Paclitaxel Associated With Lipid Nanoparticles as a New Antiscarring Agent in Experimental Glaucoma Surgery

Marcelo L. Occhiutto,[1] Fatima R. Freitas,[2] Patrícia P. Lima,[3] Raul C. Maranhão,[2,4] and Vital P. Costa[1]

[1]Department of Ophthalmology, University of Campinas, Campinas, Brazil
[2]Heart Institute, Medical School Hospital, University of São Paulo, São Paulo, Brazil
[3]Division of Anatomic Pathology, Medical School Hospital, University of São Paulo, São Paulo, Brazil
[4]Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

Correspondence: Vital P. Costa, Departamento de Oftalmologia e Otorrinolaringologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas. Rua Tessália Vieira de Camargo, 126 Cidade Universitária, 13083887 Campinas, SP – Brasil;
vp.costa@uol.com.br

Submitted: November 18, 2015
Accepted: January 20, 2016

Citation: Occhiutto ML, Freitas FR, Lima PP, Maranhão RC, Costa VP. Paclitaxel associated with lipid nanoparticles as a new antiscarring agent in experimental glaucoma surgery. *Invest Ophthalmol Vis Sci.* 2016;57:971–978. DOI:10.1167/iovs.15-18671

Purpose. To investigate the effects of paclitaxel associated with lipid nanoemulsions (LDE-PTX) on postoperative scarring in rabbits undergoing trabeculectomy.

Methods. Thirty-four rabbits that underwent trabeculectomy were allocated to four groups: LDE-PTX/SC ($n = 9$), treated with LDE-PTX (1.5 mg, intraoperative subconjunctival injection); LDE-PTX/IV ($n = 9$), treated with LDE-PTX (4 mg/kg per day intravenously) at the end of the surgery and once per week for 3 weeks; MMC ($n = 9$), treated with intraoperative 0.4 mg/mL mitomycin-C for 3 minutes; and control group (CTL, $n = 7$), without treatment. Bleb characteristics and IOP were evaluated over 4 weeks. Animals were killed on day 28. Histologic analyses were performed to assess the amount of scarring and toxicity to the conjunctiva and ciliary body.

Results. Groups were similar with respect to IOP and anterior chamber depth during the 28-day observation period. The LDE-PTX/SC, LDE-PTX/IV, and MMC groups showed greater bleb height than CTL on days 14 and 21 ($P < 0.001$). The LDE-PTX/SC, LDE-PTX/IV, and MMC groups showed longer bleb survival time than CTL ($P < 0.001$). The LDE-PTX/SC, LDE-PTX/IV, and MMC groups were equally effective in reducing fibrosis ($P < 0.001$), number of blood vessels ($P < 0.001$), and chronic inflammatory cells ($P < 0.01$) at the surgical site. However, LDE-PTX/SC and LDE-PTX/IV treatments had lower conjunctival ($P < 0.001$) and ciliary body toxicity ($P < 0.01$), compared with MMC.

Conclusions. The LDE-PTX/SC was effective in reducing the scarring process following trabeculectomy to the same extent as MMC, but with considerably less toxicity to the conjunctiva and ciliary body. The LDE-PTX/IV was somewhat less effective than LDE-PTX/SC or MMC, but could have potential as a postoperative adjuvant treatment. Therefore, the LDE-PTX preparation in both administration routes may offer promising options for wound-healing modulation in the surgical treatment of glaucoma.

Keywords: trabeculectomy, fibrosis prevention and control, wound-healing drug effects, drug carriers, nanoemulsion therapeutic use, solid lipid particles

Trabeculectomy was introduced by Cairns[1] in 1968 and is still the gold standard surgical treatment of glaucoma. The long-term success of this intervention is, however, limited by the closure of the sclerostomy resulting from excessive subconjunctival scarring,[2] which is considered the major cause of trabeculectomy failure. Fibroblastic proliferation, scarring of the trabeculectomy flap, subconjunctival fibrosis, and remodeling of collagen with contraction of tissue in and around the bleb site are the usual postoperative histopathological features that ultimately lead to the sclerostomy closure.[3,4] Subconjunctival antifibrotics, such as mitomycin-C (MMC) and 5-fluorouracil (5-FU), have been used aiming to reduce the formation of scar tissue, by inhibiting fibroblast proliferation, and interfering with many steps of the healing cascade.[2,5–7] Mitomycin-C is more effective compared with 5-FU,[2] and it is currently used in more than 85% of the trabeculectomies.[8] However, severe adverse effects and complications, such as bleb leaks, hypotony and infection may follow the use of MMC.[2,9,10] Thus, the development of alternative therapeutic strategies based on more effective and safer properties are mandatory to warrant a better prognosis after trabeculectomy.

In this regard, paclitaxel (PTX), an anticancer agent belonging to the taxane class, has been shown to enhance the success of trabeculectomy[11–14] due to its capacity of inhibiting the fibroblast migration and scar contraction,[15] similar to MMC.[14,16] However, PTX therapeutic efficacy is limited by the pharmacological instability induced by its physicochemical properties, such as hydrophobicity.[13,14] Lack of a satisfactory carrier for paclitaxel solubilization is a major drawback for the use of this agent as an adjuvant therapy for trabeculectomy. Some carriers, such as polyanhydride disks[12] and Carbopol 980 hydrogel,[16] have been attempted to achieve greater stability of PTX for subconjunctival use. However, none of those PTX formulations was adequate to introduce PTX as a valid alternative as antifibrotic agent. Moreover, the carrier used in commercial PTX formulation for intravenous cancer treat-

ment, Cremophor EL, bears intolerable side effects for ophthalmological use, such as hypersensitive reactions.[17]

As previously reported, after intravenous injection, nonprotein lipid nanoparticles termed LDE, that resemble the lipid structure of low-density lipoprotein (LDL), the lipoprotein that contains most of cholesterol in the plasma, can bind to the LDL receptors and concentrate associated drugs in malignant tumors[18–20] or in inflamed tissues as in atherosclerosis,[21,22] rheumatic disease,[23] or grafted organs.[24] This has been shown not only in animal models of disease,[25,26] but also in patients with either solid[18,19] or hematologic tumors.[20,27] In both experimental studies and clinical trials enrolling patients with cancer, PTX and other antineoplastic agents had their toxicity drastically decreased by the association to LDE, without affecting their pharmacological action.[19,20,25–27]

Overexpression of LDL receptors is the mechanism that allows the concentration of PTX associated to LDE in the targeted tissues.[19] The need for cholesterol and other lipids for building new membranes during accelerated mitosis rates determines the receptor overexpression in neoplastic and inflammatory tissues.[19,22,23] In fact, LDL receptors are also overexpressed in the activated human tenon fibroblasts,[28] which justifies the use of LDE as a PTX carrier for subconjunctival antiscarring therapy.

This study was designed to test the efficacy and safety of LDE-PTX as an antiscarring agent in rabbits undergoing trabeculectomy. We hypothesized that intraoperative LDE-PTX can be as efficacious, but less toxic, than MMC. Moreover, because systemic LDE-PTX has been shown to be safe for human cancer chemotherapy, we also decided to investigate the intravenous use of LDE-PTX as a possible novel approach in filtration surgery.

Methods

Animals

Thirty-four male New Zealand White rabbits (provided by USP Medical School Animal Care Unit, São Paulo, Brazil) weighing 3.5 ± 0.4 kg were housed in individual cages in a temperature-controlled room on a 12-hour light/dark cycle; animals were fed ad libitum during the experimental period. All animals underwent trabeculectomy, performed under anesthesia with intramuscular injection of ketamine (50 mg/kg) and xylazine (23 mg/kg). Before the surgical procedure, topical anesthetic eye drop proxymetacaine HCl 0.5% (Anestalcon; Alcon, São Paulo, Brazil) was applied. The rabbits were allocated to four groups, according to the antiscarring treatment:

1. MMC (n = 9): treated with MMC 0.4 mg/mL[29–32] administered with a sponge (Merocel PVA, Beaver-Visitec, Waltham, MA, USA), placed between the scleral flap and the conjunctiva-Tenon layer. After 3 minutes, the sponge was removed and the site was irrigated thoroughly with balanced saline solution.
2. LDE-PTX/SC (n = 9): treated with a single subconjunctival injection of LDE-PTX (1.5 mg in 300 μL volume) at the upper cul de sac, after the conjunctival suture. The choice of the 1.5-mg dose was determined by the volume (300 μL) that could regularly be injected into the superior subconjunctival space, without a noticeable leakage out of the conjunctival hole.[33,34]
3. LDE-PTX/IV (n = 9): treated with intravenous injection of LDE-PTX at the 4 mg/kg PTX, used in our previous studies,[22] at the end of the surgery and three subsequent weekly injections.
4. CTL (n = 7): control group, not treated.

The study was carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Medical Experimental Research Ethics Committee of the School of Medicine of the University of São Paulo (no. 379/13).

Trabeculectomy Procedure and Treatment Regimen

The surgical procedure was performed in all animals by a single surgeon (MLO). A superior 8-0 silk corneal traction suture was placed to allow for infraduction of the globe. After a superior limbal peritomy, a partial-thickness scleral flap (3.0 × 3.5 mm) was created, starting 2.5 mm behind the limbus and continuing until the blade was just visible in the anterior corneal stroma. The anterior chamber was entered using a 45-degree blade. A fragment of tissue containing the inner sclera, trabeculum, and peripheral cornea measuring approximately 2 × 2 mm was excised. A peripheral iridectomy was then performed and the anterior chamber was filled with balanced salt solution. The scleral flap and the conjunctiva were closed with 10-0 nylon sutures. Topical antibiotic with steroid drops and ointment (ciprofloxacin + dexamethasone) were used, once a day, until animals were killed.

After 4 weeks, animals underwent euthanasia by overdose sodium pentobarbital intravenous injection. Right eyes were then enucleated to prepare for histopathologic examination by conventional optical microscopy.

Clinical Evaluation

On the day of the surgery and on days 1, 3, 7, 14, 21, and 28 after the surgery, all animals were examined to measure IOP and evaluate the bleb characteristics. The IOP was measured at approximately 9 AM with an applanation tonometer (Tono-Pen Avia; Reichert, Inc., Depew, NY, USA), under topical anesthesia with proxymetacaine HCl 0.5%. The average of five readings was used for the analysis.

A masked observer classified the anterior chamber depth and bleb height. The anterior chamber depth was graded as deep, shallow, or flat; bleb height was graded as high, elevated, shallow, or flat.[35] Bleb survival was taken as the primary efficacy endpoint. Bleb failure was defined when a flat, scarred, vascularized bleb was observed in association with a deep anterior chamber.

Preparation of LDE With Associated PTX Oleate

The preparation of LDE-PTX oleate was made by high-pressure homogenization from a pre-emulsion obtained by ultrasonic irradiation until complete drug dissolution, according to the methods described previously.[22,25] After the homogenization cycles, the formed nanoemulsion was centrifuged and the nanoemulsion sterilized by passage through a 0.22-μm pore polycarbonate filter (Millipore, Billerica, MA, USA) and kept at 4°C until it was used. The final concentration of PTX oleate incorporated to LDE was confirmed by HPLC (Shimadzu, Columbia, MD, USA) and, as determined by this approach, the yield of association of PTX oleate to LDE was more than 95% and was not significantly changed by the passage through the 0.22-μm pore filter. As measured by laser scattering technique (Zetasizer Nano ZS90; Malvern, Worcester, UK), the average nanoemulsion particle diameter was 64 nm, with a polydispersity index of 0.162.

Histopathologic Study

Eyes were fixed in formalin for 24 hours and embedded in paraffin; 5-μm sections of the surgical sites were stained with

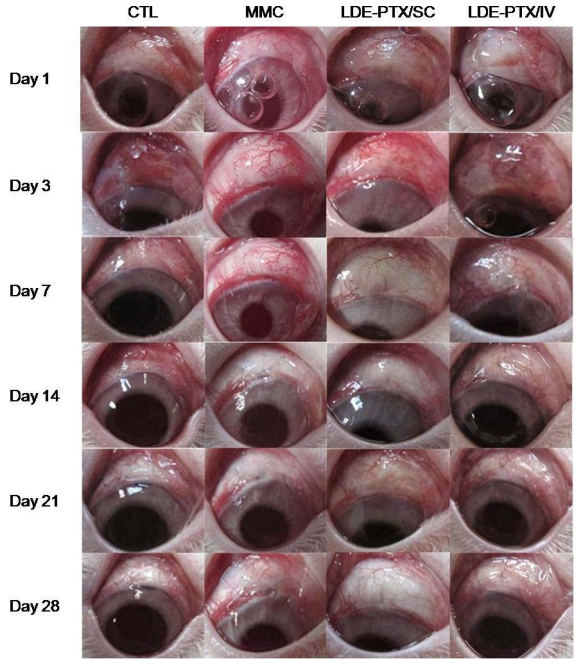

FIGURE 1. External appearance of filtering blebs of eyes treated with LDE-PTX or MMC after trabeculectomy. At the first week, all the groups exhibited elevated, diffuse, fleshy-looking blebs. Over time, these blebs became more focal (less diffuse) in appearance (outline), until their failure (flat, scarred, and vascularized bleb), earlier (in day 14) in the CTL group. The animals shown are representative of the time course of healing for all groups.

hematoxylin-eosin and with Masson Trichrome. Histological evaluations were conducted by an experienced pathologist, who was masked for the groups.

For assessment of scar formation at the subconjunctival space, the following parameters were evaluated: fibroblast count, fibrosis, collagen organization, collagen density, stromal edema, density of blood vessels, and number of mononuclear cells.

Fibroblasts were graded according to Butler et al.[36] as follows: *absent*, without fibroblasts in the tissue; *low count,* when involving less than 50% of tissue; *moderate count*, when involving between 50% and 80% of tissue; and *high count*, when involving $\geq$80% of the tissue. Stromal edema was scored according to the same scale[36] as follows: *absent*, without edema in the tissue; *mild*, when involving less than 50% of tissue; *moderate*, when involving between 50% and 80% of tissue; and *intense*, when involving $\geq$80% of the tissue. The fibrosis grading scale, according to Koz et al.,[16] was as follows: *negative*, if less than 10% of the field was constituted by fibrous tissue; *mild*, if between 10% and 25%; *moderate*, if between 26% and 75%; and *severe*, if more than 75%.

To evaluate collagen organization, the scoring system described by Ekinci et al.[37] was used: *no stromal disorganization*, with parallel mature collagen fibers; *weak stromal disorganization*, with long and discrete collagen fibers; *moderate stromal disorganization*, with shortened and focal perpendicular sequence of collagen fibers; and *strong stromal disorganization*, with collagen fibers shortened and ponting in all directions. Subconjunctival collagen density was graded according to Perkins et al.[38]: *no alteration*, with no stain; *minimal alterations*, with light blue stain; *mild alterations*, with mild blue stain; *moderate alterations*, with fairly blue stain; and *severe alterations*, with densely blue stain.

Density of blood vessels in the subconjunctival tissue was graded according to Okuda et al.,[39] with slight modifications: *no blood vessel* per image; *1 to 5 blood vessels* per image, and *6 to 20 blood vessels* per image. Mononuclear cells were counted at 10 high-power fields, and the average number of cells in these zones was analyzed.

For the assessment of conjunctival toxicity, we used the grading system described by Polak et al.,[40] slightly modified by Ekinci et al.[37]: *no histologic alteration*, normal conjunctival tissue and no inflammation; *minimal histologic alterations*, demonstrating thickening of the conjunctiva but preservation of the conjunctival epithelium; *mild histologic alterations*, demonstrating thickening of the conjunctiva, conjunctival epithelium preserved, and mild inflammatory cell infiltration; *moderate histologic alterations*, demonstrating thickening of the conjunctiva, conjunctival epithelium preserved, mild inflammatory cell infiltration, and loss of collagen fibril organization; *severe histologic alterations*, with loss of conjunctival epithelium, total disorganization, and necrosis of the underlying scleral stroma. Goblet cells were counted at 10 high-power fields, and the average number of these cells was analyzed.

For the assessment of ciliary body toxicity, we evaluated three parameters based on the grading system published by Ekinci et al.[37]: ciliary epithelium height, vascular congestion (distension of vascular lumens by erythrocytes), and edema (distension of the stromal collagen fibers) in the ciliary body. Alterations in ciliary epithelium height were scored as *minimal decrease*, *mild decrease*, *moderate decrease*, and *severe decrease*; whereas the intensity of edema and congestion were scored as *absent*, *minimal*, *mild*, *moderate*, and *severe*.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 statistical software (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA). Survival analysis of the surgery was performed using the Kaplan-Meier curve and the log rank test. The quantitative parameters were analyzed using the Kruskal-Wallis test, followed by Bonferroni correction for multiple analyses. All the semiquantitative parameters were analyzed using the Kruskal-Wallis test, followed by the test for multiple comparisons of Dunn. For all the analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Findings

No ocular complications, such as conjunctival redness, chemosis, or discharge, were observed. Body weight and hematologic profiles were not different among the treatment groups and between the day when surgery was performed and the euthanasia day (data not shown).

Bleb Survival

In all animals, trabeculectomies were successful in achieving filtration, leading to flattening of the anterior chamber and high-filtering blebs, as observed on the first day after the procedure. Flattening of the anterior chamber was maintained during the first 3 days in all groups, with deepening in gradation over time, until day 28, when the anterior chamber was deep in all groups.

In respect to the bleb height, as shown in Figure 1, all study groups showed high blebs 1 day after the surgery. During the first week, blebs were classified as elevated in all groups. By

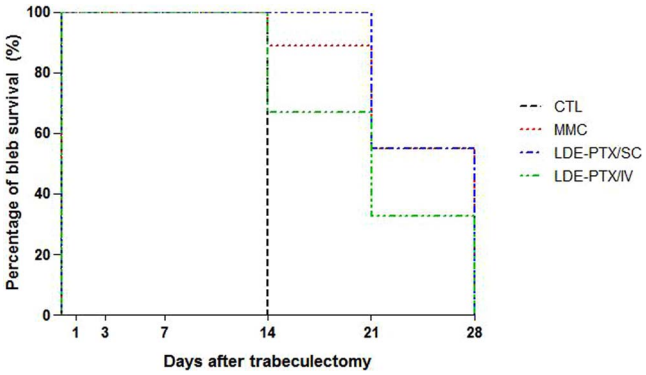

FIGURE 2. Kaplan-Meier bleb survival plot of eyes treated with LDE-PTX or MMC after trabeculectomy. Filtering blebs in the CTL group almost failed within 2 weeks. Treatment with LDE-PTX/SC and LDE-PTX/IV markedly increased the bleb survival period. In the MMC group, the bleb survived nearly 4 weeks as well in LDE-PTX/SC group. A Kaplan-Meier analysis showed a significant difference in the survival distributions among the four groups (log rank = 20.6; $P < 0.001$).

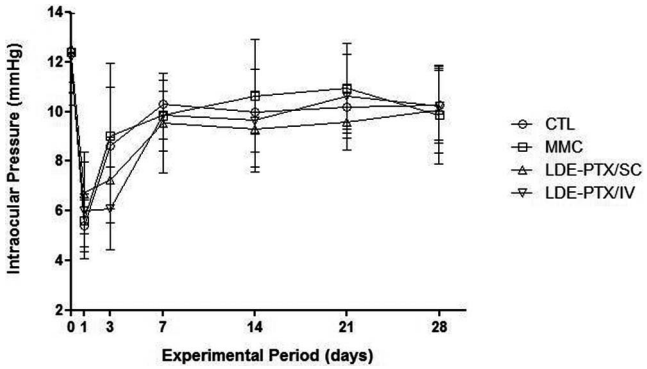

FIGURE 3. Intraocular pressure changes over time of eyes treated with LDE-PTX or MMC after trabeculectomy. Postoperative IOP was significantly lower than baseline in all groups ($P < 0.001$). The gradual increase of IOP during the study period was not significantly different among all groups.

day 14, LDE-PTX/IV and CTL groups showed shallow or flat scores in most animals, although LDE-PTX/IV showed significantly better scores than CTL ($P < 0.01$). In contrast, LDE-PTX/SC and MMC eyes still showed high and elevated scores on day 14, but this condition subsided by day 21, when most animals of these groups showed flat or shallow blebs, although still significantly better than CTL ($P < 0.01$). By day 28, most animals of all three treated groups and CTL had equally attained flat blebs.

Survival curves of the blebs are shown in Figure 2. The survival times of all treatment groups were significantly longer compared with CTL ($P < 0.001$). The median survival times were 14, 21, 28, and 28 days for the CTL, LDE-PTX/IV, LDE-PTX/SC, and MMC groups, respectively. There were no significant differences in survival times among the three treatment groups.

Intraocular Pressure

Figure 3 illustrates the IOP variation after trabeculectomy in all groups. The IOP significantly decreased on the first day after the surgery ($P < 0.001$), and gradually increased over time in all groups. There were no significant differences regarding IOP control among the four groups over the entire study period.

Histopathologic Features

Histologic analysis of the eyes, performed at the center of the sclerostomy site, as indicated by the location of iridectomy, showed several significant differences among the study groups (Table 1). The three treated groups showed lower number of fibroblasts, involving less than 50% of the tissue, compared with the CTL group ($P < 0.001$ versus MMC, $P < 0.01$ versus LDE-PTX/SC and LDE-PTX/IV). Similarly, the treated groups showed mild fibrosis, whereas the CTL group showed moderate or severe fibrosis ($P < 0.001$ versus MMC and LDE-PTX/SC; $P < 0.01$ versus LDE-PTX/IV). There were no differences in these parameters among the LDE-PTX/SC, LDE-PTX/IV, and MMC groups (Table 1).

Compared with CTL, in the three treated groups collagen fibers were more disorganized (LDE-PTX/SC, $P < 0.01$; MMC, $P < 0.001$) or tended to be more disorganized (LDE-PTX/IV, $P = 0.051$), which implies reduced fibrosis. The LDE-PTX/SC and MMC collagen fibers were equally disorganized, but LDE-PTX/IV showed less fiber disorganization than the MMC group ($P < 0.05$) (Table 1; Fig. 4).

As shown in Table 1, LDE-PTX/SC, LDE-PTX/IV, and MMC showed minimal or mild changes in collagen density, compared with CTL, where severe alterations occurred ($P < 0.01$, $P < 0.05$, $P < 0.001$, respectively). In the LDE-PTX/SC and MMC groups, the collagen density was similar, but LDE-PTX/IV showed greater collagen density than MMC ($P < 0.05$). Figure 4 shows the presence of immature fibrosis in the treated groups, in contrast to well-organized and dense fibrosis that appears in the CTL group.

As shown in Table 1, the density of blood vessels in the subconjunctival tissue was significantly lower in the treated groups than in the CTL group ($P < 0.001$ versus LDE-PTX/SC and MMC, $P < 0.01$ versus LDE-PTX/IV). Furthermore, there was more stromal edema in the subconjunctival space of treated eyes than in the CTL group ($P < 0.01$ versus LDE-PTX/SC and LDE-PTX/IV and $P < 0.05$ versus MMC) (Table 1). Moreover, the number of mononuclear cells was diminished by 60% in the treated groups, compared with CTL ($P < 0.01$ versus MMC and LDE-PTX/IV; $P < 0.001$ versus LDE-PTX/SC) (Table 1). This latter finding suggests that the chronic inflammation that follows trabeculectomy was attenuated by all the three treatments.

Regarding conjunctival toxicity (Table 2; Fig. 5), the treatment with either LDE-PTX/SC or LDE-PTX/IV did no harm to the conjunctival tissue, which remained similar to CTL. In contrast, MMC treatment elicited moderate to severe histologic alterations in the conjunctival structure, such as loss of conjunctival epithelium, loss of collagen fibril organization, and increased inflammatory cell infiltration, as compared with CTL ($P < 0.01$). The MMC treatment resulted in a pronounced, 8-fold reduction in the number of goblet cells ($P < 0.001$). Goblet cells were also reduced in the LDE-PTX/SC group compared with CTL ($P < 0.01$).

Figure 5 and Table 3 show the analysis of ciliary body toxicity. The MMC treatment produced a pronounced decrease in the epithelium height, a change in the cellular shape, from cubical to columnar, with increased congestion and edema ($P < 0.01$ versus CTL). In contrast, the ciliary body was not changed by either LDE-PTX/SC or LDE-PTX/IV treatments, as compared with the CTL group.

DISCUSSION

Rabbit trabeculectomy is the chief experimental model for this procedure in the literature.[41] Interestingly, the wound-healing

Table 1. Histopathologic Alterations in the Subconjunctival Tissue of Eyes Treated With LDE-PTX or MMC After Trabeculectomy

	CTL	MMC	LDE-PTX/SC	LDE-PTX/IV	*P*
Fibroblasts		***	**	**	<0.001
Absent	0 (0)	0 (0)	0 (0)	0 (0)	
Low count	0 (0)	9 (100)	8 (88.9)	7 (77.8)	
Moderate count	4 (57.1)	0 (0)	1 (11.1)	2 (22.2)	
High count	3 (42.9)	0 (0)	0 (0)	0 (0)	
Fibrosis		***	***	**	<0.001
Negative	0 (0)	2 (22.2)	0 (0)	0 (0)	
Mild	0 (0)	6 (66.7)	9 (100)	6 (66.7)	
Moderate	3 (42.9)	1 (11.1)	0 (0)	3 (33.3)	
Severe	4 (57.1)	0 (0)	0 (0)	0 (0)	
Collagen fiber organization		***	**	†	<0.001
No stromal disorganization	5 (71.4)	0 (0)	0 (0)	0 (0)	
Weak stromal disorganization	1 (14.3)	0 (0)	0 (0)	2 (22.2)	
Moderate stromal disorganization	1 (14.3)	4 (44.4)	6 (66.7)	7 (77.8)	
Strong stromal disorganization	0 (0)	5 (55.6)	3 (33.3)	0 (0)	
Collagen density		***	**	* †	<0.001
No alteration	0 (0)	0 (0)	0 (0)	0 (0)	
Minimal alterations	0 (0)	9 (100)	5 (55.6)	3 (33.3)	
Mild alterations	0 (0)	0 (0)	4 (44.4)	4 (44.4)	
Moderate alterations	2 (28.6)	0 (0)	0 (0)	2 (22.2)	
Severe alterations	5 (71.4)	0 (0)	0 (0)	0 (0)	
Density of blood vessels		***	***	**	<0.001
No blood vessels	0 (0)	0 (0)	0 (0)	0 (0)	
1–5 blood vessels	0 (0)	9 (100)	9 (100)	8 (88.9)	
6–20 blood vessels	7 (100)	0 (0)	0 (0)	1 (11.1)	
Stromal edema		*	**	**	0.001
Absent	5 (71.4)	1 (11.1)	0 (0)	1 (11.1)	
Mild	2 (28.6)	7 (77.8)	7 (77.8)	5 (55.6)	
Moderate	0 (0)	0 (0)	2 (22.2)	2 (22.2)	
Intense	0 (0)	1 (11.1)	0 (0)	1 (11.1)	
Number of mononuclear cells		**	***	**	0.001
	9.8 ± 1.8	4.0 ± 1.4	3.7 ± 1.9	4.3 ± 1.2	

Values are expressed as *n* (%) or mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus CTL; † $P < 0.05$ versus MMC, by Kruskal-Wallis followed by Dunn posttest or followed by Bonferroni posttest.

response at the surgical site in the rabbit is more aggressive than in human subjects.[42,43] In some studies, devices such as needle fragments connecting the anterior chamber to the subconjunctival space are used, aiming to extend the patency

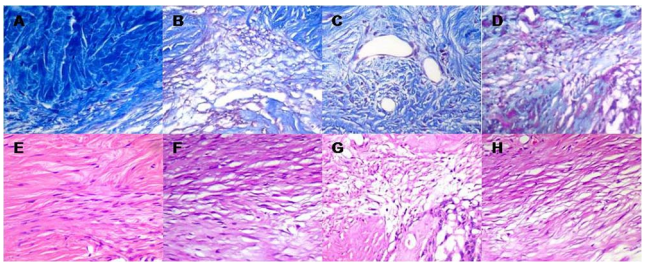

Figure 4. Collagen organization and collagen density (**A–D**) and stromal edema (**E–H**) in the subconjunctival space in CTL (**A**, **E**), MMC (**B**, **F**), LDE-PTX/SC (**C**, **G**), and LDE-PTX/IV (**D**, **H**) groups. (**A–D**) Note the *lighter blue staining* indicating the disorganized neoformed collagen fibers and less density of the tissue in the three treatment groups (**B**, **C**, **D**). (**E–H**) Note the cystic spaces throughout the subconjunctival space, demonstrating filtration through the loose collagen fibers in the all treated groups (**F**, **G**, **H**), compared with CTL (**E**), which shows minimal edema, due to more compact collagen fibers. (**A–D**) Hematoxylin-eosin stain. (**E–H**), Masson Trichrome stain. *Magnification*: ×40.

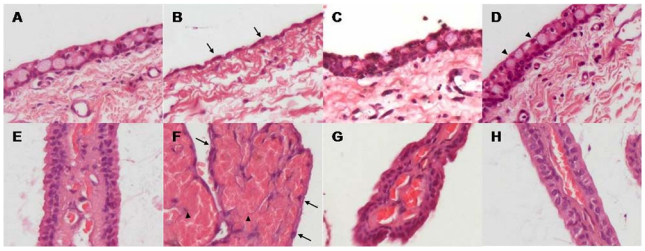

Figure 5. Conjunctival (**A–D**) and ciliary body (**E–H**) toxicity in CTL (**A**, **E**), MMC (**B**, **F**), LDE-PTX/SC (**C**, **G**), and LDE-PTX/IV (**D**, **H**) groups. (**A–D**) Note the conjunctival epithelium preserved, with minimal thickening of conjunctiva, and normal range count of healthy goblet cells in the LDE-PTX/SC (**C**), LDE-PTX/IV (**D**), and CTL (**A**) (*arrowhead*) groups. The MMC group (**B**) showed loss of goblet cells and moderate to severe alterations to the conjunctiva structure, such as loss of conjunctival epithelium, collagen fibril disorganization, and thinning of the underlying substantia propria (*black arrows*). (**E–H**) Note the normal ciliary body appearance, with preservation of ciliary body epithelium height and minimal congestion and edema in the LDE-PTX/SC (**G**), LDE-PX/IV (**H**), and CTL (**E**) groups, in contrast to the decrease of the epithelial height and disruption of the bilayered ciliary epithelium cells (*black arrows*), associated with severe congestion and edema (*arrowhead*) in the MMC (**F**) group. (**A–H**) Hematoxylin-eosin stain. *Magnification*: ×40.

Table 2. Toxic Conjunctival Changes at the Filtration Site of Eyes Treated With LDE-PTX or MMC After Trabeculectomy

	CTL	MMC	LDE-PTX/SC	LDE-PTX/IV	*P*
Conjunctival toxicity		**	†††	†††	<0.001
No alteration	0 (0)	0 (0)	0 (0)	0 (0)	
Minimal alterations	3 (42.9)	0 (0)	7 (77.8)	8 (88.9)	
Mild alterations	4 (57.1)	0 (0)	2 (22.2)	1 (11.1)	
Moderate alterations	0 (0)	8 (88.9)	0 (0)	0 (0)	
Severe alterations	0 (0)	1 (11.1)	0 (0)	0 (0)	
Number of goblet cells		***	** ††	†††	<0.001
	20.9 ± 9.7	2.5 ± 1.6	11.6 ± 3.4	16.4 ± 3.4	

Values are expressed as *n* (%) or mean ± SD. ** $P < 0.01$, *** $P < 0.001$ versus CTL; †† $P < 0.01$, ††† $P < 0.001$ versus MMC, by Kruskal-Wallis followed by Dunn posttest or followed by Bonferroni posttest.

of the surgically created fistula.[44] However, this experimental technique creates an artificial fistula, with its own particular scarring process.

This study showed that LDE-PTX/SC was as effective as MMC in preventing scarring after trabeculectomy. This statement is supported by bleb survival data and, most importantly, by the histological findings. Both LDE-PTX/SC and MMC were equally effective in reducing the fibrosis and chronic inflammatory cell counts (Table 1). Compared with the control group, microscopic appearance of the surgical site was fairly different in the animals treated with either LDE-PTX/SC or MMC. The changes were consistent with a clear antifibrotic pharmacological action of both preparations, with less-dense and disorganized collagen tissue and with reduced number of fibroblasts and mononuclear cells. Loose, less-organized collagen permits the permeation of aqueous from the anterior chamber, which accounts for the greater stromal edema that occurred in the animals treated with LDE-PTX/SC or with MMC.

Although LDE-PTX/SC showed antiscarring pharmacological action similar to MMC, the toxicity of the preparation to the conjunctiva and ciliary body was clearly smaller than that elicited by MMC treatment. The MMC group showed atrophic changes in the conjunctival epithelium and decreased count of goblet cells, in most of the animals, which did not occur in the LDE-PTX/SC group. The cell number was nonetheless within the minimal normal figure, which is approximately seven cells per high-powered field.[45] Conjunctival epithelium atrophy leads to thin-walled blebs, a commonly undesirable effect of MMC-treated eyes.[2,46] On the other hand, the depletion of goblet cells may contribute to the exacerbation of dry-eye symptoms in patients after trabeculectomy, and also to the weakening of the conjunctival structure, facilitating bleb leakages.[47,48] In this setting, the finding that LDE-PTX/SC but not MMC may preserve both the conjunctival epithelium and the goblet cells is an important advantage of this novel approach. Furthermore, with regard to ciliary body toxicity, epithelium height reduction, as well as the congestion and edema of the ciliary body were significantly more intense in the MMC group than in the LDE-PTX/SC group. All the several toxic effects of MMC observed in this study have been previously reported in the literature.[49–52]

In this study, the possibility of using LDE-PTX/IV was also explored, as the systemic use of this preparation was proven devoid of significant toxicities not only in experimental studies with animals but also in clinical trials enrolling patients with cancer.[19,20,27] Our findings suggest that LDE-PTX/IV antiscarring action is slightly inferior to those of MMC and LDE-PTX/

Table 3. Toxic Ciliary Body Changes of Eyes Treated With LDE-PTX or MMC After Trabeculectomy

	CTL	MMC	LDE-PTX/SC	LDE-PTX/IV	*P*
Ciliary epithelium height		***	††	†††	<0.001
Minimal decrease	7 (100)	0 (0)	5 (55.6)	8 (88.9)	
Mild decrease	0 (0)	0 (0)	4 (44.4)	1 (11.1)	
Moderate decrease	0 (0)	5 (55.6)	0 (0)	0 (0)	
Severe decrease	0 (0)	4 (44.4)	0 (0)	0 (0)	
Congestion		***	†††	††	<0.001
Absent	0 (0)	0 (0)	0 (0)	0 (0)	
Minimal	6 (85.7)	0 (0)	2 (22.2)	4 (44.4)	
Mild	1 (14.3)	1 (11.1)	7 (77.8)	3 (33.3)	
Moderate	0 (0)	3 (33.3)	0 (0)	2 (22.2)	
Severe	0 (0)	5 (55.6)	0 (0)	0 (0)	
Edema		**	††	††	<0.001
Absent	5 (71.4)	1 (11.1)	8 (88.9)	9 (100)	
Minimal	2 (28.6)	5 (55.6)	1 (11.1)	0 (0)	
Mild	0 (0)	3 (33.3)	0 (0)	0 (0)	
Moderate	0 (0)	0 (0)	0 (0)	0 (0)	
Severe	0 (0)	0 (0)	0 (0)	0 (0)	

Values are expressed as *n* (%). ** $P < 0.01$, *** $P < 0.001$ versus CTL; †† $P < 0.01$, ††† $P < 0.001$ versus MMC, by Kruskal-Wallis followed by Dunn posttest.

SC. The systemic administration of LDE-PTX was able to decrease the formation of fibrous tissue in the subconjunctival space and to increase the bleb survival. However, in only two of the parameters we measured, namely the degree of collagen organization and collagen density parameters, LDE-PTX/IV showed inferiority to MMC.

As a limitation of the study, the experiments were performed only in male animals. Gender can play a role in wound healing due to the different effects of androgens and estrogens on the various phases of the healing process.[53] Apparently, estrogen administration accelerates wound repair, whereas androgens have a negative effect.[54–56] Thus, experiments in female rabbits should be performed to validate the effects of LDE-PTX in female animals.

Regarding the toxicity to the eye, LDE-PTX/IV harmful effects to conjunctiva and to ciliary body were minimal, similar to LDE-PTX/SC and pronouncedly smaller than those elicited by MMC use. Those findings indicate that LDE-PTX/IV may be used as an adjuvant pharmacological tool in the wound-healing modulation in trabeculectomy. However, an important issue that arises from the use of IV LDE-PTX refers to the potential systemic toxicity, especially myelotoxicity. In this study, no hematological toxicity was observed in the rabbits (data not shown).

The current status of glaucoma surgery requires new approaches[57,58] to prolong the duration of the filtering patency by improving the efficacy and decreasing the toxicity of antifibrotic agents. The results of this study suggest that LDE-PTX may become an alternative not only for intraoperative but eventually for subsequent maintenance of the surgical fistula.

Acknowledgments

The authors thank Débora Deus, BSc, for help with the experiments.

Supported by the São Paulo State Research Support Foundation (FAPESP, São Paulo, Brazil) and a Research Carrier Award from the National Council for Scientific and Technological Development (CNPq, Brasilia, Brazil) (RCM).

Disclosure: **M.L. Occhiutto**, None; **F.R. Freitas**, None; **P.P. Lima**, None; **R.C. Maranhão**, None; **V.P. Costa**, None

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