proteineous keratopathy, Bietti's band-shaped nodular dystrophy and chronic actinic keratopathy [1].

The cause of CDK is unknown, but certain factors such as solar radiation, aging, low humidity, micro trauma from the wind sand and ice and possibly extremes of temperature and previous

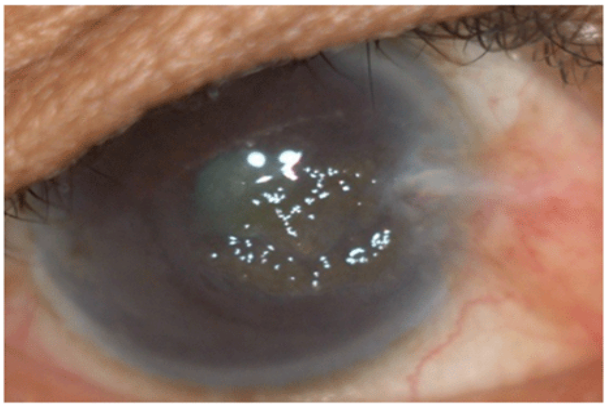

Figure 1: Climatic droplet keratopathy Aggregates of large yellow-golden globules patient with CDK

corneal inflammation seem to be related to its progress. These factors, plus others that are possibly genetic, cause an elastotic degeneration of the collagen in the cornea and conjunctiva and deposits that consist of an exudates of fibroblastic cells of the cornea and conjunctiva [2].

Fraunfelder and Hannna [3] described two types of CDK. The first is a primary corneal type which is bilateral and related to the aging not to corneal disease of any kind. The other type is secondary bilateral or unilateral and related to corneal disease (glaucoma, herpes and dystrophies) or chronic climatic insults or both. Climatic Droplet Keratopathy (CDK) most often affects men who work outdoors.

The problem with this classification is that CDK is not well defined and any division into types is likely to be arbitrary. Climatic factors certainly influence the type occurring in the aged, though they are probably less influential in old than in the young patients. Local corneal inflammation may act to enhance the predilection to deposit the materials.

Three grades of severity have been described: grade 1, in which only the peripheral inter palpebral zone of the cornea is affected, grade 2 in which the opacity spreads to the pupillary

area and grade 3 in which large yellowish opalescent nodule elevate the epithelium and reduce vision to less than 20/200 [1].

Histopathologically, the changes begin with sub-epithelial accumulation of opalescent droplets that coalesce to form a yellowish grey band beneath the epithelium. Larger aggregates of droplets accumulate later and elevate the corneal epithelium. They are extracellular and autofluoresent with ultraviolet light [3].

The histopathology composition of spheroidal degeneration has long been unclear and to some extends controversial. Different workers have described the material variously as being hyaline, colloid, high tyrosine protein, lipid, keratin like, a Secretory product of abnormal fibrocytes, and elastotic degeneration of collagen. It seems clear however that the material is acidophilic and amorphous by light microscopy, finely granular by electron microscopy, extracellular and proteinaceous [3].

Histopathological investigations led by Garner, Morgan and Tripathi [4] concluded that the material represents incomplete form of keratin originates from the epithelium however, Hanna and Fraunfelder [5] were of the opinion that the substance is of stromal origin, consisting of a fibrocyte-derived granular protein deposited on adjacent collagen fibrils. Both groups of workers agreed that the composition is not lipid; despise the oil droplet clinical appearance.

Most workers now think that the process is basically stromal one and that epithelium involvement occurs only secondarily after destruction of Bowman's membrane.

Climatic Droplet Keratopathy (CDK) being occur in the inter palpebral zones of the cornea and conjunctiva suggesting that actinic exposure plays a role in its development. The degraded protein material may then be deposited in the superficial stroma. Ultra Violet light is widely accepted to be the main etiological factor in the pathogenesis of CDK [5].

Sector iridectomy, corneal epithelial debridement, lamellar keratoplasty, and penetrating keratoplasty have all been employed in the treatment of visually incapacitating CDK [6].

Methods

The study included 22 patients having climatic droplet keratopathy. Ten cases were treated by keratectomy and corneal transplantation and 12 cases were treated by corneal scraping. All tissue specimens were presented in saline to the histopathology laboratory. Two cases were shifted to 2.5% glutaraldehyde to be fixed for electron microscopy and the remaining 20 cases were preserved in 10% formaldehyde and processed with hematoxylin and eosin stained sections for immuno fluorescent study. Specimens were preserved in 2.5% glutaraldehyde in 100 ml Phosphate Buffer Saline (PBS) at pH 7.0 for 24 hours.

Post fixation specimen was transferred to 1% osmium tetroxide in 100 ml phosphate buffer saline for two hours at 4°C. Specimens were washed at least five times in distilled water to remove all excess phosphate ions to prevent Uranyl Acetate (UA) from being precipitated. Dehydration in 30% acetone for 15 minutes, followed by 50% acetone for 15 minutes, 70% acetone for 15 minutes, 90% acetone for 15 minutes and finally in three changes of 100% acetone, each for 30 minutes.

Resin embedding was done with Epoxy resin which consists of four ingredients, the monomeric resin, a hardener, an accelerator, and a plasticizer. Propylene oxide was used for clearing two changes, each for 15 minutes. Resin infiltration 2:1 mix of propylene oxide to resin, was used for one hour, then 1:1 mix of propylene oxide to resin was used for one hour then 1:2 mix of propylene oxide to resin was used for one hour, then 100% resin was used overnight followed by the use of fresh resin for one hour. Once infiltration was done, tissue samples were placed in an appropriate mold (polyethylene capsules) filled with resin for 24 hours and allowed to be polymerized by heat ($60C^0$ to $70C^0$).

Block trimming and ultra-thin sections of 80 nm were prepared. Staining by uranyl acetate and lead citrate was done by placing droplets of the stain on a Petri dish; place the grid section side down on the droplets for 10 minutes, followed by rinsing the grids in three changes of distilled water [7].

For specimen preserved in neutral buffered 10% formalin and routinely processed, the cut sections were placed on slides at five microns. Paraffin was removed by immersing the slides in Xylem to be washed three times 5 minutes each. The slides were rehydrated by different grades of alcohol, 100% Ethanol two washes for 10 minutes each, 95% Ethanol two washes for 10 minutes each, 70% Ethanol two washes for 10 minutes each, 50% Ethanol two washes for 10 minutes each and finally water, two washes for 5 minutes. A circle on the slide was made around the tissue with a hydrophobic barrier pen or with rubber cement.

For antigen retrieval, a microwave was used, the slides were boiled in 10 ml sodium citrate buffer (pH 6.0) and maintained at a sub-boiling temperature for 10 minutes, and then the slides were cooled on the bench-top for 30 minutes. Sections were washed by immersing them in distilled water for 5 minutes, followed by blocking the endogenous peroxidase activity. The tissue sections were incubated with 3.0% hydrogen peroxide in methanol for 15 minutes followed by washing the sections in distilled water twice for 5 minutes.

Blocking of any non-specific binding was done by incubating the tissue sections with 5% animal serum in PBS for 30 minutes at room temperature. Adding primary antibody diluted in 1% animal serum PBS and incubated at room temperature for 1-2 hours followed by storing the slides overnight at 4°C in a humidified chamber. Washing sections twice with 1% serum PBS for 10 minutes each was done. Adding a secondary antibody labeled with fluorescent dye (Ig G, Ig M, Ig A and C3) diluted in 1% serum PBS and incubate at room temperature for 1-2 hours was done. Finally, sections were washed twice with 1% serum PBS for 10 minutes each with addition of mounting medium with cover slip and the slides were examined by fluorescent microscope. Slides were stored between-20°C and 4°C in a dark slide box for 2 weeks [8].

The collected data were coded, tabulated and analyzed as the minimum, the maximum and the median for quantitative parametric data, while numbers and percentage were used for qualitative data.

Results

Hematoxylin and eosin (H&E) stained sections of the received corneal specimens showed homogeneous eosinophilic amorphous and globular deposits of variable sizes that are noted just under the corneal squamous epithelium and in the superficial cornea stromal layer (Figure 2 a, b, c, d). The surrounding corneal stroma showed myxoid like changes and fibrosis. The deeper corneal stromal layer showed scattered small blood vessels with fibrosis and scattered inflammatory cells, mainly macrophages and lymphocytes (Figure 3 a, b, c, d).

Examination of immuno fluorescent stained slides for Ig G, Ig M, Ig A, and C3 were all negative in all examined cases. Electron microscopic photos revealed electron dense granular material in the cornea (Figure 4 a, b).

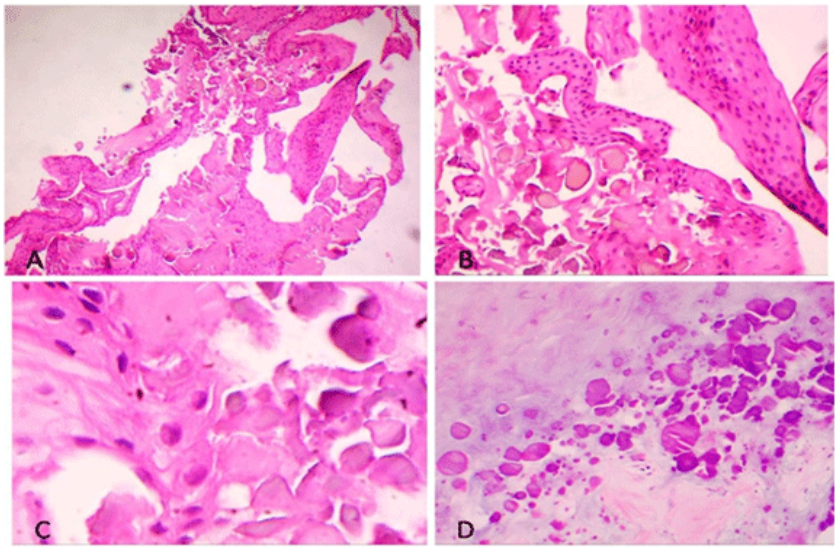

Figure 2 (A,B,C,D): A,B and C: Climatic droplet keratopathy showed many globular deposits of variable sizes just beneath the corneal stratified squamous epithelium, H&E x40 x100 and x400 **(D):** The deposits in the superficial corneal stroma associated with stromal fibrosis and myxoid change H&E x100

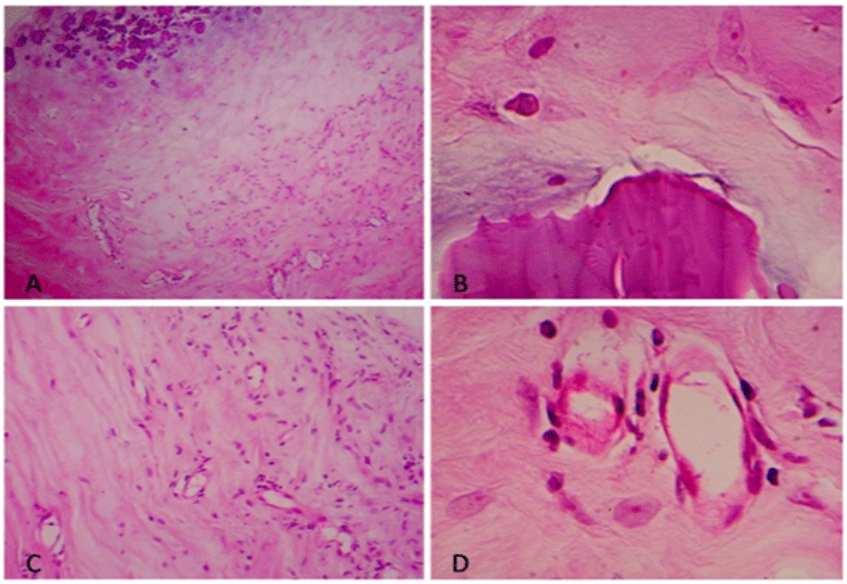

Figure 3 (A,B,C,D): A,B: Climatic droplet keratopathy showed extensive stromal fibrosis with myxoid changes around the deposits, H&E x40, x400. **(C, D):** The deeper corneal stroma showed extensive fibrosis with small blood vessels proliferation associated with mild inflammatory cells as lymphocytes and macrophages, H&E, x100, x400.

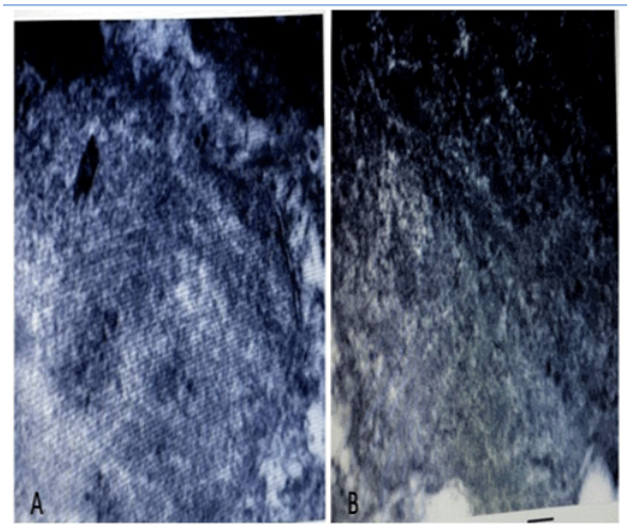

Figure 4(A and B): An electron micrograph showing the extracellular deposition of electron Figure 4 dense round and oval globules in the corneal stroma just beneath the epithelium (uranyl acetate and lead citrate x6000).

Discussion

Climatic Droplet Keratopathy (CDK) is an acquired and potentially handicapping cornea degenerative disease that is highly prevalent in certain rural communities around the world. It predominantly affects males over their forties.

The exact etiology and pathogenesis of CDK are unknown, but they are possibly multi factorial such as corneal micro traumas, low humidity and lack of adequate protection for exposure to Ultraviolet Radiation (UVR). This degenerative corneal disease leads to progressive opacities of the anterior layers of the cornea [9]. Currently, no pharmacological treatment for CDK is available leaving corneal transplantation, the only choice of treatment for advanced stages of the disease. Recurrence of CDK has been observed in grafted patients both with lamellar and penetrating keratoplasty between 3½ years and 7 years after surgery [10].

Few researches were performed in the previous years; try to elucidate the molecular mechanisms involved in the pathogenesis of CDK. In the past, most authors imply that it is formed in situ, from structural components of the cornea [11], however, Johnson and Overall proposed that degenerate elastotic material formed from collagen beneath the adjacent conjunctiva might be diffusing into the cornea [12].

In 1986, Tabbara [13] found that staining with the Masson trichrome stain revealed reddish gray color of spherules (not greenish blue as collagen) however, most aggregates stain positively with Verhoeff-van Gieson stain for elastic tissue. In 2007, Kaji Y et al. [14] found advanced glycation end-products (AGEs), which are the final products of the reaction between sugars and proteins in these amorphous deposits of CDK.

In 2011, Holopainen et al. [15] have also considered the importance to investigate the biological features of matrix metallo pretenses (MMPs) and their inhibitors TIMPs in patients with CDK, since these molecules control the degradation of the corneal epithelium and stroma. Preliminary studies showed enhanced MMP-2 and -9 levels and a decreased expression of TIMP-1 in CDK patients' tears.

In 2013, Schurr et al. [16] reported that the golden yellow droplets that seen in CDK patients are generally lack positive staining for fat or calcium but proteinaceous constituents have been found. In 2017, Serra et al. [9] rreported approximately 105 proteins (mainly) secreted Extra-Cellular Matrix (ECM) proteins and plasma proteins, being a subset of the droplet composition confirmed by immuno histochemistry.

Despite the fact that some of its constituents were identified in the droplets [13-17] the exact nature and composition of these droplets remain unclear [9,16].

Finding out the exact pathogenesis of the disease may provide more therapeutic modalities for these patients. This research was designated to find out any associated humoral immune response (immune complex formation) in climatic droplet keratopathy cases through immuno fluorescent study for IgG, IgM, IgA and C3 especially those electron dense deposits appeared on electron microscopic study simulate the electron dense humps that seen in immune mediate glomerular nephritis.

Our Hematoxylin and eosin histo pathological findings were similar to what reported by Tabbara [13] and by Bissau et al [18] that showed many extracellular eosinophilic amorphous and globular deposits of variable sizes in the superficial corneal stroma just beneath the epithelium.

As similar amorphous eosinophilic extracellular material is seen in amyloidosis, many studies perform Congo red stain to assess the amyloid nature of the material but the Congo red stain did not support the presence of amyloid, nor has previous electron micrographs [19] The fibrillar structure of amyloid is not seen, but only electron dense material was found. Meanwhile, some cases of CDK showed foci of Congo red positive stain with birefringence using polarizing light that suggests the presence of amyloid in association with some CDK cases [13].

In the present study, in addition to the presence extracellular eosinophilic globules, revascularization and inflammatory cells including lymphocytes were found in the corneal stroma. With electron microscopy, these globules revealed aggregates of extracellular electron dense round to oval deposits, among the collagen fibrils of the superficial corneal stroma. These findings are in accordance with the work done by Schurr et al. They reported that electron microscopy has shown that the corneal globules in CDK are round, electron-dense and sharply demarcated structures and adjacent to disorganized collagen fibrils. Also Serra et al. [10] reported the presence of globular deposits of different sizes under the corneal epithelium by means of light and electron microscopy in CDK.

Also in this study, extracellular electron dense granular material was found in the corneal stroma on electron microscopic

examination but its appearance in photos was different from what was seen by Tabbara. He used magnification of 1000 and showed large globular electron dense deposits. We used magnification of 6000 and showed granular materials inside these large electron dense deposits itself.

Since it is well known that the extracellular electron dense deposits that are seen in many nephrotic and nephritic associated glomerular lesions are immune complexes, [20] so in this study we try to examine the possibility of any associated specific humoral immune reaction (immune complexes) which if confirmed, would be of a great value in patient treatment. Immune suppressive drugs could have a role in the disease course to slow down its progression and the need for surgical intervention.

Some studies examine the presence of inflammatory mediators in CDK and showed significant presence of them. Serra et al. [21] and Ordonez et al. [20] reported that hyper-sensitive reaction occurred in the cornea with the initial participation of important pro-inflammatory components of the innate immune system. Holopainen et al. [22] found significant increase in pro-inflammatory cytokine secretion in the tears from CDK patients and demonstrated that the corneal epithelium could participate in CDK development as a source of cytokines and gelatinases.

The present study was not able to show associated immune complexes in the examined CDK cases as the immune fluorescent study for all examined markers (Ig G, Ig M, IgA and C3) were negative.

Declarations

Ethical Approval

All procedures in this study were adhered to Declaration of Helsinki Ethical Principles for Medical Research involving Human Subjects and approved by Faculty of Medicine, Ain Shams University Research Ethical Committee (FMASUREC). A Written informed consent was obtained from all individual participants in this study

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