Subconjunctival and topical application of recombinant tissue plasminogen activator in rabbits

Uso tópico e subconjuntival de ativador de plasminogênio tecidual recombinante em coelhos

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ABSTRACT

Purpose: To quantify fibrin degradation products after topical and subconjunctival administration of recombinant tissue plasminogen activator in rabbits.

Methods: Fibrin formation was induced in the anterior chamber in 25 rabbits. Subsequently, five rabbits received an injection of r-TPA (positive control) in the anterior chamber, another 10 received a subconjunctival injection of r-TPA, and the remaining 10 received instillations of topical r-TPA. Afterwards, samples of aqueous humor were collected and semi-quantitative analysis of fibrin degradation products (FDP) was performed.

Results: No statistical differences were noted between the treatment and control groups at any time point. Fibrin degradation products semi-quantification showed statistical improvement in the control group and the subconjunctival group.

Conclusion: Fibrin degradation products were observed in the anterior chamber after subconjunctival administration of r-TPA. However, it was probably not sufficient to cause fibrin degradation. Topical r-TPA did not effectively absorb anterior chamber fibrin.

Keywords: Tissue plasminogen activator; Anterior chamber; Hyphema; Inflammation; Postoperative complications; Animals; Rabbits

RESUMO

Objetivo: *Quantificar produtos de degradação de fibrina (PDF) após uso tópico e subconjuntival de ativador de plasminogênio tecidual recombinante (r-TPA) em coelhos.*

Métodos: *Formação de fibrina foi induzida na câmara anterior em 25 coelhos. Cinco coelhos foram submetidos a injeção intracameral de r-TPA (controle positivo). Dez coelhos foram submetidos a injeção subconjuntival de r-TPA e dez coelhos foram submetidos a instilação tópica de r-TPA. Amostras de humor aquoso foram coletados e uma análise quantitativa dos produtos de degradação de fibrina foi realizada.*

Resultados: *Não foi observado diferença estatisticamente significativa na degradação de fibrina em nenhum dos momentos estudados quando comparados com o controle. Porém foi observado diferença estatisticamente significante na quantificação do produtos de degradação de fibrina no grupo controle e no grupo subconjuntival.*

Conclusão: *Produtos de degradação de fibrina foi observado nas amostras do grupo subconjunctival, porém, provavelmente não foi suficiente para degradar a fibrin presente. r-TPA tópico não foi efetivo em absorver fibrina na câmara anterior.*

Descritores: *Ativador de plasminogênio tecidual; Câmera anterior; Hifema; Inflamação; Complicações pós-operatórias; Animais; Coelhos*

INTRODUCTION

The development of exuberant fibrinous exudation or inflammation is a rare but serious complication after ocular surgery. It can cause numerous sequelae including membranes, anterior or posterior synechiae, secondary glaucoma, and pupillary block. Previous studies showed that recombinant tissue plasminogen activator (r-TPA) is effective and safe for improving the clinical course of fibrinous related complications in cases of traumatic hyphema[1-3], severe post cataract fibrinous membranes in pediatric[4], fibrin formation after cataract surgery[4-6], glaucoma surgery[7-9], subretinal hemorrhage[10], and endophthalmitis[11].

Intracameral r-TPA has been shown to be effective for fibrin degradation. However, the effectiveness of topical[12], intravitreous[13], and subconjunctival[7,9] r-TPA remains controversial. Most studies regarding absorption or anterior chamber dosing of r-TPA were non-quantitative. To the best of our knowledge, no study has compared intracameral, subconjunctival and topical application of r-TPA using the same quantitative methods.

Therefore, the purpose of this study was to quantify fibrin degradation products (FDP) after topical and subconjunctival administration of r-TPA in rabbits.

METHODS

This prospective double-blind experimental study was performed in the Setor de Técnica Cirúrgica da Santa Casa de São Paulo under veterinary supervision. It was approved by the Animal Ethical Committee and Institutional Board of the Hospital das Clínicas and the Faculdade de Medicina da Universidade de São Paulo. All procedures followed proper legislation for the protection of animals (EU Directive 2010/63/EU) and adhered to the Association of Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

Twenty-five New Zealand male rabbits weighing 3.5 to 4.0 kg were enrolled in this study. All rabbits underwent an ocular examination and slit lamp documentation (Nikon, FS3 slit lamp, Japan). All rabbits with ocular abnormalities were excluded from the study.

Anesthesia was induced in each rabbit using intramuscular injection of a mixture of 0.3 mL/kg tiletamine hydrochloride and zolazepam (Zoletil 50®, Laboratoire Virbac, France). General anesthesia was achieved with an intramuscular injection of a mixture of 0.3 mL/kg tiletamine hydrochloride and zolazepam, and a mixture of 0.4 mL/kg fentanyl and droperidol (Innovar-Vet®, MTC Pharmaceuticals, Ontario, Canada).

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After appropriate anesthesia was attained, 5.0 mL of blood was collected from the ear vein and centrifuged to isolate the plasma. Each rabbit received topical 0.5% procaine chloridrate (Anestalcon®, Alcon, São Paulo, Brazil), and then a lid speculum was used for paracentesis in the superior portion of the cornea with a 25 gauge needle. Keeping the needle in the anterior chamber, 0.1 mL of aqueous humor was aspirated and 0.1 mL of plasma citrate was injected.

After 24 h, all rabbits were sedated and photographed under a slit lamp. All eyes with fibrin in the anterior chamber were graded according to the classification described by Lim et al.[14] (grade 0: no visible fibrin; grade 1: few visible fibrin filaments and clear details of the iris; grade 2: presence of a fibrin clot and blurry details of the iris; grade 3: presence of a fibrin clot, no visible details of the iris). All eyes with fibrin levels of grade 1 and 2 of fibrin in the anterior chamber were excluded from the study. Sampling calculations were based on a previous pilot study and statistical analysis was performed using the WINPEPI program with the COMPARE2 module (Version 2.68) to avoid sampling error. The sample size was increased in 20% due to possible loss to follow-up.

Rabbits were divided into three study groups: group 1 (n=5; positive control) received intracamerular injection of r-TPA; Group 2 (n=10) received subconjunctival injection of r-TPA; Group 3 (n=10) received topical r-TPA.

One eye from each rabbit in group 1 was randomly chosen (positive control) to receive an injection of 0.1 mL of 0.25 µg/mL r-TPA (TPA-Alteplase®, Ophthalmos, São Paulo, Brazil) in the anterior chamber after instillation of topical 0.5% procaine chloridrate (Anestalcon®, Alcon, São Paulo, Brazil). The other eye from the same rabbit was injected with 0.1 mL balanced salt solution using the same technique.

One eye from each rabbit in group 2 was randomly chosen to receive a subconjunctival injection of 0.1 mL of 0.25 µg/mL r-TPA after instillation of topical 0.5% procaine chloridrate. The other eye was injected with 0.1 mL balanced salt solution using the same technique.

One eye from each rabbit in Group 3 was randomly chosen to receive one drop of topical r-TPA at 1 mg/mL, 9 times at intervals of every 5 min after instillation of topical 0.5% procaine chloridrate. The other eye received one drop of 0.1 mg/mL balanced salt solution in the same regimen.

Each rabbit was examined, classified, and documented under sedation using a slit lamp (Nikon, model FS3, Japan) at the moment of the procedure (M0), and 30 min (M1), 60 min (M2), 7 h (M3) and 24 h (M4) after the procedure.

After 24 h, each rabbit was euthanized with intramuscular 1 mg/kg acepromazine and intravenous 30 mg/kg thiopental sodium. All eyes underwent paracentesis and aspiration of samples of aqueous humor using a 25-gauge needle. Samples were immediately transported to our laboratory for qualitative and semi-quantitative analysis of FDP and fibrinogen using the macro-latex slide agglutination test (FDP Plasma®, Diagnostica Stago Inc, France) according to the manufacturer`s instructions. This test involves multiple microlatex particles coated with mouse monoclonal anti-human FDP antibodies[15]. Presence of FDPs causes agglutination of the latex particles as the FDPs bind to the antibodies. Then these agglutinated particles are detected visually. The detection limit of this test is 2.5 µg/mL.

Qualitatively, the agglutination pattern was interpreted as negative when transparent and positive when blurred. Quantitatively, a positive agglutination pattern was interpreted as recommended in the manufacturer`s instructions.

All results were analyzed statistically with the Wilcoxon test ($p<0.05$).

All animal care and procedures were performed under the supervision of a single veterinarian to avoid bias and factors that could influence the hemodynamics and neuroendocrinological conditions of the animals (anesthesia technique, stress, food, etc.). All animals were kept in individual cages and given water and food *ad libitum*. Light, temperature, and humidity were controlled and monitored.

RESULTS

Table 1 shows the averages and standard deviations of anterior chamber fibrin grading following Lim et al.[14] after application of r-TPA.

In group 1 (intracameral administration), no statistically significant difference was noted at M1 compared to the control group ($p=0.06$). However, anterior chamber fibrin grading showed statistically significant improvement at M2, M3, and M4 compared to the control group ($p=0.0079$).

In group 2 (subconjunctival administration), no statistically significant difference was noted at any time point (M1, M2, M3 and M4) compared to the control group ($p>0.05$).

In group 3 (topical administration), no statistically significant difference was noted at any time point (M1, M2, M3 and M4) compared to the control group ($p>0.05$).

Table 2 shows the results of the semi-quantification of the FDPs following the different applications of r-TPA compared to the control group.

FDP semi-quantification showed statistically significant improvement in groups 1 ($p=0.0079$) and 2 ($p=0.0052$), but no statistically significant difference was observed in group 3 ($p=0.48$).

DISCUSSION

Fibrinous exudation is a serious complication of intraocular surgery and endophthalmitis. These complications can preclude fundus examination and interfere with the performance of a second intervention such as vitrectomy. These fibrinous membranes are usually difficult to manage with conventional steroid therapy. Typically, intraocular fibrin can be surgically removed, treated with an argon laser, or

Table 1. Distribution of the average and mean deviation of anterior chamber fibrin grading following criteria according to Lim et al.[14], after application of recombinant tissue plasminogen activator

		Examination time points															
		M1				M2				M3				M4			
		A	±	SD	p	A	±	SD	p	A	±	SD	p	A	±	SD	p
Group intracameral	Control	3.00	±	0.00	0.06	3.00	±	0.00	0.0079**	2.80	±	0.45	0.0079**	2.00	±	0.00	0.0079**
(0.25 µg/mL)	r-TPA	1.80	±	0.84		1.60	±	0.55		1.00	±	0.00		0.20	±	0.45	
Group subconjunctival	Control	2.90	±	0.32	0.97	2.90	±	0.32	1.00	2.70	±	0.48	1.00	2.10	±	0.32	0.80
(0.25 µg/mL)	r-TPA	3.00	±	0.00		2.90	±	0.32		2.70	±	0.48		2.00	±	0.67	
Group topical	Control	3.00	±	0.00	1.00	3.00	±	0.00	1.00	2.50	±	0.53	0.74	2.20	±	0.42	0.74
(1 mg/mL)	r-TPA	3.00	±	0.00		3.00	±	0.00		2.60	±	0.52		2.30	±	0.48	

r-TPA= recombinant tissue plasminogen activator; A= average; SD= standard deviation; M1= anterior chamber fibrin grading scale 30 min after r-TPA application; M2= anterior chamber fibrin grading scale 60 min after r-TPA application; M3= anterior chamber fibrin grading scale 7 h after r-TPA application; M4= anterior chamber fibrin grading scale 24 h after r-TPA application.

Table 2. Distribution of average and mean deviation of the dilution of a positive agglutination pattern of fibrin degradation products as determined by semi-quantification analysis using the macro-latex slide agglutination test (FDP Plasma®, Diagnostica Stago Inc., France). Semi-quantitatively, a positive agglutination pattern was interpreted as positive when the examiner visually detected agglutinated particles

		Level of FDP (µg/mL)			
		A	±	SD	p
Group intracameral	Control	2.50	±	0.00	0.0079**
(0.25 µg/mL)	r-TPA	12.00	±	4.47	
Group subconjunctival	Control	2.50	±	0.00	0.0052**
(0.25 µg/mL)	r-TPA	4.50	±	1.05	
Group topical	Control	2.50	±	0.00	0.48
(1 mg/mL)	r-TPA	2.75	±	0.79	

r-TPA= recombinant tissue plasminogen activator; FDP= fibrinogen degradation products; A= average; SD= standard deviation; Wilcoxon test (**P<0.005).

occasionally fibrinolytic drugs such as streptokinase and urokinase are used[16,17]. However, intraocular toxicity was often observed with these drugs[18].

R-TPA is a genetically cloned serine protease that promotes the degradation of fibrin only at the clot surface[19]. It has minimal side effects or toxicity when applied to the eye. It is considered a clot-specific fibrinolytic agent, used for the treatment of deep vein thrombosis[20] and acute myocardial infarction[21]. In ophthalmology, it was described as effective in thrombolytic therapy in patients with branch or central vein occlusion of the retina[22], glaucoma filtration surgery[23], or intracameral fibrinolysis following ocular surgery[5,6].

In our study using rabbit eyes, we found that intracamerular r-TPA (0.25 µg/1 mL) more effectively degraded anterior chamber fibrin compared to the control group. Other studies yielded similar results[2,24,25] with similar control groups.

There was no statistically significant difference in the degradation of anterior chamber fibrin in rabbit eyes with subconjunctival r-TPA compared to the control group. Although some degradation products were present in the anterior chamber, they were insufficient to indicate effective fibrin degradation. Additional studies are necessary to clarify whether a different concentration of subconjunctival r-TPA could actually increase fibrin degradation in the anterior chamber. The benefits and indications of r-TPA are evident especially in complications of glaucoma surgery involving the conjunctiva[7,9,23].

Compared to the control group, there was also no statistically relevant difference in the degradation of anterior chamber fibrin in rabbit eyes following r-TPA injection at 1 mg/mL. Previous studies were controversial[12,26-28]. The inefficacy of this method is probably related to poor penetration beyond the surface of the clot. Higher concentrations of r-TPA were not administered because toxicity to the epithelium was observed during a pilot study (data not shown).

The minimum concentration necessary to promote degradation of anterior chamber fibrin varied in previous studies. Based on these studies, an approximate dose of 25 µg/1 mL is suggested to effectively degrade fibrin with no intraocular toxicity[2,3]. There is no evidence of lens abnormalities in animal models following the use of this concentration[2,29]. Other authors observed good results using 10 µg/mL r-TPA for total hyphema in rabbit eyes[30]. Subconjunctival administration of r-TPA may be a novel method for promoting fibrin degradation.

The use of r-TPA in the eye has been considered safe with very few complications. The potential complications related to the use of r-TPA are corneal edema and anterior chamber turbidity, which results from immediate fibrinolysis products, prostaglandins, and leukocyte hydrolytic enzymes[31]. Previous studies did not show clinically relevant abnormalities in the cornea, lens, or retina[2,29], intraocular pressure changes, or an inflammatory response or rebleeding when intracameral or vitreous r-TPA was used at doses under 25 µg/1 mL. However, some studies suggested that it can increase the risk of intraocular rebleeding, ocular toxicity, and corneal abnormalities. Our study showed promising results with a lower dose of intracameral r-TPA. However, further studies are required to determine whether higher doses could be more effective.

FDPs were observed in the anterior chamber after 0.25 µg/mL subconjunctival r-TPA; however, this dose was probably insufficient to cause fibrin degradation. Topical 1 mg/mL r-TPA did not effective degrade anterior chamber fibrin.

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