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Retinal safety of intravitreal rtPA in healthy rats and under excitotoxic conditions

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Purpose: Intravitreal recombinant tissue plasminogen activator (rtPA) is used off-label for the surgical management of submacular hemorrhage, a severe complication of neovascular age-related macular degeneration. rtPA is approved for coronary and cerebral thrombolysis. However, in ischemic stroke rtPA is known to increase excitotoxic neural cell death by interacting with the N-methyl-D-aspartate (NMDA) receptor. We therefore investigated the retinal toxicity of rtPA in healthy rats and in a model of NMDA-induced retinal excitotoxicity.

Methods: First, rtPA at three different doses (2.16 µg/5 µl, 0.54 µg/5 µl, and 0.27 µg/5 µl) or vehicle (NaCl 0.9%) was injected intravitreally in healthy rat eyes. Electroretinograms (ERGs) were performed at 24 h or 7 days. Annexin V-fluorescein isothiocyanate (FITC)-labeled apoptotic retinal ganglion cells (RGCs) were counted on flatmounted retinas at 24 h or 7 days. Next, NMDA + vehicle or NMDA + rtPA (0.27 µg/5 µl) was injected intravitreally to generate excitotoxic conditions. Apoptotic annexin V-FITC-labeled RGCs and surviving Brn3a-labeled RGCs were quantified on flatmounted retinas and radial sections, 18 h after treatment.

Results: In healthy rat eyes, the number of apoptotic RGCs was statistically significantly increased 24 h after the administration of rtPA at the highest dose (2.16 µg/5 µl; $p = 0.0250$) but not at the lower doses of 0.54 and 0.27 µg/5 µl ($p = 0.36$ and $p = 0.20$), compared to vehicle. At day 7, there was no difference in the apoptotic RGC count between the rtPA- and vehicle-injected eyes ($p = 0.70$, $p = 0.52$, $p = 0.11$). ERG amplitudes and implicit times were not modified at 24 h or 7 days after injection of any tested rtPA doses, compared to the baseline. Intravitreal administration of NMDA induced RGC death, but under these excitotoxic conditions, coadministration of rtPA did not increase the number of dead RGCs ($p = 0.70$). Similarly, the number of surviving RGCs on the flatmounted retinas and retinal sections did not differ between the eyes injected with NMDA + vehicle and NMDA + rtPA ($p = 0.59$ and $p = 0.67$).

Conclusions: At low clinical equivalent doses corresponding to 25 µg/0.1 ml in humans, intravitreal rtPA is not toxic for healthy rat retinas and does not enhance NMDA-induced excitotoxicity. Vitreal equivalent doses ≥200 µg/0.1 ml should be avoided in patients, due to potential RGC toxicity.

Submacular hemorrhage (SMH) is a rare but devastating complication of neovascular age-related macular degeneration (AMD) [1]. The overall annual incidence of SMH among patients with exudative AMD has been estimated at 0.14% [2]. Damage to the sensory retina and the RPE by SMH has been attributed to limited nutrient diffusion from the choroid toward the retina, the shrinkage of the outer retinal layers due to clot formation, and the accumulation of toxic substances, such as fibrin and iron [3,4].

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Recombinant tissue plasminogen activator (rtPA) is a serine protease of 70 kDa that, in the presence of fibrin, catalyzes the conversion of plasminogen to plasmin, breaking down the fibrin clot into degradation products [5-7]. Intravenous rtPA is approved for coronary and cerebral thrombolysis in myocardial infarction and ischemic stroke, respectively. Off-label rtPA is currently used in ophthalmology to perform fibrinolysis in SMH [8]. The vitreoretinal procedure consists of either intravitreal or subretinal injections of rtPA, followed by gas tamponade meant to displace the dissolved blood collection [9-15]. The two routes showed similar efficacy regarding hematoma displacement and similar complication rates [16]. However, the exact visual benefits of the procedure cannot be ascertained: Although rtPA efficiently contributes to dissolving the fibrin clot, rtPA may induce neuronal damage [17-19]. In the central nervous system,

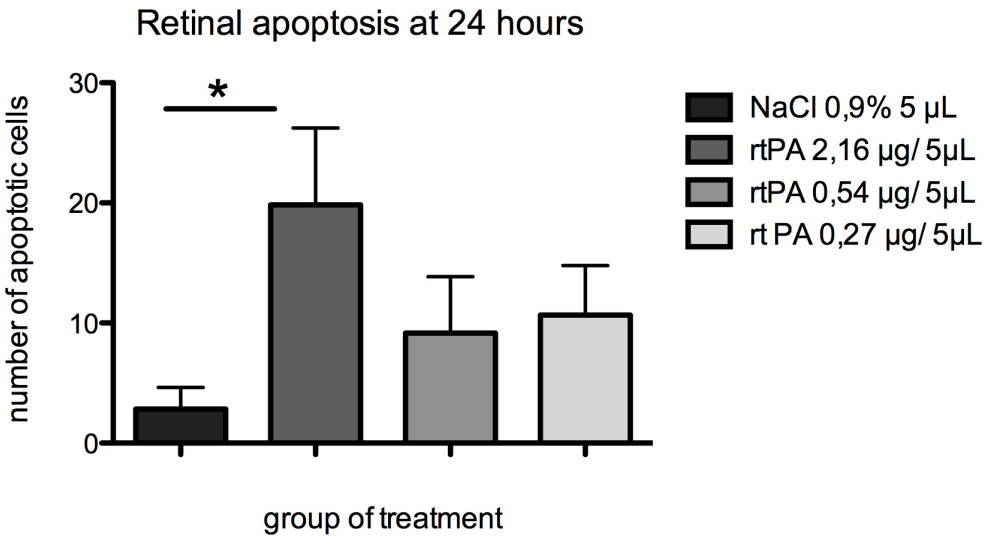

Figure 1. Number of apoptotic retinal ganglion cells in flat-mounted healthy retinas 24 h after intravitreal administration of rtPA (2.16, 0.54, and 0.27 µg in 5 µl) or vehicle (NaCl 0.9%, 5 µl). There was a statistically significantly higher number of apoptotic cells in the eyes that received 2.16 µg/5 µl compared to vehicle. * $p<0.05$.

tissue plasminogen activator (tPA) can potentiate excitotoxic neuronal death [20], via the N-methyl-D-aspartate receptor (NMDAR), inducing calcium influx and activation of extracellular signal regulated kinases 1/2 (ERK 1/2) with subsequent neuronal death [21-24]. However, at low concentrations, tPA can activate NMDAR via a plasminogen-independent mechanism, turning on the ERK 1/2-CREB-Atf3 prosurvival pathway that protects neurons from excitotoxicity [25-27]. Depending on the excitotoxic conditions and concentration, rtPA thus may be either pro- or antiapoptotic. In ophthalmology, there is no consensus regarding the appropriate dose of rtPA administered, which varies from 10 to 100 µg/0.1 ml.

Retinal safety studies in healthy rabbits and cats did not show toxicity when rtPA was injected intravitreally at a concentration of 25 µg/0.1 ml [28-30]. However, whether non-toxic concentrations remain safe under retinal excitotoxic conditions mimicking SMH remains to be determined. As retinal ganglion cells (RGCs) display N-methyl-D-aspartate (NMDA)-dependent neurotoxicity [17,18,31-35], we investigated the retinal toxicity of rtPA in healthy rats and in a rat model of NMDA-induced retinal excitotoxicity.

METHODS

The experimental flowchart is provided in Appendix 1.

Animals: Forty-two male Long Evans rats (Elevage Janvier, Le Genest Saint-Isle, France; age: 6 weeks) were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Paris Descartes University (Paris, France, reference: 00377.01). Rats were anesthetized by intramuscular injection of 100 µl/100 g of a solution containing ketamine (40 mg/ml; Ketamine 1,000®, Virbac, Carros, France) and xylazine (4 mg/ml; Rompun 2%®, Bayer Santé, Puteaux, France). Pupillary dilatation was obtained by instillation of 2 drops of tropicamide (Mydriaticum 0.5%®, Thea, France) with a 5-min interval. Topical ocular anesthesia with 1 drop of tetracaine chlorohydrate (Tetracaine 1%®, Faure, France) was performed 2 min before injection. Animals were euthanazied by an overdose (150 mg/kg) of intraperitoneal pentobarbital and eyes enucleated for analysis.

Tested compounds: Intravitreal injections were performed on anesthetized rats after pupillary dilatation as described. Animals were installed on an operating table with a surgical eye drape. Under microscope visualization, injections were performed into the vitreous in the inferior quadrant of the eye, using 300-µl sterile syringes fitted with 30-Gauge needles (Microfine©, Becton Dickinson AG, Meylan, France). A lubricant eye gel (Lacrigel®, Europhta, Monaco) was administered at the end of the procedure.

First, we analyzed the potential toxicity of increasing doses of intravitreal rtPA in healthy rats (n = 24 animals), assessed at 24 h and 7 days. Five microliters of solution containing 0.27, 0.54, or 2.16 µg rtPA (Actilyse® 10 mg, Boehringer-Ingelheim, Germany) were injected into the vitreous (n = 6 eyes per dose and per time point). Control eyes received 5 µl of saline solution (NaCl 0.9%, n = 6 eyes per time point), which is employed clinically to resuspend rtPA before administration, and served as the vehicle for the treated eye in the present experiment. The three doses of intravitreal rtPA selected for this experiment were calculated by analogy with the doses used clinically. A literature survey indicated that 25 to 50 µg/0.1 ml of rtPA is the most common

dose injected into the vitreous [16]. Given the average eyeball volume of Long Evans rats (0.05 ml) [36], the equivalent doses resulting in similar vitreal concentrations in rats are 0.27 and 0.54 µg/5 µl, respectively. We also tested a dose of 2.16 µg/5 µl (4 × 0.54 µg) to estimate the toxicity threshold.

Then, we evaluated the potential additive toxicity of rtPA in NMDA-induced excitotoxic conditions (n = 18 animals). For this experiment, the lowest clinical equivalent dose of rtPA (0.27 µg/5 µl) was selected to assess its safety. Intravitreal injection of 45 nmol of NMDA (Sigma, Poole, UK) in 6 µl of 1× Dubelcco's phosphate-buffered saline (PBS; 1X; 138 mM NaCl, 2.7 mM KCl, 8 mM $NaPO_4$, 1.5 mM KPO_4, pH 7.0-7.3) was performed to induce RGC death at 18 h, as previously described [37,38], with (n = 18 eyes) or without (n = 18 eyes) intravitreal rtPA coadministration at the time of NMDA injection.

Assessment of rtPA-induced retinal toxicity:

Electroretinography—Electroretinograms (ERGs) were performed before and 24 h and 7 days after the rtPA injections. Full-field ERGs were performed on anesthetized rats (Rats were anesthetized by intramuscular injection of 100µl/100 g of a solution containing ketamine (40 mg/ml; Ketamine 1,000®, Virbac, Carros, France) and xylazine (4 mg/ml; Rompun 2%®, Bayer Santé, Puteaux, France). Topical ocular anesthesia with 1 drop of tetracaine chlorhydrate (Tetracaine 1%®, Faure, France) was performed 2 min prior to injection under dim red light. Gold wire ring electrodes were placed on the corneas and inserted into the forehead, and served as the working and reference electrodes, respectively. A stainless steel needle electrode was subcutaneously inserted at the base of the animal tail for grounding. Measurements were performed using the commercial VisioSystem

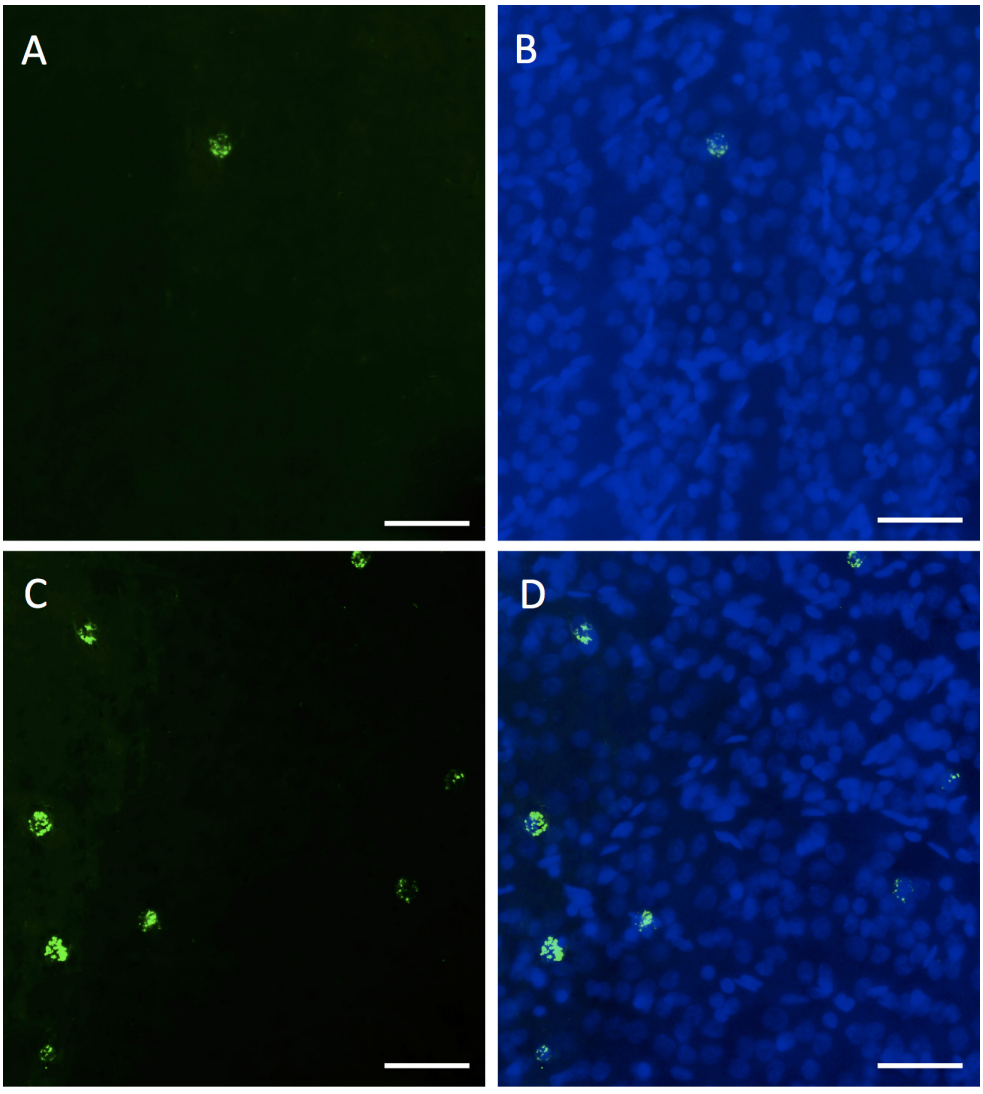

Figure 2. Flatmounted retinas from rats euthanized 24 h after intravitreal administration of rtPA (2.16 µg/5 µl) or vehicle (NaCl 0.9%, 5 µl). Apoptotic retinal ganglion cells labeled with intravitreal annexin V-fluorescein isothiocyanate (FITC) (**A**–**C**) or annexin V-FITC and 4',6-diamidino-2-phenylindole (DAPI) (**B**–**D**) before the animals were euthanized. **A**, **B**: Control eye (NaCl 0.9%, 5 µl). **C**, **D**: Eye treated with intravitreal recombinant tissue plasminogen activator (rtPA; 2.16 µg/5 µl) displaying a higher number of annexin V-FITC labeling, colocalizing with retinal ganglion cell nuclei. Green = Annexin-V-FITC staining; Blue = DAPI staining. Bar = 50 µm.

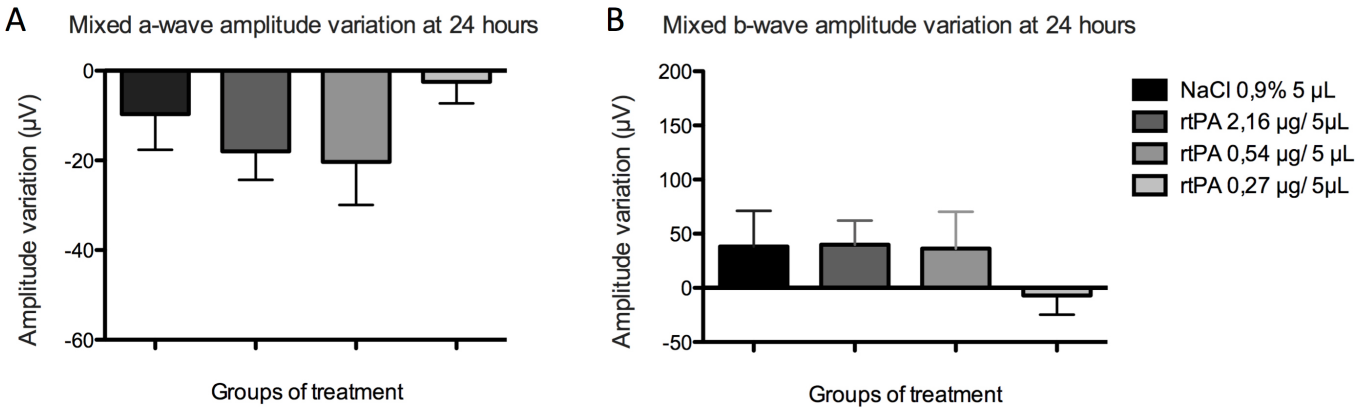

Figure 3. Mixed electroretinogram showing rod- and cone-driven responses 24 h after intravitreal injection of saline solution or three different doses of rtPA (2.16, 0.54, and 0.27 µg in 5 µl) in healthy rats. **A**: Variation in the a-wave amplitudes (µV) from the preinjection recordings. **B**: Variation in the b-wave amplitude (µV) from the preinjection recordings. No statistically significant difference was observed between the recombinant tissue plasminogen activator (rtPA)- and vehicle-injected eyes.

device (Siem Biomedicale, Nîmes, France). For the scotopic recordings, animals were dark-adapted overnight. Flash intensities ranged from 0.0003 to 10 cd.s/m^2. Five flashes per stimulation intensity were applied at a 0.5 Hz frequency, under scotopic conditions, and the corresponding responses were averaged. The flash duration was 10 ms (–30 to 0 dB) except for 10 cd.s/m^2, which was 30 ms (0 dB). For the photopic recordings, the animals were light-adapted for 5 min with a rod-suppressing background light of 25 cd/m^2. A light flash was then applied, and the light intensity was 10 cd.s/m^2 (flash duration 79 ms). The amplitudes (in µV) and latencies (in ms) of the a- and b-waves were measured. For each eye, we calculated the variation (the difference between the post-treatment and pretreatment values) of each ERG parameter (amplitudes, implicit times). Data obtained from all eyes in the same experimental group were averaged, and the treatment groups were compared.

Quantification of apoptotic cells—The apoptotic cells were labeled in vivo with an intravitreal injection of 2 µg/10 µl of annexin V-fluorescein isothiocyanate (FITC; Santa Cruz Biotechnology, Inc., Dallas, TX) 24 h or 7 days after the rtPA injection in the healthy rats and 18 h after the NMDA ± rtPA injection in the excitotoxicity model. The animals were euthanized (an overdose 150 mg/kg intraperitoneal pentobarbital) 1 h after the annexin V-FITC injection, and the eyes were removed immediately and fixed for 15 min in 4% paraformaldehyde (PAF) at room temperature. Then the retinas were flatmounted, fixed in acetone at −20 °C for 10 min, and permeabilized with PBS/0.3% Triton for 20 min. 4',6-diamidino-2-phenylindole (DAPI) staining (1:5,000; Sigma-Aldrich, St. Louis, MO) was performed for 10 min. After rinsing with PBS, the retinas were finally flatmounted between a slide and a coverslip using mounting medium (Vectashield©, Vector Laboratories Inc., Burlingame, CA). They were visualized on a fluorescence microscope (BX51, Olympus, Rungis, France). Twelve photographs were taken following a cross pattern centered on the optic nerve. Annexin V-FITC-positive cells were counted using ImageJ software (Version 1.47p, National Institutes of Health, Bethesda, MD).

Quantification of living RGCs—To better discriminate the potentiating effect of rtPA on NMDA toxicity, living RGCs were counted at 18 h on flatmounted retinas of rats treated with NMDA± rtPA, using overnight incubation at 4 °C with a goat-anti Brn3a (C-20) antibody (Santa Cruz Biotechnology, Heidelberg, Germany) 1:100 in blocking buffer (PBS, 2% bovine serum albumin, 2% Triton). Quantification of living RGCs was also performed on the radial retinal sections.

Radial sections: The enucleated eyes were immediately fixed with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 1× PBS for 2 h at room temperature. The eyes were rinsed for 2 h in 1× PBS and dehydrated at room temperature with increasing ethanol concentrations before being incubated overnight at 4 °C with infiltration solution provided in the Leica Historesin Embedding kit (Rueil-Malmaison, France). Samples were embedded in resin (Leica), and 5-µm-thick histological sections passing through the optic nerve head were performed along the nasotemporal plane of the eye using a microtome (Leica). Sections were stuck on gelatin-coated slides, stained for 2 min with 1% toluidine blue solution, and observed with light microscopy under an Aristoplan light microscope (Leica) coupled with a Leica DFC480 camera. Photographs of the whole section were made, and the ganglion cell layer nuclei were then counted using ImageJ software.

Statistical analysis: Statistical analysis was performed on GraphPad Prism (Version 5.0, GraphPad Software Inc., La Jolla, CA). Results were expressed as mean ± standard deviation (SD), and the non-parametric Mann–Whitney test was used to compare quantitative values between different groups. P values of less than 0.05 were considered statistically significant.

RESULTS

High-dose intravitreal rtPA induced ganglion cell death in healthy rats at 24 h: Twenty-four hours after intravitreal injection, no statistically significant difference in the number of apoptotic ganglion cells was observed between the eyes that received 0.54 or 0.27 µg of rtPA and the control eyes that received the vehicle (p = 0.36 and p = 0.20, respectively). However, there was a statistically significantly higher number of apoptotic ganglion cells in eyes that received 2.16 µg of rtPA compared to the control eyes (19.83±6.405 versus 2.83±1.797 cells, p=0.025; Figure 1 and Figure 2).

The variation in the ERG amplitudes and culmination times induced by the rtPA injections were compared between each treatment group and the control group 24 h after injection. In the mixed ERG responses (cumulated cone- and rod-driven responses), there was no statistically significant difference in the a- and b-wave amplitude variations 24 h after injection (Figure 3). None of the tested intravitreal doses of rtPA (2.16, 0.54, and 0.27 µg) led to statistically significant changes in the culmination times of the a- or b-waves. The scotopic and photopic ERGs were not modified in any group 24 h after treatment.

Absence of ongoing apoptotic process 7 days after intravitreal rtPA in healthy rats: The same evaluations were repeated 7 days after the intravitreal injections of rtPA or vehicle. The number of apoptotic RGCs did not differ between

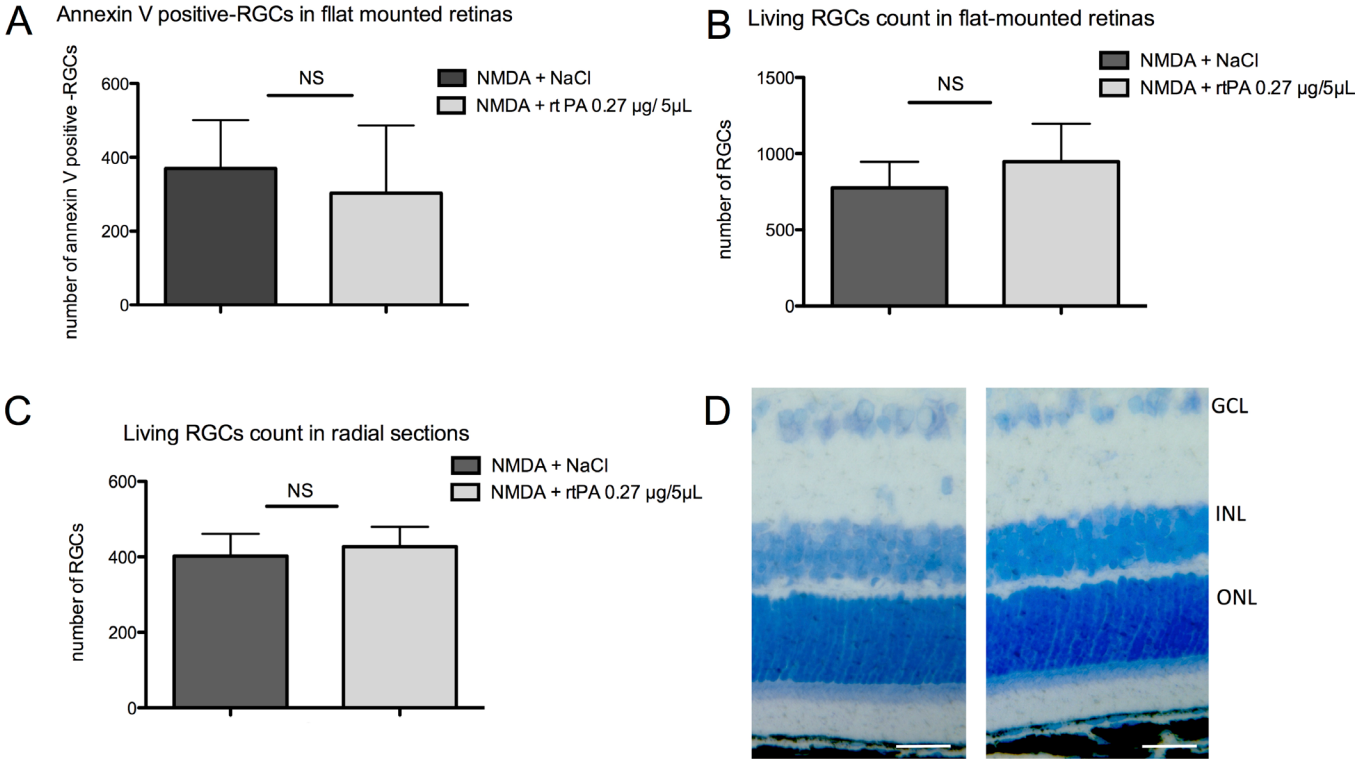

Figure 4. Assessment of retinal toxicity 18 h after intravitreal administration of rtPA in rat eyes under excitotoxic conditions. Animals received either N-methyl-D-aspartate (NMDA; 45 nmol/6 µl) + saline solution or NMDA (45 nmol/6 µl) + recombinant tissue plasminogen activator (rtPA; 0.27 µg/5 µl). **A**: Number of annexin V-positive retinal ganglion cells quantified on flatmounted retinas, showing no difference between the eyes that received NMDA + saline and the eyes that received NMDA + rtPA. **B**: Number of Brn3a-positive living retinal ganglion cells, quantified on flatmounted retinas, showing no difference between the eyes that received NMDA + saline and the eyes that received NMDA + rtPA. **C**: Number of living retinal ganglion cells, quantified on the toluidine blue–stained histologic radial retinal sections, as the number of cell nuclei visualized at the level of the retinal ganglion cell layer, showing no difference between the eyes that received NMDA + saline and the eyes that received NMDA + rtPA. **D**: Toluidine blue–stained retinal radial section illustrating the similar density of retinal ganglion cells in the eyes that received NMDA + saline (left) and the eyes that received NMDA + rtPA (right). GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Bar = 20 µm.

Table 1. Observations of tPA-related modulation of retinal toxicities reported in the literature.

Protocol			Model	Main Results	Ref
Injected compound	**Dose**	**Time of sacrifice**			
NMDA	30 nM	12 h after injection	tPA −/− and wild-type mice	TUNEL-positive cells in the GCL and INL in tPA−/−mice after intravitreal injection of NMDA were significantly fewer than in wild-type mice	[37]
NMDA	30, 120 and 240 nM	6, 12, 24, 72 h, 1 or 2 weeks	tPA −/− and wild-type mice	At 12 h, TUNEL-positive cells in both GCL and INL were significantly fewer in tPA-deficient mice than in wild-type mice, only in the case of the lower dose of NMDA (30 nmol/mouse), but not at higher doses (120 or 240 nmol/mouse)	[17]
Kainic acid (KA)	10, 20, and 40 nM	48 h	Adult CD-1 mice	KA-injected eyes showed a dose-dependent and time-related increase in tPA activity in the retina, witch was associated with TUNEL-positive cells the GCL and subsequently in the INL and ONL.	[18]
Commercial rtPA containing L-Arginine (Actilyse ®)	1 µl of 250 µg/ml tPA (containing L-arginine)	24 h	ICR mice	Severe morphologic damages appeared in the retinal cell layers with a vitreous injection of the tPA (containing L-arginine) compared to the control eye	[46]
	5 mM of L-arginine alone			Similar histopathologic results were observed in the retinal areas receiving L-arginine (5 mM)	
	10 µg/400 µl tPA (containing L-arginine)	-	Primary retinal cells culture	Exposure of cultured retinal cells to tPA (containing L-arginine) or L-arginine led to a marked increase in nitrite, a nitric oxide intermediator	

GCL=ganglion cell layer; INL=inner nuclear layer; ONL=outer nuclear layer.

the rtPA-treated eyes (2.16, 0.54, or 0.27 µg rtPA) and the vehicle-treated eyes (p = 0.70, 0.52, and 0.11, respectively). The mixed, scotopic, and photopic ERG responses did not show any statistically significant modification from baseline to 7 days post-injection in terms of the amplitudes or implicit times.

Absence of additive toxicity of 0.27 µg of intravitreal rtPA in an NMDA-induced excitotoxicity model at 18 h: The lowest clinical equivalent dose of rtPA (0.27 µg/5 µl), which was safe for healthy rat eyes, was selected to assess whether rtPA could potentiate NMDA-induced retinal damage in excitotoxic conditions.

In the flatmounted retinas from the animals euthanized 18 h after injection, the number of annexin-V-positive RGCs did not differ between the eyes that received intravitreal NMDA + vehicle and the eyes that received intravitreal NMDA + rtPA (0.27 µg; p = 0.70). To confirm these findings, we quantified the number of living RGCs in each group on the flatmounted retinas and the radial sections. The number of living RGCs did not differ between the two groups in the flatmounted retinas (p = 0.59) and radial sections (p = 0.67; Figure 4).

DISCUSSION

To dissolve blood collected in the subretinal space, and facilitate its displacement by gas tamponade, rtPA is commonly used off-label in the surgical management of SMH complicating neovascular AMD. However, optimal doses and conditions of use have not been evaluated by the manufacturer for intraocular administration. The reported doses in clinical studies vary from 10 to 100 µg/0.1 ml [9,11,13,15,16]. Retinal toxicity has been suspected after a single intravitreal administration of 100 µg of rtPA [11] or two injections of 50 µg at a 48-h interval [39]. Controversially, others studies reported no deleterious effects with 100 µg [9,15,40] or 50 µg [13,41-43] of intravitreal rtPA.

In the present study, we observed that a transient increase in RGC death was induced in healthy rat eyes 24 h after intravitreal injection of the highest dose of rtPA (2.16 µg/5 µl, corresponding to 200 µg/0.1 ml in the human vitreous), which was not associated with functional modifications on ERG. Injection of lower doses (0.27 and 0.54 µg/5 µl) did not induce RGC death or changes on the ERG at any time point.

The exact mechanisms of rtPA-induced RGC toxicity remain uncertain. Proposed mechanisms for the retinal toxicity of tPA are summarized in Table 1. L-arginine, contained in the commercial formulation of rtPA, has been incriminated in the toxicity of high-dose rtPA because L-arginine is a substrate for nitric oxide (NO) synthases. Different types of NO synthases are present in RGCs and photoreceptors [44,45], and NO produced by the inducible or constitutive form of NO synthases could exert toxic effects. Activation of the NR1 subunit of the NMDAR by rtPA could enhance calcium influx into the cell [21-23] and subsequently accelerate neuronal cell death, as shown to occur in the brain. In the present experiments, the variations in the ERG signals did not statistically significantly differ between the treatment and control groups because the proportion of damaged cells necessary to alter responses to full-field ERG might not have been reached. This also suggests that retinal toxicity might be overlooked in the clinical setting. In healthy rats, doses of 0.27 and 0.54 µg/5 µl, equivalent to 25 and 50 µg/0.1 ml in the human vitreous, did not exert retinal toxicity. These results are in accordance with previous studies performed in other animal models, such as cats and rabbits. Harach et al. observed fundus pigmentary alterations in cat eyes that received intravitreal doses ≥50 µg/0.1 ml. On electroretinography, the scotopic b-wave amplitudes were reduced 14 days after treatment with doses ≥50 µg/0.1 ml, while the a-wave amplitudes were reduced with doses ≥75 µg/0.1 ml [29]. In rabbit eyes, Johnson et al. found no evidence of retinal toxic reaction in eyes that received 25 µg/0.1 ml of intravitreal rtPA [30]. Similarly, Rowley et al. did not observe retinal toxicity at increasing doses up to 50 µg/0.1 ml. However, with doses of 200 µg/0.1 ml or more, there was evidence of retinal toxicity on electroretinography, ophthalmoscopy, and histology [28].

Clinically, rtPA is administered in eyes with advanced, uncontrolled, or refractory neovascular AMD complicated by SMH. In these severely affected eyes, retinal neurons and supportive cells are submitted to an intense, multifactorial cellular stress. As enhanced rtPA toxicity has been reported in the brain submitted to excitotoxic stress, we also evaluated the retinal toxicity of intravitreal rtPA, in excitotoxic conditions. We used two quantitative methods to assess RGC apoptosis and survival on flatmounted and sectioned retinas. In this experimental setting, we did not observe any potentiating neurotoxic effect of the low clinical equivalent dose of rtPA. Although this finding could be related to the low dose used, and should be confirmed with other doses and other routes of administration, it is consistent with the reported paradoxical neuroprotective effect of low-dose tPA in excitotoxic conditions [25].

In summary, these results confirm that human equivalent doses of rtPA injected into the vitreous (corresponding to 25–50 µg/0.1 ml in the human eye) are safe for the retina. The lowest dose (corresponding to 25 µg/0.1 ml) does not potentiate the neurotoxicity of excitotoxic conditions. This

finding carries statistically significant clinical implications as it indicates that this dose can be used safely in patients with submacular hemorrhage due to AMD or other retinal vascular disorders, and those with frequently associated neurodegenerative diseases, such as glaucoma.

APPENDIX 1.

To access the data, click or select the words "Appendix 1."

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