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行政院國家科學委員會專題研究計畫成果報告

組織胞漿素原活化劑治療腦室內出血時脈絡叢及腦室壁之顯微結構研究

Microscopic changes of the choroid plexuses and cerebral ventricular walls after treatment with tissue plasminogen activator for intraventricular hemorrhage

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中文摘要

本研究旨在探討利用組織胞漿素原活化劑(tPA)治療腦室內出血(IVH)，所引起脈絡叢及腦室壁的傷害，是否經由破壞其上皮細胞或內皮細胞上的基底層與緊密接合所致。本實驗係依據前次國科會計畫”組織型胞漿素原活化劑對大白鼠腦室出血之療效探討”(計畫編號：NSC89－2314－B－075A－010)為繼續進行之研究。本實驗仍是以大白鼠為實驗對象，採用立體定位法於大腦側腦室中注入 30 μl 自體血液，產生腦室內出血，2 小時後將上次實驗治療認為適當劑量 300 單位和 1200 單位 tPA 分別注入腦室中治療，24 小時後取下鼠腦作切片。腦部形態計量係以數位影像分析系統計算腦室及腦室內殘餘血塊體積。另外進行螢光免疫組織染色，以螢光顯微鏡檢視脈絡叢及腦室壁上的閉鎖小帶(ZO-1)、第四型膠原纖維與基質金屬蛋白酶(MMPs)之表現和分佈情形。

實驗結果顯示，在動物模式中，300 單位 tPA 是治療腦室出血的最佳選擇劑量，它能減小腦室中血塊之大小，比較不會破壞腦室周邊細胞的緊密結合，並防止脈絡叢的傷害。1200 單位 tPA 雖然有溶解血塊的效果，但不能改善腦室周邊的水腫，反而造成腦室內白血球的聚集與脈絡叢的傷害，同時亦破壞腦室上皮細胞或血管壁的基底膜和緊密接合。本實驗數據可提供臨床醫師在使用 tPA 治療腦出血或腦室內出血時，作為評估安全劑量之重要參考。

關鍵詞：脈絡叢、腦室出血、基質金屬蛋白酶、緊密接合、組織胞漿素原活化劑、腦室壁、第四型膠原纖維

Abstract

This study aimed at exploring the possible mechanism of choroids plexus and cerebral ventricular walls while tissue plasmininogen activator (tPA) was used to treat intraventricular hemorrhage (IVH). The present investigation has been made by observing whether tPA caused the damage of

a tight junction of basal laminia on epithelial or endothelial cells. This study was followed by a previous project from the National Science Council (NSC89－2314－B－075A－010).

Thirty μl autogenous blood was injected into the ventricle of the Sprague-Dawley rat to simulate an IVH. Two hours later, 300 or 1200 international units of tPA was injected into the lateral ventricle of the rat brain. 24 hours later, the rat brain was removed and dissect into 2 mm-slices. The digital-image analytical system was used to calculate the remaining volume of clot among the ventricle of brains. In addition, a fluorescence microscope was applied to examine the collagen type IV, zonula occludents-1(ZO-1), and matrix metalloproteinases (MMPs) in the choroid plexus and the ventricular walls.

Our results indicated that 300 international units tPA was considered to be the optimal dose of the treatment of intraventricular hemorrhage in rat. It was not only diminishe the clot in rat brains, but also to cause less damage the tight junction between intraventricular of epithelial cells and vascular walls of basal lamina. On the other hand, though 1200 u of tPA dissolved the clot, it could not relief the edema induced by IVH, and also resulted in more choroid plexus edema and intraventricular leukocytosis. The data may provide an important data for clinicians to assess the optimal dosage treated by tPA.

Keywords: choroids plexus, collagen type IV, intraventricular hemorrhage, matrix metalloproteinase (MMPs), tight junction, tissue plasminogen activator, ventricular wall, zonula occludents-1(ZO-1)

Introduction

Intracerebral hemorrhage (ICH) is still a disease with high mortality and morbidity. When the hemorrhage extends into all ventricles, the mortality rate may up to 80%. The clinically poor outcome may be due to obstruction of outflow of the cerebrospinal fluid by the intraventricular clots, progressive ventricular dilatation and increased intracranial pressure.

Various therapeutical modes for IVH have been proposed. Ventricular drainage alone may not effectively remove the intraventricular clot when the IVH is relatively large. Tissue plasminogen activator is a serum protease normally secreted by vascular endothelia. It directly converts plasminogen to plasmin that degrades fibrin. TPA is much more fibrin-specific and about 10 times more effective than urokinase for in vitro fibrinolysis. In clinical practice, tPA has been widely used for thrombolysis in acute myocardial infarction and acute ischemic stroke. However, symptomatic intracerebral hemorrhage occurs in 6-7% of patients given by tPA. Recently, tPA was reported to mediate brain inflammation and be involved in neuronal damage caused by cerebral ischemia, traumatic brain injury or excitotoxin injection. All these findings further provoke our concern about the safety of tPA in treating IVH. According to our previous study, although tPA could effectively lyse the intraventricular hematoma, high doses of it may aggravated the periventricular edema induced by IVH and cause additional choroid plexus edema and

2

intraventricular leukocytosis. It suggests that products derived from tPA reaction or tPA itself may increase damage to the blood-brain, blood-CSF or CSF-brain barriers, leading to increased permeability of these barriers and cerebral inflammation. Thus, the goal of this study is to further investigate the mechanisms by which choroid plexuses and ventricular walls are damaged during tPA treatment for IVH.

Taken together, in this study, we further investigated the mechanism by doses of tPA causing damage to the choroid plexuses and periventricular tissues. The findings of immunofluorescence, immunoelectron microscopy and digital image analysis also utilized to examine and evaluate the above changes.

Materials and methods

The experimental protocols of this study were in conformity with the Guide for the Care and Use of Laboratory Animals formulated by the National Science Council of the Republic of China and was approved by the review committee in Taichung Veterans General Hospital. During the experiments, appropriate anesthesia was given to prevent the animals from suffering pain, as detailed below.

Animal preparation

Adult male Sprague-Dawley rats weighing 330-380 g was divided into four groups: control, saline, T300 (t-PA 300 u) and T1200 (t-PA 1200 u). The animals were anesthetized with chloral hydrate (400 mg/kg,

i.p.). Each group of rat produced intraventricular blood injection but except control group, than treat by saline or different two dose of tPA. A femoral vein and a femoral artery were catheterized with polyethylene tubing. The former was used for maintaining the anesthetic condition by continuous infusion of chloral hydrate (85 mg/kg·hr, i.v.) and the latter for withdrawal of blood. The body temperature was monitored and kept at 37°C by a temperature controller. The head was fixed prone in a stereotaxic frame. Using aseptic techniques, the scalp was incised to expose the skull and a burr hole was on the left parietal bone with a dental drill. A cannula (o.d. 0.7 mm) with a fine needle inside was inserted through the burr hole into the left lateral ventricle. The cannula was fixed to the skull with anchoring screws and dental acrylic.

Build-up of experimental IVH

After surgery, the rat was allowed to equilibrate for 2 hours. Then 0.2 ml of the autogenous blood was aspirated from the femoral artery, filled into a glass microsyringe, and incubated at 30   for 7 min to make the blood viscous. 30 μl of the incubated blood was injected through the stereotaxically inserted needle into the left lateral ventricle with a microinfusion pump. The infusion rate was set at 4 μl/min lasting for 7.5 min.

TPA administration

Two hours after the build-up of IVH, infusion of Ringer's saline or 0.5 or 2 μg of tissue plasminogen activator (expressed as T300 and T1200) through the intraventricular needle was initiated. These two dose of T300 and T1200 were selected for study dose based

on a prior project, "Tissue plasminogen activator in the treatment of intraventricular hemorrhage in rats", founded by National Science Council. The total dose was equally divided into three subdoses and given at one-hour intervals. Each subdose in 2 µl solution was infused into the intraventricular hematoma at the rate of 2 µl/min and the needle was then be capped. After one-hour capping, the lysed blood and injected solution was aspirated with a syringe as mildly as possible for each subdose. After completing the three cycles of tPA infusion and aspiration of the liquefied blood, the animal was care to recover.

Brain morphometry

24 hours after the build-up of IVH, the rats was transcardially perfused with heparinized saline and the brains be removed and fixed in 10% phosphate-buffered formalin for 4 hours. The fixed brains were coronally sectioned at 2 mm intervals. Six or seven coronal slices are usually obtained for each brain. Both sides of each slice were photographed with a digital camera and the images were evaluated with planimetry using an image analyzing software. The mean areas (mm^2) of the residual intraventricular blood clots and cerebral lateral ventricles of each slice were first measured. The volume (mm^3) was then be obtained by multiplying the sum of all mean areas by 2 mm. After the morphometrical study, the brain slices was subjected to cryosectioning for immunohistochemistry or immunoelectron microscopy as described below.

Immunohistochemistry for collagen type IV, ZO-1 and MMP-9

Collagen type IV is a major constituent of basement membranes. ZO-1 is a peripheral membrane protein associated with tight junctions (zonula occludentes), which are located at the cytoplasmic surface of epithelia and endothelial cells. MMPs are important protein hydrolysis when extracellular matrix and basal lamina are in the process of dissolution, and MMPs are released when damaged cells require reconstruction. The post-fixed brain slices was embedded in Tissue-Tek OCT compound, frozen in 2-methylbutane/dry ice, and stored at -80°C for cryostat sectioning. 10 µm cryostat sections were cut from each brain slice. After rinsed with PBS, the sections were serially blocked with PBS for 10 min and 5% skim milk in PBS for 30 min. Then they were incubated with the primary antibody [against collagen type IV (Serotec), ZO-1 (Zymed Laboratories, Inc), and MMP-9 (Chemicon)] overnight at 4°C. Subsequently, the sections were rinsed with PBS and incubated with Tetramethyl Rhodamine Isothiocyanate (TRITC)- conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 hour at room temperature. The immunofluorescence was examined under a fluorescence microscope.

Data analysis

Data of brain morphometry was expressed as mean ± standard error of means (SEM). Differences among groups was first compared by one-way analysis of varianc (ANOVA) and then analyzed by Duncan's test. $p < 0.05$ is considered to be statistically significant.
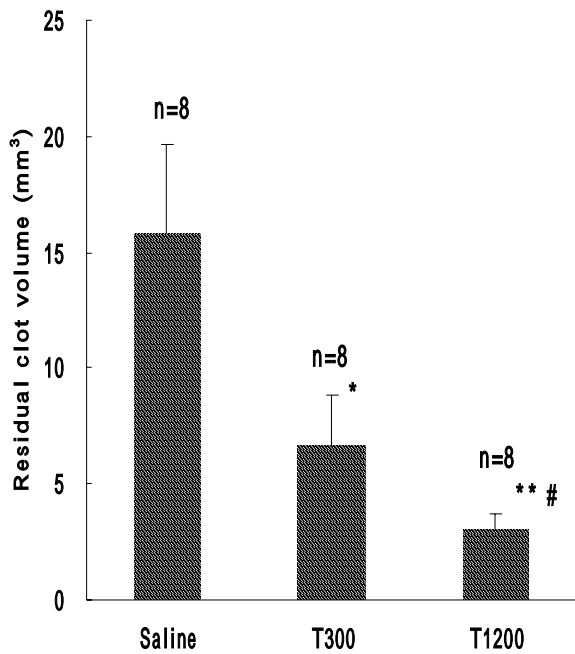
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Results and Discussion

Fig. 1. Residual clot volume in the lateral ventricles 24 hours after the onset of intraventricular hemorrhage (IVH). Animals were treated with Ringer's saline or 0.5 and 2 μg of tissue plasminogen activator (expressed as T300 and T1200) 2 hours after the onset of IVH. n = animal number. *p<0.01 and **p<0.01 as compared to the saline group; #p<0.01 as compared to the tPA-low group.

Figure 1 shows the variations of residual blood clot size in the lateral ventricles 24 hours after the onset of IVH in different groups. The remaining blood clot of the control group was measured 15.8 mm^3 of size, and the remaining clot size of T300 and T1200 were 6.7 mm^3 and 3.1 mm^3 respectively. The treatment of T300 and T1200 reduced IVH by 58% and 81%. The results of this experiment support those of the prior project.

The distribution and expression of collagen type IV on the lateral ventricular walls could be evaluated, but not on the choroid plexuses in this study. collagen type IV was found to has faint staining on the choroid plexuses, which may due to the factors of detecation or/and reaction of collagen type IV antigen and antibody. Figure 2 showed the periventricular tissue of the lateral ventricle of the rat brain. The red fluorescence stains accounted for the integrality of the collagen type IV on blood vessels presented on the normal brain (Fig. 2A, white arrows). The traumatized brain tissue treated by saline revealed no stain (Fig. 2B). Comparison between the 300 u (Fig. 2C, white arrows) and 1200 u (Fig. 2D) of tPA, the red fluorescence stains of the blood vessel was more inconspicuous by tPA 1200 u than 300 u. It may indicated 300 u provided the repair of blood vessel of the ventriculsr wall better than 1200 u.

The distribution and expression of ZO-1 on the changes of tight junction of periventricular blood capillary could be evaluated (Fig.3), but still not on the choroid plexus in this study also. ZO-1 for chroid plexus was found not only to have a faint staining and need high magnification to examination, which was difficult for evaluation. The tight junction of blood capillary of periventricular wall in normal rat's brain was stained by ZO-1 (Fig. 3A, white arrows). ZO-1 did not stained in saline treated brain (Fig.3B), which indicated the tight junction of the periventricular wall.
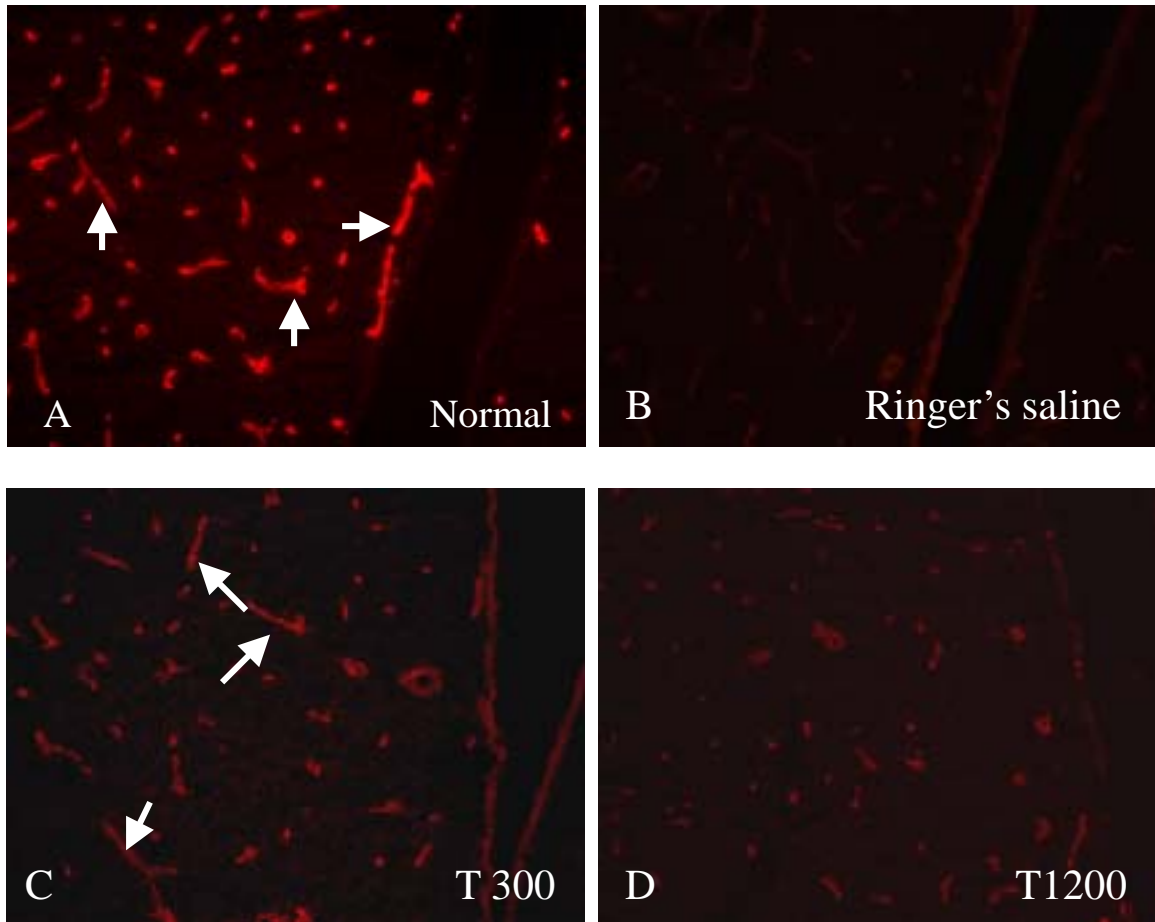
Fig. 2. Immunofluorescence of collagen type IV (red) shower that periventricular tissue of the left lateral ventricle form rats 24 hours after the build-up of intraventricular hemorrhage (200X magnification). (A) Normal brain (*white arrows*)　(B) Ringer's saline treated brain with the destruction of the basal lamina underlying the vascular endothelium and epidermal cells of collagen type IV; (C) 0.5 µg tPA treated brain with the reduction damage of collagen type Ⅳ (*white arrows*).；(D) 2 µg tPA treated brain with demolished the intraventricular of epithelial cells and vascular walls of collagen type IV .

Fig.3C showed the red fluroscence staining on the capillary of periventricular wall, which indicated the tigh junction of cells were restored. Fig.3D demonstrated a poor red fluroscence staining . In comparison of 300 u and 1200 u of tPA, 1200 u presented no effective contriution of repair the tight junction between epithelial cells and vascular tissue of ventricular wall.

The stain of MMP-9 on choroids plexuses revealed poor stain, red fluorescence, slight red fluorescence, similar red fluorescence in normal, saline, T300 and T1200, respectively. Figure 4 shows the expression of MMP-9 by different treatments on choroid plexuses. Poor stain was shown as to the choroid plexuses of normal rat brains that MMP-9 didn't exist (Fig. 4A). Red fluorescence stain was found on choroid plexuses treated by saline, representing damaged brain tissue that plenty of MMPs released (Fig. 4B, white arrows). Slight red fluorescence stain has been observed by 300 u of tPA treatment (Fig. 4C, white arrows).
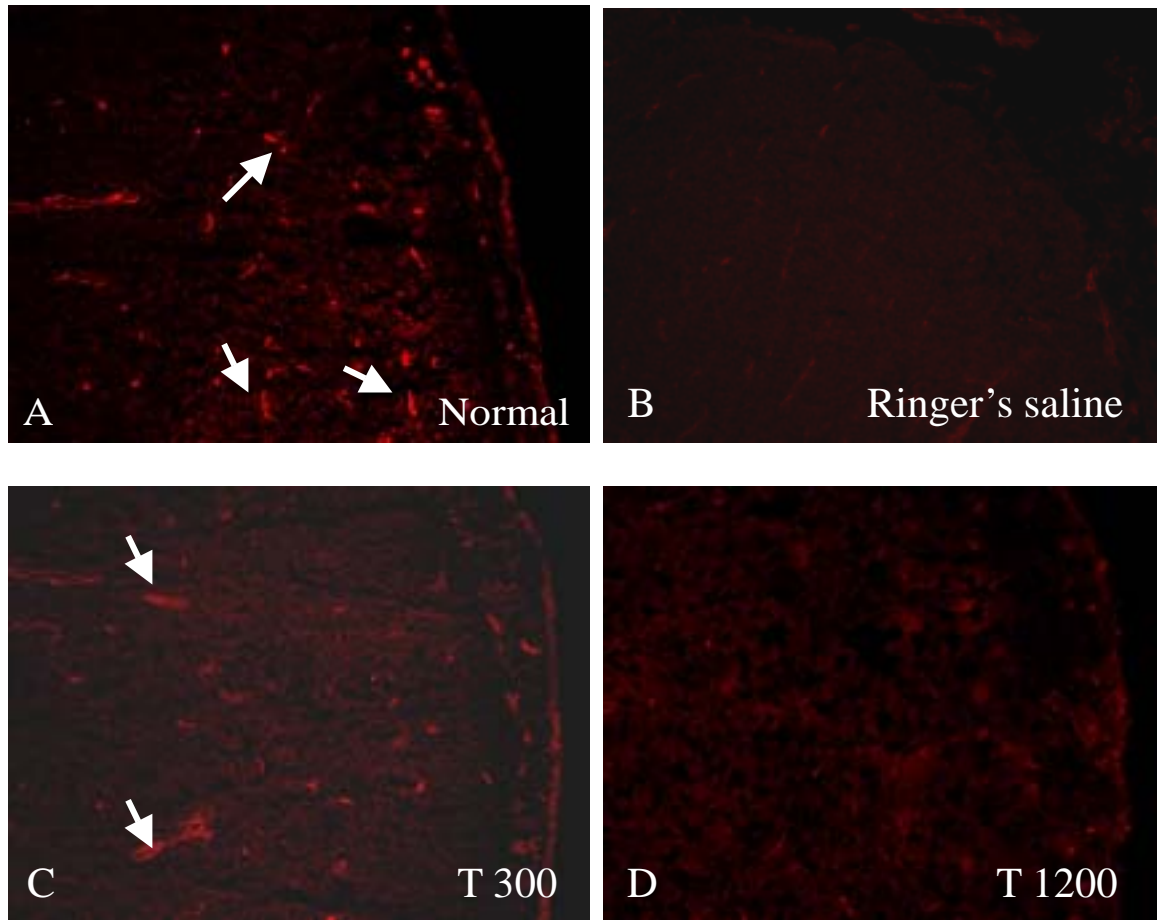
Fig.3. Immunofluorescence of zonula occludents-1（ZO-1 staining, red）shower that periventricular tissue of the left lateral ventricle form rats 24 hours after the build-up of intraventricular hemorrhage（200X magnification）. (A) Normal brain (*white arrows*) (B) Ringer's saline treated brain with the damage in both choroid plexus epithelial cells and in epidermal cells ; (C) 0.5 μg tPA treated brain with the reduction damage of ZO-1 (*white arrows*) ; (D) 2 μg tPA treated brain with. the damage of tight junction of basal laminia on epithelial or endothelial cells of ZO-1.

Howere the fluorescence stain of T1200 (Fig. 4D, white arrows) also presented similar red as Ringer's saline. The findings as indicated 300 u of tPA causing less damage on the cellular matrix of choroid plexuses which in comparison of T1200.

In summary, the reduction of IVH treated by T300 and T1200 was 58% and 81% respectively in this rat study. Immunofluorescence stain of collagen type IV showed somewhat red stain for blood vessels in the lateral ventricular wall by T300 but poor stain by T1200. The immunofluorescence stain of ZO-1 revealed somewhat red stain for tight junction in the lateal ventricular wall by T300, and but no stain of tight junction by T1200. Immunofluorescence stain of MMP-9 showed bright red stain in the choroids plexuses, treated by T1200 rather than T300. It may indicate that the damage of the choroids plexuses induced by IVH, which couldn't be repaired by T1200. Those results demonstrated how tPA best dissolved the blood clots as well as two different dosages affect or even harm choroid plexuses and cerebral ventricular walls in treatment of intraventricular hemorrhage. This data
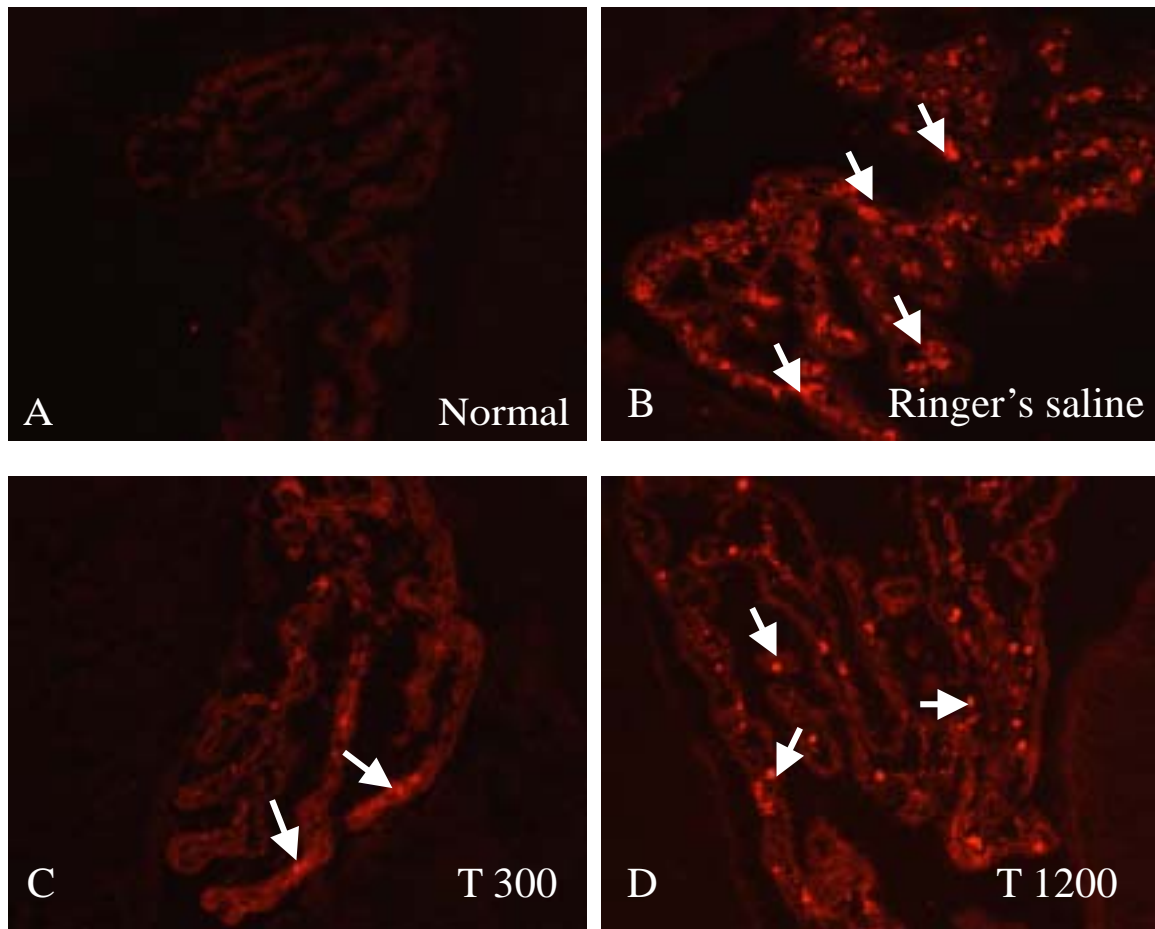
Fig.4. Immunofluorescence of matrix metalloproteinase-9 (MMP-9 staining, red) shower that the choroid plexuses of the left lateral ventricle of rats 24 hours after the build-up of intraventricular hemorrhage (200X magnification). (A) Normal brain   (B) Ringer's saline treated brain with the Numerous immunosignals for MMP-9 (*white arrows*) represented by the choroid plexuses and the extracellular connective tissue; (C) 0.5 µg tPA treated brain with the reduction damage of the choroid plexuses and the extracellular connective tissue (*white arrows*) ; (D) 2 µg tPA treated brain with the damage of the choroid plexuses (*white arrows*)

suggests that T300 may provide an optimal dose of intraventricular injection for IVH, and T1200 did not good contribution of treatment as T300 in rat.

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