Original Article

Role of concanavalin A lectin in recognition of pterygium remnant after surgical excision: Preliminary results of a prospective study

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Background: Pterygium is one of the most common conjunctival diseases among ophthalmic pathologies. The frequency of recurrences is high, either after surgical treatment or after treatment combined with mitomycin C or beta-radiation therapy.

Aims: The purpose of this study was to determine whether concanavalin A (ConA) lectin bound to the pterygial surface can be used to detect recurrence or remnants of pterygium after surgical excision.

Materials and Methods: This was a prospective study on 20 patients with pterygium, divided in five stages, pre-surgery, early post-surgery (24h), late post-surgery (seven days), very late post-surgery (four weeks) and two months after the procedure.

A drop of fluorescein-marked Con A (35 µg/mL) was instilled in the lower conjunctival eyelid sac and the eye was exposed to the light of a Wood's lamp for an average of five seconds.

Results: Out of the 20 patients, eight patients were found to have fluorescent stretch marks over the scar corresponding to residual pterygial tissue at four weeks; two months after the procedure of re-surgery we observed no fluorescent remnants. All residual pterygia were confirmed through histochemistry studies.

Conclusion: It was possible to detect remnants of pterygium in postoperative patients and recurrences in early pre-clinical stages through the visualization of fluorescent ConA bound to the pterygial surface.

Key words: Concanavalin A lectin, pterygium

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The etiology of pterygium is not known but currently, it is proposed to be associated with several factors, like exposure to solar light, increased P53 expression and the presence of oncogenic viruses[1] such as human papilloma virus (HPV) and herpes simplex virus (HSV).[2] It has been observed through microscopy that the pterygium is covered by a highly differentiated cylindrical epithelium with tubular-glandular tissue and "goblet" cells underneath. The histopathology and ultrastructural studies reveal a conjunctival type of epithelium that covers a highly vascular structure with elastotic degeneration of the collagen, including destruction of the Bowman stratum.[3]

Concanavalin A (ConA) is a lectin extracted from *Cannavalia ensiformis* (Jack bean) with selectivity for methyl α-D-mannopyranoside and which recognizes trimannoside core-linked N-glycan, Manα1-6[Manα1-3]Man.[4] Concanavalin A has been used to study glycosylation of healthy conjunctival tissue and keratoconus.[5]

There is evidence that changes in glycosylation are based on lower Galβ-3-GlcNAc, sialyltransferase activity in the pterygium as compared to healthy conjunctiva.[6] We found that ConA binds to the pterygial surface *in vivo*, therefore we used ConA marked with fluorescein isothiocianate (FITC) to detect the remnants of pterygial human tissue *in vivo* as well as the early recurrences. The cytochemical activity can be studied using ConA.[7] The aim of the present pilot study was to know whether ConA can be used to detect recurrence of pterygium in the early postoperative period after pterygium surgery.

Materials and Methods

Concanavalin A III (Sigma, Chemical Co, USA.) was activated with minerals according to Lootiens' method.[7] First, the hemagglutination activity was standardized to a 1:256 dilution. Concanavalin A was labeled with FITC (Sigma Chemical Co, USA) according to Coligan's technique.[8] The hemagglutination activity was standardized to a final 1:64 dilution and the pH adjusted to 7.4 with hydrochloric acid. It was then sterilized by filtration through 0.2-µm Millipore membranes (Millipore Corporation, Bedford, MA, USA). The final lectin concentration

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(35 μg/mL in 0.9% NaCl) was used to instill a drop on the conjunctival surface.

Before its use on humans, ConA was tested on 10 albino rats (*Rattus sprague*). All the rats had a drop of ConA (around 1.5 μg) placed in their conjunctival sac, which completely impregnated the conjunctiva. The application was performed at different times, from 0 to 72h. Once the lectin's harmlessness was proven in the conjunctiva of albino rats, the sensitivity on the lower lip of 30 healthy human volunteers was tested.

The selected volunteers, ranging from 18 to 60 years of age, had no history of allergies or ocular pathology. They were not taking any medication. Observation criteria followed those published elsewhere, such as application of an allergen on the labial surface,[9] allergic patterns and hypersensitivity surveillance for the application of allergens on mucous epithelia,[10] immediate, intermediate and late reactions of allergic responses and local and generalized allergic reactions. After ConA application on the surface of the lower lip, the volunteers were closely watched for 72h.

This study was divided into five stages: pre-surgery; early post-surgery (24h); late post-surgery (seven days); very late post-surgery (four weeks) and two months after the procedure. Twenty patients with primary pterygia, aged 18 to 60 years, were selected from the "Dr. Aurelio Valdivieso" General Hospital from the health services of the state of Oaxaca, Mexico. These patients presented different degrees of pterygial growth and they were scheduled for surgical excision of the pterygium. The ConA was not used during the surgical procedure because we eliminated other causes of post-surgery inflammation.

One drop of FITC-labeled ConA (35 μg per mL of 0.9% NaCl) was instilled in the lower conjunctival sac. Ten minutes later, the eye was exposed to a halogen light with a cobalt blue filter from a SL-2E Slit-Lamp (Topcon, Japan), 25X and 40X; fluorescence was magnified with a Wood's lamp (John, China), which emits a 365 nm-wavelength light in the ultraviolet spectrum. Findings were documented with photographs. This was the first stage, i.e. pre-surgery treatment.

Resection of the pterygium was done under local anesthesia using the Ziegler technique.[11] Patients were carefully monitored during the postoperative period. A drop of ConA-FITC, at the mentioned concentration, was instilled 24h, a week, a month and two months after the surgery. Findings were also documented with photographs. All 20 surgical patients were exposed to FITC-marked ConA for around 10min and then the eye was tangentially exposed to a Wood's lamp during an average of six seconds. Finally, the conjunctival tissue was washed in all patients, with two drops of 0.1M α-methyl-mannopyranoside.

To confirm the interaction between pterygial epithelium and ConA, we performed an *in vitro* histochemical study. The surgically excised pterygium was preserved in 4% paraformaldehyde (ICN Biochemicals, Cleveland, USA); and, once embedded in paraffin wax, 5-μm sections were cut and affixed to glass slides by warming to 55°C for 1h, for its histopathological study according to the method described elsewhere.[12] To confirm ConA binding to the pterygial epithelium, we followed Kiernan's method.[13] Briefly, the glass slides were deparaffinized, immersed in 70% alcohol and endogenous peroxidase activity was quenched by immersing the slides in a solution of four parts methanol and one part 3% hydrogen peroxide for 30 min. Then, the slides were washed three times with 0.15 M phosphate buffered saline, pH 7.4, incubated with milk for 5 min to suppress nonspecific binding of proteins (Powdered Milk, Epitope Inc. Beaverton, USA). Sections were rinsed with Tris-buffered saline (150 mM NaCl, 10 mM Trizma base, pH 7.4). Afterwards, sufficient ConA (1 mg/ml with 0.1M $CaCl_2$) was used to completely cover the sections that were then incubated at 4°C overnight. The sections were washed with phosphate buffered saline, pH 7.4, for 5 min; incubated with 1 mg/ml peroxidase,[14] pH 10 (Sigma Chemical Co, USA). The sections were washed again with phosphate buffered saline, pH 7.4 and diaminobenzidine (Sigma FastTM DAB Peroxidase substrate Tablet Set with 0.7 mg/ml of 3,3′diaminobenzidine, 1.6 mg/ml urea hydrogen peroxide and 0.06M tris-buffer) was added for 10 min. To intensify the sensitivity of the chromogen, we added a 0.3% solution of cobalt chloride (Sigma Chemical Co, USA) in 0.1M Tris buffer, pH 7.6, for 10 min.[15] To contrast and observe the goblet cells, the slides were stained with 1% Alcian Blue (Sigma Chemical Co, USA) in 3% acetic acid, pH 2.5.[16] Results were documented with photographs. Some experiments were made with lectin incubate with manose 200 mM during 30 min before preparing glass slides as specificity controls.

This project was reviewed and approved by the "Dr. Aurelio Valdivieso" General Hospital Ethics Committee. An informed written consent was signed by all patients or their responsible relatives.

Results

Regarding the albino rat tests, neither the activated nor the fluorescent ConA produced any reaction in the immediate region. The pH of the ConA and its concentration were important to avoid possible adverse reactions in the immediate region.

Regarding ConA on the surface of the lower lip test, in humans, no incidents of regional or systemic hypersensitivity were observed in any patient at the end of the observation period.

In pterygial remnants, an intense affinity to ConA was observed with the SL-2E Slit-Lamp, 25X and 40X; the intensity was directly proportional to the degree of pterygial tissue

Table 1: Patients subjected to pterygium excision by the Ziegler technique. The proportion of patients yielding positive test with ConA-FITC

Pterygium	Number of patients before surgery	Number of patients after surgery			Proportion of patients at two months
		One week	Four week	Two months	
Positive tests with ConA-FITC	20	5	3	0	0
Negative test with ConA- FITC	0	15	17	20	20

FITC = Fluorescein isothiocianate

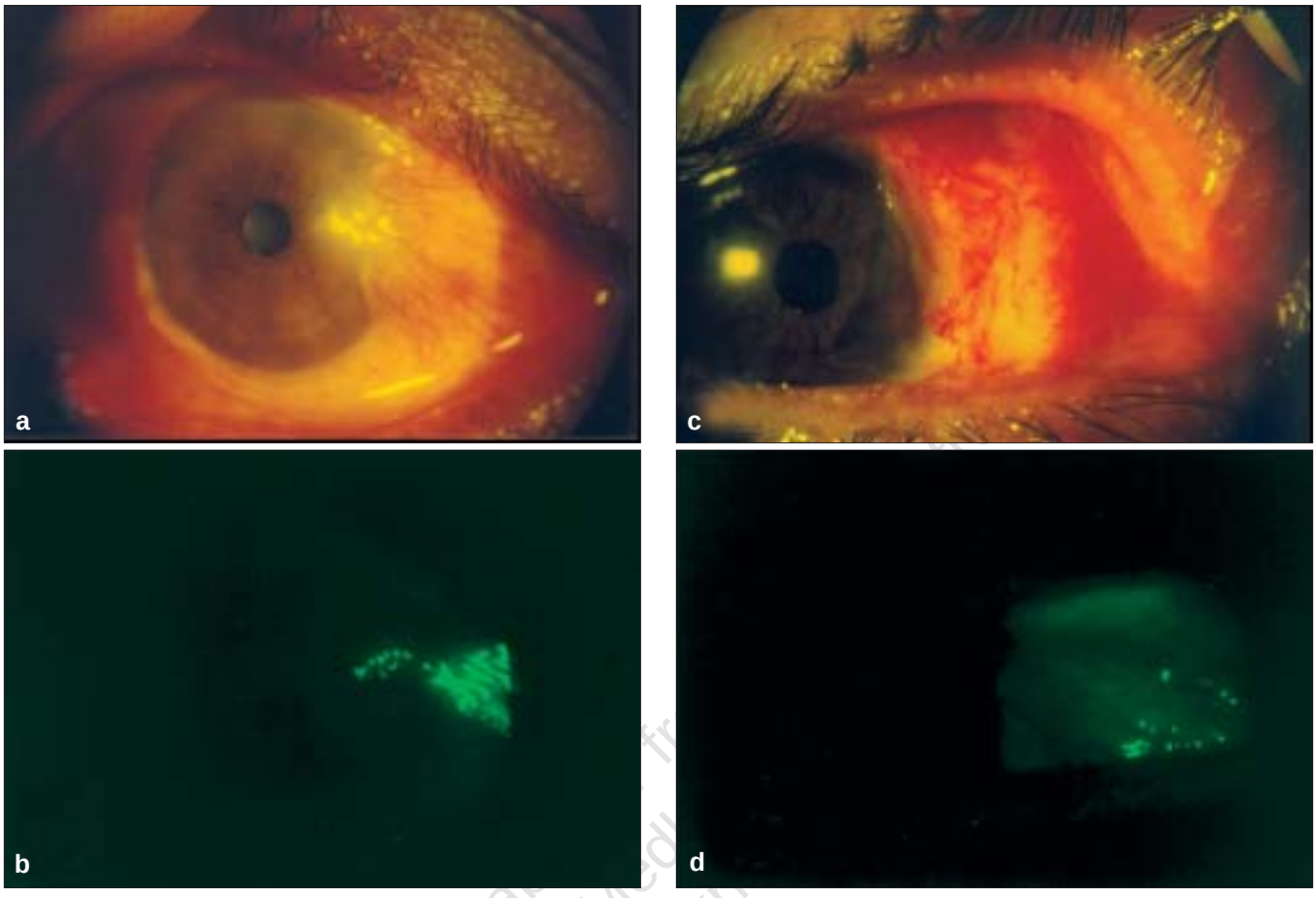

Figure 1: Photographs, 25x, using a slit-lamp camera; one of the five patients who showed pterygial remnants a week after the operation. a) Patient before pterygium resection; b) The same patient after using ConA-FITC; c) The same patient a week after pterygium resection; d) The same patient a week after pterygium resection using ConA-FITC.

growth, *i.e.* cases with a severe and invasive pterygium showed a very intense affinity to the lectin. The fluorescence was just as intense on the surface as on the extension of the affected conjunctiva; conversely, cases with an incipient pterygial growth showed a lesser fluorescence and extension.

Exploration with the slit-lamp, 24h after surgery, yielded negative results in all patients. One week after the surgical treatment, five patients were observed having small, fluorescent stretch marks over the scar corresponding to residual pterygial tissue. Four weeks after surgical treatment, three patients, amongst the five patients noted at one week, showed the presence of few fluorescent stretch marks in the resected pterygial area [Table 1]. After removal of recurrent tissue, it was subjected to histopathological analysis and it was confirmed that these stretch marks correspond to pterygial tissue. These stretch marks clinically and histologically corresponded to pterygial recurrence. When we detected pterygial remnants in a patient, the remnants were surgically removed following observation of fluorescent staining.

Two months after the first surgery we observed no fluorescent remnants. Fig. 1 depicts the fluorescent zone that corresponds to the pterygial surface. The FITC-labeled lectin image was revealed with a slit-lamp at an amplification of 25X.

The histochemical technique revealed epithelium alone in 16/20 pterygial tissue and goblet cells in 8/20 pterygial tissue. After one and four weeks of the first surgery epithelium in the five samples was observed and the three re-surgery tissue samples also had epithelium. The epithelium was found bound to ConA in all tissues. Fig. 2 shows the *in vitro* interaction, revealing the affinity of this lectin towards the epithelium of

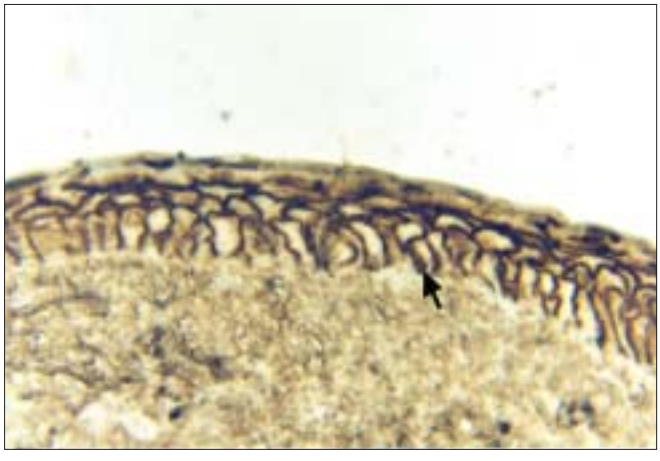

Figure 2: Photomicrograph of a pterygial tissue section, 500x. The complete depth of the columnar epithelium is observed with a darker shade, corresponding to ConA binding (in black)

pterygial tissue (photomicrograph with 500x amplifications) and the resected pterygial tissue.

In all the resected tissues, the conjunctival mucous membrane, characterized by columnar epithelium and layers of normal basal cells, was observed. The fibro-vascular, sub-epithelial tissue showed wide, irregular hyalinization with degeneration of basophiles. Infiltration of lymphocytes was observed in most of the resected tissues. In some tissues, goblet cells were also observed.

Discussion

As mentioned earlier, the frequency of recurrences after surgical treatment of the pterygium is high despite the techniques used after the surgical excision such as β-therapy with Sr-90 or topical application of mitomycin C in low doses (even though the latter produces multiple secondary risks). In this preliminary study the proportion of recurrences was 8/20, similar to other reports.[17] The pterygium continues to be a treatment problem for the general physician and the ophthalmologist. The opportune detection of recurrences or remnants in postoperative patients in early preclinical stages offers the possibility of re-intervening in time to prevent greater, additional discomforts. Concanavalin A -FITC lectin has proven to be a valuable tool because of its affinity for the pterygial surface, which allows detecting even small areas of pterygial tissue. Furthermore, this method offers the possibility of doing an easy, fast and macroscopical analysis in the ophthalmology office. Pterygial remnants or relapses could be detected at an early time and eventually treated. This is an advantage which offers the possibility of reliable monitoring, cuts down on evolution times and improves the prognosis of these patients.

On the other hand, the changes in glycosylation of the conjunctival tissue are noteworthy. The healthy conjunctiva does not express oligosaccharides rich in mannoses, as inferred from the type of lectins that can bind to it. However, the pterygium is rich in mannosidic structures, which allow ConA binding to this tissue, as observed in this work. These findings suggest the possibility of exploring changes in glycosylation pattern expression that are differentiated from other kind of conjunctival pathologies. In particular, in pterygium we studied mannosidic residues that could be implicated in pterygial proliferation, in order to avoid observing reactivity to this lectin in healthy conjunctiva.

With respect to ConA toxicity, several reports are available,[18] as for example the level of ConA toxicity in rat embryos exposed to ConA at 12.5 to 100 μg/ml, which inhibited the early migration of neural crest cells;[19] however, there are some reports that refer to the positive aspects of ConA, for example in postnatal rat cochlear explant cultures, ConA protects hair cells from aminoglycoside ototoxic damage.[20] The possibilities of using ConA therapeutically are limited by its unusual toxicity, especially at the supraoptimal dose level. The optimal ConA dose with respect to blastogenic response is about 30 μg/ml,[21] whereas the optimal response to induce synthesis of small amounts of Ig is around 6 μg/ml.[22] We used very small ConA doses, around 1.75 μg by drop, to prevent any reaction in the eye.

On the other hand, upon modifying the pH of the lectin, we observed that the lectin preparation instilled in rats and on human lips produced no reaction, even though the dose was repeated throughout a period longer than 72h. In spite of this, we used N-acetyl-D-mannopyranoside, the specific sugar, to unbind the ConA from the epithelial surface.

With regard to exposing the surface of the eyeball to the ultraviolet radiation from Wood's lamp, even though the emitted light is type-A ionizing radiation, according to its wavelength spectrum, it had no harmful effects on the superficial structures, such as the conjunctiva of the cornea. Besides, exposure to the ocular surface lasted only very short periods of time (five to seven seconds).

We consider that the use of the ConA assay to recognize pterygial tissue in the postoperative eye or to detect early recurrences, although easy for ophthalmologists to obtain and use, needs more tests to establish the sensitivity, specificity, positive predictive value and negative predictive value of this assay.

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