Acta Scientific Ophthalmology (ISSN: 2582-3191)

Volume 4 Issue 1 January 2021

Research Article

Histopathologic and Immunohistochemical Analysis of Conjunctivochalasis

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Received: October 30, 2020

Published: December 14, 2020

Abstract

Purpose: To histopathologically and immunohistochemically investigate conjunctivochalasis (CCh) and compare it to normal conjunctival tissue.

Methods: 20 consecutive conjunctivochalasis specimens and 5 age-matched controls were submitted for pathologic evaluation between 2010 and 2017.

Control tissue was harvested from age appropriate Eye Bank donors with no ocular or ophthalmic surgical history. Specimens were stained with Hematoxylin and Eosin (H&E), Verhoeff-von Gieson (VVG), M2A (D2-40) lymphatic endothelial marker and CD68 macrophage marker. Collagen interfibril distance, elastin fiber density, lymphatic vessel density and macrophage density were measured using Aperio Imagescope software. Patient identifying information was masked from observers. Statistical analysis performed using Mann-Whitney Test.

Results: H&E Control: mean collagen interfibril distance 4.10 microns compared to CCh: mean interfibril distance 7.54 microns ($p = 0.018$). In the VVG Control: mean 22.63 elastic fibrils per 10 collagen fibers compared to CCh: mean 8.92 elastic fibrils per 10 collagen fibers ($p = 0.007$). In the M2A (D2-40) subset Control: 0.012 lymphatic vessels per square millimeter; CCh: 0.027 lymphatic vessels per square millimeter ($p = 0.0124$). CD68 subset Control: 0.038 macrophages per square millimeters to CCh: 0.12 macrophages per square millimeters ($p = 0.045$, z-score -2.00), all comparisons showed statistically significant differences.

Conclusion: Statistically significant increased collagen interfibril distance, decreased elastic tissue density, higher lymphatic vessel density and increased CD68 macrophage count occurred in the CCh subset compared to controls. These findings support the influence of mechanical and inflammatory factors as being central to causation of conjunctivochalasis.

Keywords: Conjunctivochalasis; Histopathology; Lymphangiectasis; Elasticity; Infiltration

Abbreviations

CCh: Conjunctivochalasis; ATD: Aqueous Tear Deficiency; BLICK: Blink dynamics, Lid malposition, Imbrication, Conjunctivochalasis, Kissing puncta; LIPCOF: Lid-Parallel Conjunctival Folds; SLK: Superior Limbic Keratoconjunctivitis; H&E: Hematoxylin and Eosin; VVG: Verhoeff-von Gieson

Introduction

Wendell Hughes first coined the term conjunctivochalasis (CCh) in 1942 [1]. CCh is characterized by redundant, loose, non-edematous inferior bulbar conjunctiva interposed between the globe and the lower eyelid. The condition is usually bilateral with a slight female preponderance and increases in severity with age [2]. Patients may be asymptomatic or have symptoms ranging from

tearing due to punctal occlusion to exposure-related pain and irritation secondary to nocturnal lagophthalmos and dellen formation [3]. Clinically, patients often present with the ability to localize their area of discomfort compared to other dry eye states where the chief complaint is a generalized ocular discomfort. They also commonly note a worsening of pain on down-gaze [4]. Not uncommonly, there is a history of cataract surgery (phacoemulsification), which can lead to a worsening of CCh [5].

Mechanical factors have been implicated in CCh pathophysiology. Hughes originally proposed that CCh occurs due to aging changes in the conjunctival elastic supporting tissues [1]. Mechanical forces have been hypothesized to explain the higher prevalence of CCh in the lower versus upper conjunctiva. CCh is thought to be more predominant on the lower eyelid secondary to both a greater effect of gravity on the lower eyelid and stress on the conjunctiva during down gaze where there is minimal movement of the lower lid [6]. This contrasts with the movement of the eye on up gaze when the upper eyelid moves upward with the globe, reducing the stress on the superior conjunctiva [4]. These gaze and pressure-dependent changes of CCh have also been shown to increase with duration of hard and soft-contact lens use [7]. CCh shows a high prevalence in patients with autoimmune thyroidopathy [8] and has also been found in patients with superior limbic keratoconjunctivitis (SLK) [9]. Treatment of redundant superior conjunctiva in SLK has led to symptomatic relief, suggesting that SLK may be considered a type of superior conjunctivochalasis [9]. Histologically, elastin fiber concentration has been evaluated as a measure of mechanical effects on the conjunctiva, with most studies showing decreased fibers [10,11], while others show no alteration [1,12] or even increased fiber density [13].

CCh is strongly associated with tear film abnormalities and dry eye. In moderate cases, CCh has been shown to exacerbate dry eye symptoms in patients with aqueous tear deficiency [4] and has been clinically linked to epiphora [14]. Decreased tear meniscus recovery rates have also been seen in symptomatic CCh patients [15]. Diagnostically, measurement of lid-parallel conjunctival folds (LIPCOF) has proven an accurate measure of dry eye [16] and presence of CCh cited as an important component in the evaluation of epiphora (BLICK mnemonic) [17]. In severe stages, CCh is linked to subconjunctival hemorrhage and exposure keratitis [6].

Prior histopathologic studies have also attempted to investigate the enzymatic and inflammatory factors involved in the pathogenesis of CCh. Enzymatic changes, including increased MMP-3 and MMP-9 concentrations [13], have been linked to decreased collagen fiber density [11,13]. Lymphangiectasis [11,18] and inflammatory cell infiltration [11,19,20] have also been investigated as possible evidence of an inflammatory etiology.

We set out to elucidate and quantify the histopathologic and immunohistochemical properties of CCh in an effort to determine its ultimate etiology. Prior studies have been limited by absence of significant controls or absence of age-matched controls [11], small sample size [1,9] and lack of detailed, fully quantitative methodology [10,12,13,20]. We hope by showing a well-defined case with clear clinical and histopathological features there will be a greater clarity and better understanding of CCh. Lastly, this will allow for a better appreciation and understanding of the detailed histologic features in this disease entity.

Case Report

The patient was a healthy 78-year-old woman who presented with complaints of bilateral burning and tearing in eyes, foreign-body sensation, epiphora and point-tenderness of the right eye. She was managed initially with topical lubricating drops but reported persistent foreign-body sensation, point tenderness and epiphora. Topical corticosteroids were also added without improvement and after several months of treatment surgery was discussed. Stage 3 CCh with punctal occlusion was noted (Figure 1 and 2). The involved inferior conjunctiva showed severe lissamine green staining, signaling to the physician that CCh surgery may be beneficial when coupled with the symptom of point tenderness (Figure 2). Surgical repair was by inferior conjunctival resection with glued amniotic membrane grafts on each eye, after which the patient reported significant improvement of her symptoms without need for further treatment (Figure 3). Hematoxylin and Eosin (H&E) 20X stain showed a prominent increase in interfibril distance, a myriad of lymphatic channels and focal collections of inflammatory cells, especially when compared to controls (Figure 4). Verhoeff-von Gieson (VVG) elastin stain showed a diffuse loss of elastic tissue in the substantia propia with positive staining limited to the blood vessel walls (Figure 5). M2A lymphatic endothelial marker, and CD68 macrophage marker (Figure 6 and 7) helped to confirm the diagnosis as conjunctivochalasis especially obvious compared to control. This case illustrates some of the main clinical features and frequently missed histopathologic findings in conjunctivochalasis. These histological findings possibly being misdiagnosed as normal and the need for a more systematic approach to conjunctivochalasis diagnosis led to the conception of this study.

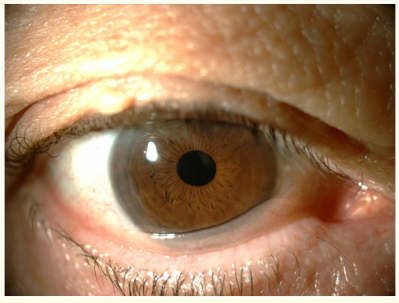
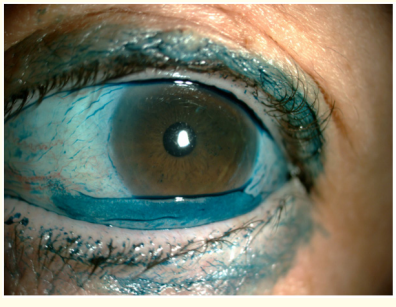


Figure 1



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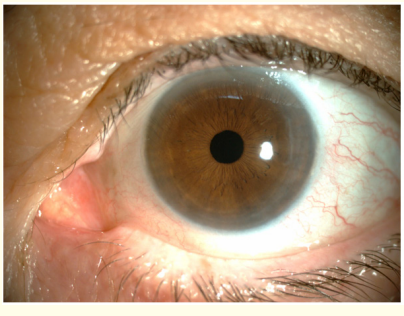

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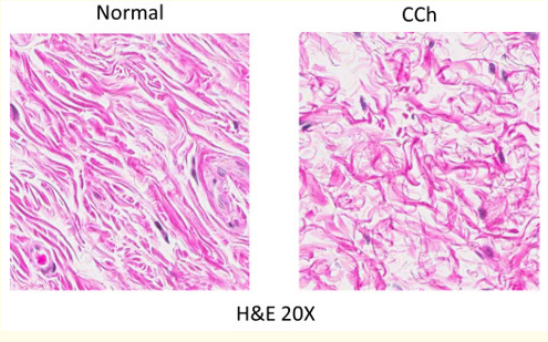

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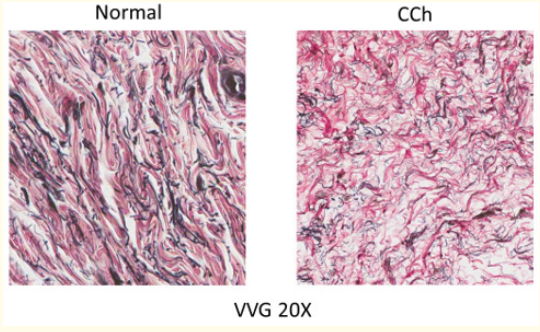

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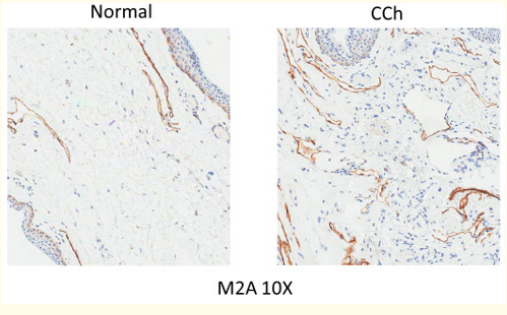

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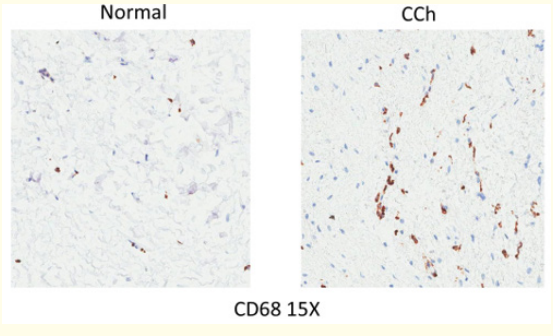

Figure 7

Materials and Methods

20 consecutive pathology specimens and 5 age-matched controls were prospectively submitted for pathologic evaluation between 2010 and 2017. Control tissue was harvested from age matched Eye Bank donors with no ocular or ophthalmic surgical history. All specimens were immersed into 10% buffered formaldehyde immediately following collection and processed within 24 - 48 hours following fixation. Specimens ranged from 3 - 6 mm to 4 - 8 mm in diameter, were sectioned at 2 - 3 mm intervals, and were

entirely submitted for pathologic evaluation to ensure representative and adequate tissue sampling. Two to three sections from each block were placed on each glass slide. Consecutive specimen sections, each 4 microns thick, were stained with H&E, VVG, M2A and CD68. Both study cases and controls had 4 representative slides from each stain for evaluation. Collagen interfibril distance, elastin fiber density, lymphatic vessel density and macrophage density were then measured using Aperio Imagescope (Leica Biosystems, Illinois, USA) software. For each specimen, the software was used to isolate and examine the entire conjunctival stroma. Patient identifying information was masked from observers. Statistical analysis performed using Mann-Whitney Test. All p-values of < 0.05 were interpreted as statistically significant.

Collagen interfibril distance was determined on H&E stained preparations by measuring the mean of 3 densely populated "hot spot" areas of collagen and 3 loosely populated "hot spot" areas of collagen. Eyeballing the entire tissue at the 10x to 20x objective magnification utilizing the Aperio Imagescope identified maximally dense and loose collagen and elastic fiber aggregates. In each "hot spot," the distance between 10 consecutive collagen fibers was measured. The mean value of these 10 distances was then calculated for each "hot spot". The elastin fiber density was assessed on VVG stained preparations by calculating the mean of 3 densely populated "hot spots" of elastic fibers and 3 loosely populated "hot spots" of elastic fibers. Each "hot spot" was determined by counting the number of elastic fibrils per 10 consecutive collagen fibers. Each elastin fiber had to be adjacent to or in direct contact with a collagen fiber in order to qualify toward the official count. M2A immunohistochemical stain was used to determine the lymphatic channel density in each specimen by counting the number of lymphatic vessels per micrometer-squared. We chose this measurement because the variability of arrangement and orientation of lymphatics in the tissue precluded accurate assessment of mean channel diameter. The CD68 immunohistochemical stain was used to determine the macrophage density in each specimen by counting the number of macrophages per micrometer-squared. Statistical analysis was performed using the Mann-Whitney test.

Results

In the H&E control group, the mean collagen interfibril distance was 4.11 microns and in the CCh group 7.54 microns. This was a statistically significant difference (z-score 2.58, $p = 0.018$, $p < 0.05$). In the VVG control group, a mean of 22.63 elastic fibrils per 10 collagen fibers compared to the CCh group's mean of 8.92 elastic fibrils per 10 collagen fibers. This was statistically significant difference (z score -3.36, $p = 0.0078$, $p < 0.05$). In the M2A subset control group, 0.013 lymphatic vessels per square millimeter were measured. In the CCh group, 0.028 lymphatic vessels per square millimeter was a statistically significant difference (z-score 3.23, $p = 0.001$, $p < 0.01$) In the CD68 control group: 0.038 macrophages per square millimeters were compared to the CCh subset, 0.12 macrophages per square millimeters ($p = 0.046$, z-score -2.00).

Discussion

One of the key findings in our study was the finding of a significant decrease in elastic fibrils in CCh when compared to age-matched controls. The prior data on elastic fibers in CCh has been, thus far, controversial. Hughes [1] and Hashemian [12] found no alteration in elastic fibers in CCh tissues. Ward., *et al*. [13] demonstrated increased accumulation of elastic fibers in the conjunctival stroma of CCh tissues on transmission electron microscopy as well as "marked accumulation" of elastic fibers on VVG compared to controls. In contrast, Zhang., *et al*. [10] documented decreased elastic fibers by VVG staining in CCh. Watanabe., *et al*. [11] further observed "fragmented elastin fibers" on VVG. These contradictory results stem, in part, from lack of formal methodology in quantification of elastic fiber density. In this study, we conducted a systematic image analysis-assisted quantification of elastic fiber density in CCh and control tissue. Our study results support observations of Zhang [10] and Watanabe [11]. This finding also parallels floppy eyelid syndrome where there is a significant decrease in elastic fibers in the affected tarsus [21].

With regards to collagen density, Ward., *et al*. [13] and Watanabe., *et al*. [11] showed "sparse assemblages" of collagen fibers but did not quantify their results. Our study revealed a statistically significant decrease in collagen fibers in CCh when compared to age-matched controls. Collagenolytic activity has been linked to MMP-3 and MMP-9 increases in CCh [13]. Furthermore, Ehlers-Danlos syndrome, classic type, has been associated with CCh, suggesting a link between abnormal collagen genotypes and conjunctival hyperelastosis. However, the authors of this study state that this effect may result from a secondary predisposition to elastotic degeneration due to UV light exposure on the inferior conjunctiva [22].

Watanabe., *et al*. [11] found 39 of 44 specimens to have "microscopic stromal lymphangiectasia and edema". However, one case-

control study [18] demonstrated statistically significant incidence of lymphangiectasia in their CCh as compared to their control. To our knowledge, no one has previously evaluated the density of lymphatic channels in CCh stroma. We have observed decreased collagen density and increased lymphatic density in CCh tissue, supporting the potential role of enzymatic and inflammatory factors in the pathogenesis of CCh.

There are a few studies that explore the role of inflammation in CCh but without the formal quantification of inflammatory cell density. Our software-assisted image analysis documented an almost four-fold increase in CD68 expressing macrophages, as compared to the control tissue. With regards to other inflammatory mediators, Kinoshita., *et al*. [19] found "negligible" infiltrating inflammatory cells in four patients with CCh. In their study, the specimens sporadically stained weakly positive for cell markers CD3, CD4, CD8 and HLA-DR, while all 4 specimens stained weakly positive for CD68. Watanabe., *et al*. [11] found "no evidence of inflammation". The authors also found no evidence of goblet cell hyperplasia (a marker of chronic inflammation) in any of their 44 CCh specimens. Francis., *et al*. [20] showed normal conjunctival histology in 22 of 29 (76%) CCh specimens, inflammatory changes in 4 cases (14%) and elastotic degeneration in 3 remaining cases (9.7%). The authors concluded that the results confirmed the likely multifactorial nature of CCh, but believed it was of interest that all the eyes with inflammatory changes showed signs of nasolacrimal duct obstruction, supporting the delayed tear hypothesis. This hypothesis is confirmed by the fact that higher levels of TNF-alpha are found in CCh patients. The combined data in prior studies and in our study confirms the multifactorial nature of CCh, with mechanical factors and aging likely inducing secondary inflammatory changes.

There are several limitations to our study. First, this is not a true stereologic study to sampling only small areas of each slide being evaluated. Also, since the specimens were handled at different times, fixation and processing differences may occur. Lastly, the concept of "hot spots" may lead to bias. Our study has the advantage of being the largest age-matched control cohort and the first systematic, software-assisted, objective quantification of various histopathologic and immunohistochemical parameters that help distinguish CCh from control conjunctival tissue. Decreases in elastic fibers and collagen density combined with increases in lymphatic and macrophage density appear to characterize the mechanical and inflammatory factors that distinguish CCh from normal conjunctiva.

Conflict of Interest Statement

There are no conflicts of interest to declare for any of the authors.

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