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Journal of the Neurological Sciences 247 (2006) 121 – 129

Journal of the Neurological Sciences

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Effect of combined therapy with thrombolysis and citicoline in a rat model of embolic stroke

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Received 28 April 2005; received in revised form 3 January 2006; accepted 3 March 2006
Available online 22 June 2006

Abstract

An approach combining reperfusion mediated by thrombolytics with pharmacological neuroprotection aimed at inhibiting the physiopathological disorders responsible for ischemia-reperfusion damage, could provide an optimal treatment of ischemic stroke. We investigate, in a rat embolic stroke model, the combination of rtPA with citicoline as compared to either alone as monotherapy, and whether the neuroprotector should be provided before or after thrombolysis to achieve a greater reduction of ischemic brain damage. One hundred and nine rats have been studied: four were sham-operated and the rest embolized in the right internal carotid artery with an autologous clot and divided among 5 groups: 1) control; 2) iv rtPA 5 mg/kg 30 min post-embolization 3) citicoline 250 mg/kg ip ×3 doses, 10 min, 24 h and 48 h post-embolization; 4) citicoline combined with rtPA following the same pattern; 5) rtPA combined with citicoline, with a first dose 10 min after thrombolysis. Mortality, neurological score, volume of ischemic lesion and neuronal death (TUNEL) after 72 h and plasma levels of IL-6 and TNF-α, were considered to assess ischemic brain damage. Compared with controls, the use of citicoline after thrombolysis produced the greatest reduction of mortality caused by the ischemic lesion ($p<0.01$), infarct volume ($p=0.027$), number of TUNEL positive cells in striatum ($p=0.014$) and plasma levels of TNF-α at 3 h ($p=0.027$) and 72 h ($p=0.011$). rtPA induced reperfusion provided a slight non-significant reduction of infarct volume and neuronal death, but it reduced mortality due to brain damage ($p<0.01$) although an increase in the risk of fatal bleeding was noted. CiT as monotherapy only produced a significant reduction of neuronal death in striatum ($p=0.014$). The combination of CiT before rtPA did not add any benefit to rtPA alone. The superiority of the combined treatment with rtPA followed by citicoline suggests that early reperfusion should be followed by effective neuroprotection to inhibit ischemia-reperfusion injury and better protect the tissue at risk.

Keywords: Thrombolysis; Neuroprotection; Experimental stroke; Cerebral ischemia; rtPA; Citicoline

1. Introduction

Increasing knowledge about the pathophysiological processes underlying ischemic brain damage [1–6] and the recognition of potentially recoverable tissue after focal cerebral ischemia [7,8] have led to investigation of therapeutic strategies aimed at protecting and restoring the endangered tissue, in order to minimize the clinical consequences of ischemic stroke [9]. The first approach to the treatment was

DOI of original article: 10.1016/j.jns.2006.05.042.
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doi:10.1016/j.jns.2006.03.022

restoration of blood flow with thrombolytics such as recombinant tissue plasminogen activator (rtPA), that have proven efficacy when used early enough in selected subjects [10–13]. However, there is experimental and clinical evidence that reperfusion alone may only be capable of detaining the ischemic cascade if it occurs at very early stages of cerebral ischemia; otherwise it may be insufficient to stop injury mechanisms and may even potentiate them [1,2]. For this reason, research has also been focused on pharmacological inhibition of the biochemical disorders responsible for the progression of damage, which has been called neuroprotection [9]. Citicoline (CiT), a naturally occurring nucleoside essential for the formation of phosphatidyl-choline and subsequently for maintenance of membranes, has demonstrated different neuroprotective actions when administered exogenously in experimental models of focal cerebral ischemia [14–17] and has proven beneficial effects in patients with an acute ischemic stroke [18–20]. However, many other pharmacological approaches investigated for neuroprotection have shown disappointing clinical results [21–23]. This indicates that neuroprotection alone is not sufficient to resolve ischemic damage. Considering the pathophysiology of cerebral ischemia, it seems reasonable to suggest that the best approach to the treatment would be a combination of early reperfusion with effective neuroprotection in order to inhibit all the mechanisms responsible for ischemia-reperfusion damage. Based on this hypothesis, we designed an experimental study in a rat model of embolic stroke aimed at analyzing the effect of thrombolysis with intravenous rtPA or intraperitoneal CiT as either monotherapy or in combination, giving CiT before or after thrombolysis. Our objective was to establish whether combined therapy is superior to either reperfusion or neuroprotection alone, and if the optimal pattern for neuroprotector administration is before or after reperfusion. Clinical, morphological and biochemical parameters were used as outcome measures to be compared between treatment groups.

2. Materials and methods

The procedure was carried out at the Cerebrovascular Research Unit La Paz University Hospital in Madrid, following the recommendations of the Ethics Committee for Animal Experimentation that are in accordance with national and international guidelines for the ethical use of animals for investigation. The experiments were designed to use the smallest number of animals and to minimize their suffering.

2.1. Subjects

Adult male Long Evans rats (250–350 g) were obtained from our breeding colony derived from a stock originally purchased from Janvier (France). Animals were housed with free access to food and water at a room temperature of 21 ±2 °C, relative humidity of 45±15% and light/dark cycle of 12 h (7:00 to 19:00). Rats were subjected to focal cerebral ischemia by autologous clot embolization in the right internal carotid artery.

2.2. Surgical procedure and embolization

Anesthesia was induced by a solution of ketamine (25 mg/ml), diazepam (2 mg/ml), and atropine (0.1 mg/ml) at a dose of 2.5 ml/kg by intraperitoneal injection. Analgesia was provided by meloxicam 2 mg/kg by a subcutaneous route. The femoral vein and artery were cannulated (Centracath Vygon 19G) for administration of substances and for extraction of samples and control of blood pressure and heart rate respectively, as was the external carotid artery (ECA) to introduce the embolus. This consisted of a thrombus 3 mm long by 0.4 mm wide obtained from arterial blood coagulated in a polyethylene tube (Centracath Vygon 19 G, inside diameter 0.5 mm) at 37.5 °C for 40 min. As previously described [24,25], it was introduced in the internal carotid artery (ICA) through a catheter located in the ECA, enabling the blood flow to impel it up to the bifurcation of the intracranial ICA where it impacts due to its diameter, causing interruption of blood flow to middle cerebral artery (MCA) (Fig. 1). Location of clot and proper occlusion of MCA were verified by an angiography (see later). In only a few cases the embolus failed to produce complete interruption of blood flow through MCA and these

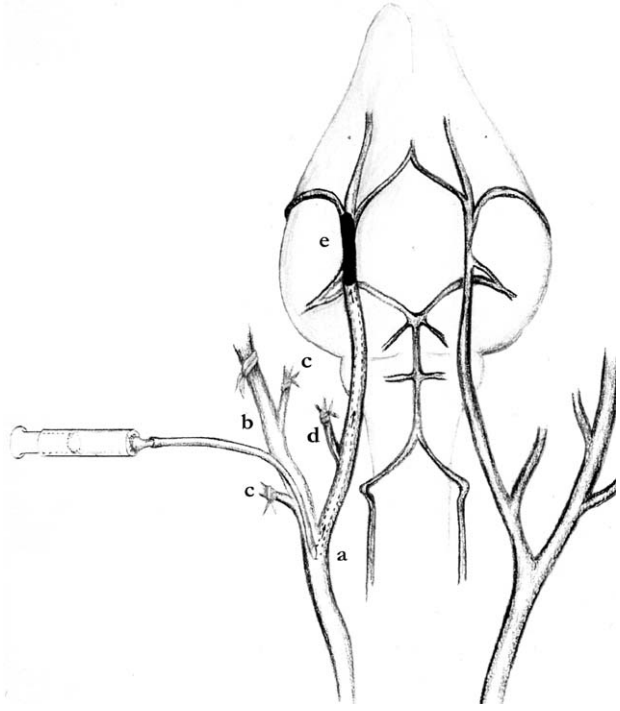

Fig. 1. Surgical procedure for embolization and location of embolus. The clot is released into ICA (a) through a catheter located in external carotid artery (b) after ligature of its branches (c). Pterygopalatine branch of ICA is also ligated (d). Blood flow impels the clot distally up to the bifurcation of ICA where impacts due to its size, occluding MCA at its origin (e). From Sánchez C, Alonso de Leciñana M et al [43] with permission.

Table 1
Study groups and treatments

Group	Ischemia	*n*	Treatment 1	Treatment 2
Sham	−	4	Saline	Saline
Control	+	34	Saline	Saline
rtPA	+	19	rtPA 5 mg/kg i.v.	Saline
Citicoline (CiT)	+	27	Citicoline 250 mg/kg i.p.	Saline
Combination CiT–rtPA	+	12	Citicoline 250 mg/kg i.p.	rtPA 5 mg/kg i.v.
Combination rtPA–CiT	+	13	rtPA 5 mg/kg i.v.	Citicoline 250 mg/kg i.p.

animals were rejected. All animals considered for the study had a complete occlusion of MCA.

2.3. Experimental protocol

Animals were randomly distributed in 6 groups, increasing the sample as necessary to obtain the same number of survivors at 72 h in all groups (Table 1). Sham-operated animals underwent the entire surgical procedure except for embolization and the controls were subjected to embolism, but not to active treatment. These animals received a saline solution instead of the study drugs.

The rats assigned to treatment with rtPA received, beginning 30 min after embolization, an intravenous infusion of 5 mg/kg during 60 min (Actylise. Boehringer Ingelheim). Those treated with CiT (Ferrer International) received 3 intraperitoneal doses of 250 mg/kg; the first was 10 min after the embolization, and the others at 24 and at 48 h later. In the two groups of combined treatment, rtPA was administered in the same way described above. In one of the combined treatment groups (CiT–rtPA) the first dose of CiT was also administered 10 min after embolization (20 min before rtPA) and in the other (rtPA–CiT) the first citicoline dose was administered 10 min after the end of rtPA infusion; the second and third doses of CiT were administered at 24 and 48 h. The animals in the monotherapy groups received a saline solution instead of the drug (Fig. 2, Table 1).

Glycemia, blood gases and blood pressure (Schiller AG CH-6340BAAR) were continuously monitored. Temperature was maintained at 36.5±0.5 °C. Animals with values outside normal limits were rejected.

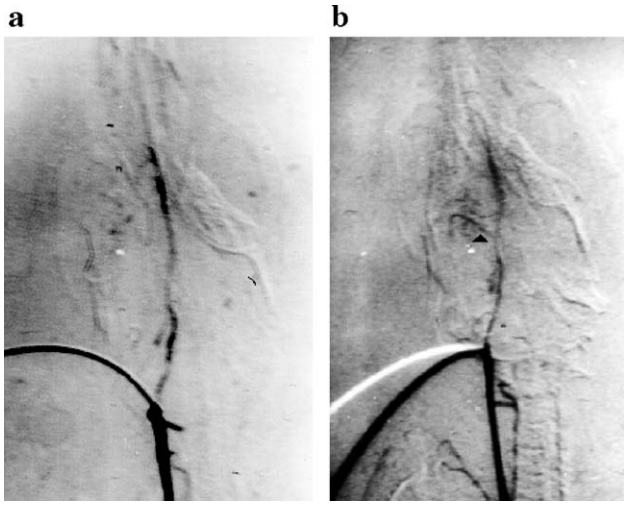

Fig. 3. Example of an angiography vía ECA as performed in all animals. (a) MCA occlusion as shown in all animals considered for the study. (b) Example of recanalization after thrombolytic treatment.

After 72 h of survival, animals were re-anesthetised for sacrifice by transcardial perfusion with a saline solution followed by a fixing solution (4% paraformaldehyde and 0.1% glutaraldehyde in 10% buffered formaline phosphate).

2.4. Angiography

An angiography (Stenoscop General Electric. Exposure: 40 Kv, 0.5 mA) with 0.3 ml of non-ionic contrast (Iohexol. Omnitrast 300. Schering) was performed at 20 min after embolization to verify the arterial occlusion (Fig. 3a) and again at 120 min to check whether recanalization had occurred (Fig. 3b). Animals that did not present arterial occlusion after the first angiography, those that spontaneously recanalized (animals not receiving rtPA that show MCA patency on the second angiogram) or those without recanalization after thrombolysis were rejected. Using these criteria, we ensured that all animals included in the study had a MCA occlusion, that reperfusion only occurred in those receiving rtPA and that all animals receiving the thrombolytic showed MCA recanalization. Therefore we could accurately investigate neuroprotection and thrombolysis as monotherapy and the combination of both, as proposed.

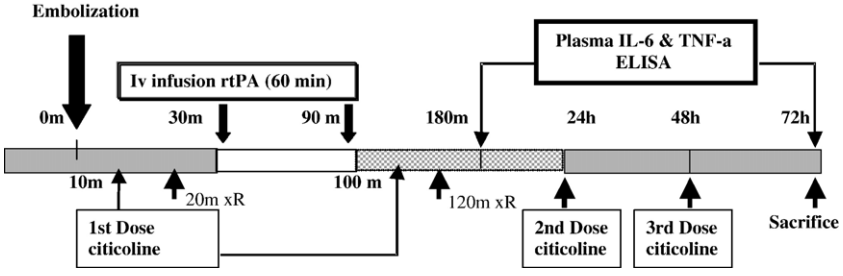

Fig. 2. Schematic representation of the experimental protocol.

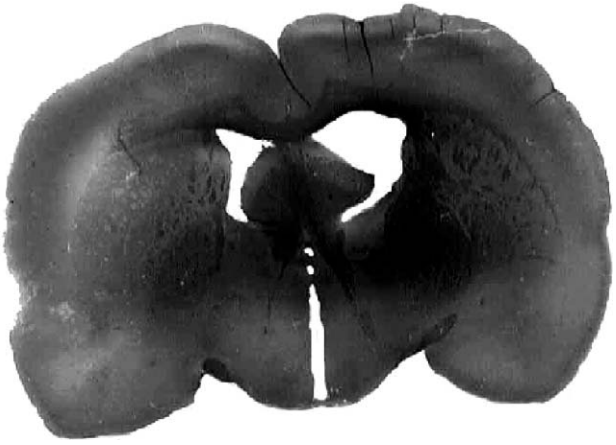

Fig. 4. Coronal section of brain stained with H&E. Infarct is identified as a pale edematous area affecting cortex and caudoputamen in the right hemisphere. As is shown, there was not any ischemic lesion in the contralateral hemisphere.

2.5. Morphological evaluation. Neuropathology and immunohistochemistry

Measurement of lesion size and quantification of neuronal death (necrosis and apoptosis) were considered as the morphological parameters of brain damage in this model. They were only determined in animals that survived 72 h from each group of treatment in order to consider lesions at the same evolutionary stage.

After sacrifice, the brains were removed and postfixed in 10% buffered formaline for 24 h at 4 °C. Brains were sectioned at the optic chiasma and at the infundibular stalk. The resultant blocks of brain between these two cuts were then embedded in paraffin and sectioned in coronal slices 5 μm thick. Every twentieth slice, which means a total of four of these slices (number 1, 21, 41 and 61), separated 100 μm from each other, were stained with haematoxylin-eosin (H&E). H&E staining permits identification of the ischemic lesion as a well-defined pale area (Fig. 4). A digitalized image was obtained from these slices (scanner Epson Perfection 1260) to automatically measure the ischemic area (Image Pro plus 4.0, Media Cybernetics, USA). Morphometric determination of infarct volume and healthy tissue volume were obtained by means of an unbiased estimator of volume based on Cavalieri's principle [27,28]. Using the four slices, volumes of infarct and normal tissue in the right hemisphere were estimated by integrating the partial measures derived from the cross-sectional areas and the distance between sections. The ratio between the volume of the lesion and the total volume of embolized hemisphere (sum of the volumes of healthy and affected tissue in right hemisphere) multiplied by 100, indicated the percentage fraction of embolized hemisphere affected by ischemia. The existence of any damage in contralateral (non-embolized) hemisphere was also investigated.

Neuronal death was evaluated by marking the fragmented nuclear DNA *in situ* using the TUNEL method (TdT mediated biotin dUTP nick-end labelling; TdT-FragEL DNA Fragmentation Detection Kit, Oncogene Research Products) following the methodology indicated by the supplier. TUNEL marks free 3'OH ends of DNA simple chains, thus detecting the DNA fragments in individual necrotic or apoptotic cells that can be distinguished using morphological criteria [29]. TUNEL positive cells (Fig. 5) were counted in all animals in the same predetermined slice of brain located in the central area of infarct (slice number 46) in five microscopic (400×) random fields in the frontal, lateral and piriform areas of the cortex, and in the medial and lateral striatum. The mean of the five counts in each area was calculated. Counts were made both in the embolized and contralateral hemispheres. The results were presented as the total for the embolized hemisphere and then separately for the cortex and striatum.

2.6. Clinical evaluation. Mortality

Before the procedure and at 24 and 48 h after embolization, each animal was given a score on the neurological scale described by Rogers [26]: 0 = No deficit; 1 = failure to extend left forelimb; 2 = decreased grip of the left forelimb while tail pulled; 3 = spontaneous movement in all directions, contralateral circling if pulled by the tail; 4 = circling or walking to the left; 5 = movement only when stimulated; 6 = unresponsive to stimulation; 7 = death. An autopsy was performed on animals that did not survive 72 h to determine the cause of death. The existence and severity of ischemic brain lesion and the occurrence of any bleeding, either intracranial or systemic, were investigated as potential causes of early death.

2.7. Quantification of biochemical markers of brain damage

The pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were considered as

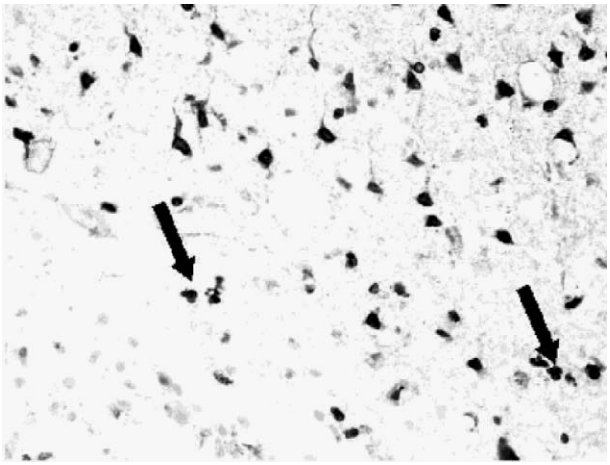

Fig. 5. Tunel positive cells at the infarct border-zone. There were not any TUNEL positive cells in the contralateral hemisphere.

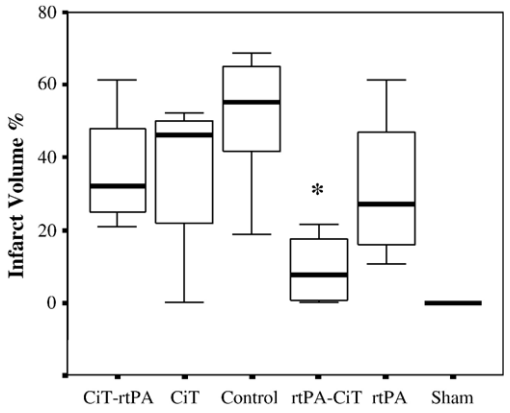

Fig. 6. Infarct volume in each treatment group expressed as a percentage of the embolized hemisphere. Data are median and interquartile ranges. * Significant difference compared with controls (Mann–Whitney test $p=0.027$).

biochemical parameters of brain damage and were determined in plasma by ELISA (Inmunogenetics, S.A.U.) at 3 and 72 h after ischemia.

2.8. Statistical analysis

Quantitative data are shown as medians and interquartile ranges and qualitative data as a percentage. Since data followed a non-normal distribution, non-parametric tests were selected for comparisons between groups. The Kruskal–Wallis test followed by the Mann–Whitney test were used to compare the values of physiological parameters, size of the lesion, number of TUNEL positive cells and plasma levels for inflammatory cytokines between controls and each treatment group. Mortality was compared using the χ^2 test. Values of $p<0.05$ were considered significant. (Statistical software SPSS 10.0 for Windows).

3. Results

The study was performed on one hundred and nine animals pre-assigned to treatment groups as specified in Table 1. Physiological parameters were maintained within normal values; a deviation of less than 25% from normal mean values was accepted. There were no significant differences between groups. There was no correlation between physiological parameters and the different lesion markers nor any significant differences between the animals that died prematurely and the survivors.

In all cases considered for study, the embolus impacted at the same location as demonstrated by angiography. The angiograms showed stop of contrast at the intracranial bifurcation of the ICA with interruption of flow to the MCA at its origin (Fig. 3a). Animals that did not show MCA occlusion at its origin were rejected and not considered for the study. The second angiogram verified complete recanalization of MCA (Fig. 3b) in all the animals treated with rtPA and in none of the animals that did not receive the thrombolytic agent.

3.1. Morphological evaluation. Lesion size and quantification of cell death (TUNEL)

Morphological study included only the animals that survived 72 h and completed the experimental protocol (5 controls and 4 from each of the other study groups). No brain damage (areas of ischemia identified by H&E or neuronal death using the TUNEL method) was observed in the sham group nor in the left non-embolized hemisphere. Volume of infarct expressed as the percentage of affected tissue in right hemisphere was 55.20%, 23.23% (median, interquartile range) in the control group. Compared to controls, all the study treatments reduced the size of the lesion, although this effect was not significant in the groups receiving either rtPA (26.90%, 21.35%) or CiT as monotherapy (45.95%, 16.10%) or in the combination group in which CiT was administered before thrombolysis (31.95%, 13.98%). However, the administration of CiT after reperfusion produced an important reduction in the size of the lesion (7.55%, 14.75%, $p=0.027$) (Fig. 6).

All treatments reduced the number of TUNEL positive cells (Table 2). Considering the whole lesion, differences compared to the controls did not reach statistical significance nor did they when the cortex was studied separately. However, in the striatum, CiT alone and especially when administered after thrombolysis, reduced the number of TUNEL positive cells ($p=0.014$).

3.2. Clinical evaluation, mortality and haemorrhagic complications

The score obtained on the neurological scales at 24 and 48 h is shown in Fig. 7. All sham-operated animals scored 0. There were no significant differences between the treatment groups, although there was a tendency toward a more favourable outcome in animals in whom reperfusion occurred and in those with combined therapy. No correlation was found between neurological score and lesion size, except for the rats that died from brain damage; the lack of correlation is probably due to the variability in lesion size.

Table 2
Number of TUNEL positive cells in the whole lesion and separately in the cortex and in the striatum

Group	N	Total	Cortex	Striatum
Sham	4	0	0	0
Control	5	41.0, 13.0	48.0, 45.0	50.0, 21.0
rtPA	4	27.5, 16.3	35.0, 40.8	21.5, 36.3
CiT	4	16.5, 8.5	29.5, 9.5	10.5, 10.8 *
CiT–rtPA	4	15.5, 9.8	23.0, 25.5	21.0, 21.3
rtPA–CiT	4	6.0, 11.0	0.0, 20.3	4.5, 11.5 *

Data are expressed as median, interquartile range.
* Significant difference (Mann–Whitney test $p=0.014$).

Table 3
Number of deaths and percentage of deaths due to severe ischemic brain damage or haemorrhages in each study group

Group	*n*	Global mortality (%)	Hemorrhage (%)	Cerebral damage (%)	Other causes (%)
Sham	4	0 (0)	0 (0)	0 (0)	0 (0)
Control	34	29 (85.29)	0 (0)*	29 (85.29)	0 (0)
rtPA	19	15 (78.95)	8 (42.11)	7 (36.84)*	0 (0)
Citicoline (CiT)	27	23(85.19)	0 (0)*	20 (74.7)	3 (11.11)
CiT–rtPA	12	8 (66.67)	3 (25)	4 (33.33)*	1 (8.33)
rtPA–CiT	13	9 (69.23)	5 (38.46)	4 (30.77)*	0 (0)

* Significant difference compared to control group ($p<0.01$, χ^2 test).

Mortality rates and causes of death are shown in Table 3. All the study treatments reduced mortality due to brain damage when compared with the control group. This reduction was only significant ($p<0.01$) in the animals in which reperfusion actually occurred and was more significant in the two groups with a combined treatment. Severe ischemic brain damage was the cause of death in all the animals from the control group, while only 36.84% died due to this cause in the rtPA-treated group and approximately 1/3 in the combined treatment groups. Intraperitoneal bleeding as the cause of death occurred in 42.11% of the animals treated with rtPA. There were no intracranial haemorrhages and only slight bleeding at the surgical wounds that was not clinically relevant. Surviving animals from the rtPA-treated groups showed no bleeding complications. Animals not receiving rtPA had no bleeding complications.

3.3. Inflammatory cytokines

There were no differences in plasma levels of TNF-α and IL-6 between the sham and control groups, especially 72 h after ischemia when both cytokines were highest (Figs. 8 and 9).

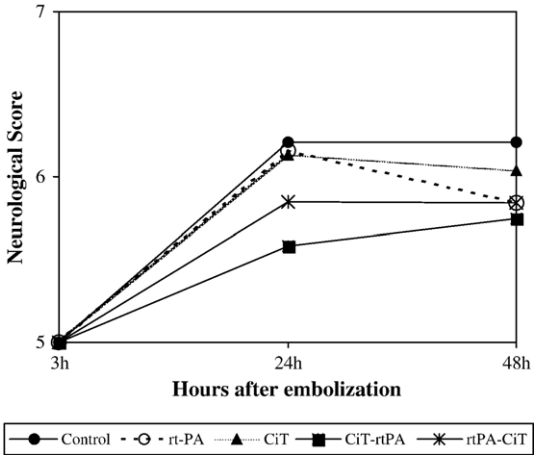

Fig. 7. Mean neurological score at 24 and 48 h in each treatment group.

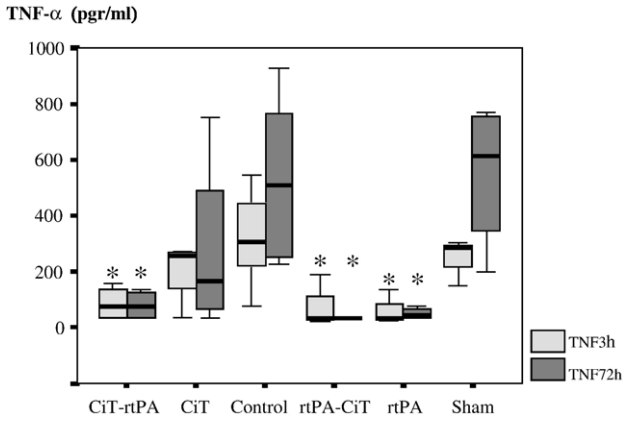

Fig. 8. Plasma levels of TNF-α (median, interquartile range). * $p<0.05$ comparing with controls (Mann–Whitney test).

At 3 h after ischemia, TNF-α was higher in the controls (304.0, 225.0)(median, interquartile range) than in any of the treatment groups. It was lower after reperfusion in animals treated with rtPA (34.0, 28.0, $p=0.027$), the combination of CiT–rtPA (75.0, 92.5, $p=0.049$) and rtPA–CiT (34.0, 42.0, $p=0.027$) and there was no significant difference with those treated with CiT in monotherapy (256.0, 75.0). At 72 h after ischemia, it increased most in the controls (510.0, 514.2) and somewhat less in the CiT monotherapy group (164.0, 275.5) but remained unchanged or had even dropped in the animals treated with rtPA (34.0, 25.5, $p=0.014$), CiT–rtPA (73.0, 84.0, $p=0.014$) or rtPA–CiT (34.0, 0.0, $p=0.011$) (Fig. 8).

There were no significant differences between groups in blood IL-6 at either 3 h (control: 920.0, 1005.4; CiT: 607.5, 451.0; rtPA: 281.5, 208.0; CiT–rtPA: 318.0, 161.5; rtPA–CIT: 325.0, 120.0) or 72 h (control: 1400.0, 1318.6; CiT: 558.0, 433.0; rtPA: 542.0, 453.8; CiT–rtPA: 726.5, 283.8; rtPA–CiT: 374.5, 95.3) (Fig. 9).

There was no correlation between inflammatory cytokines and histological damage (infarct volume or TUNEL) in any group.

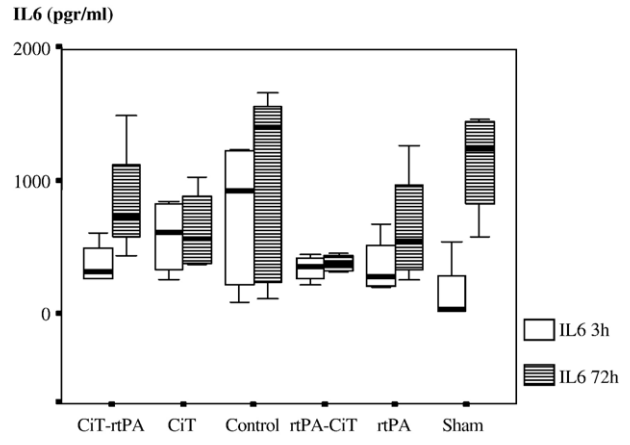

Fig. 9. Plasma levels of IL6 (median, interquartile range).

4. Discussion

The results of our study sustain the hypothesis that the optimal treatment of ischemic stroke should associate early thrombolysis with neuroprotection provided after reperfusion. The combination of citicoline after systemic thrombolysis with rtPA achieved better results than either each one as monotherapy or than the combination of CiT before rtPA. The benefits include significant reduction of infarct volume and neuronal death together with a reduction of mortality due to brain damage and a moderate improvement in clinical outcome. Thrombolysis with rtPA as monotherapy shows moderate benefits in mortality reduction that are partially masked by haemorrhagic complications. CiT as monotherapy at the dose given shows the lowest benefit when considering the combination of morphological, clinical and biochemical parameters used in this study as outcome measures, as it only reduced the number of TUNEL positive cells in striatum. The combination of CiT before rtPA does not add any benefit to rtPA alone.

The election of CiT was based on its well-known mechanisms of action, which define it as a potentially effective neuroprotector that may act both in early and late stages of ischemic damage. CiT stabilizes and repairs membranes [14], favours the synthesis of nucleic acids, proteins, acetylcholine and other neurotransmitters, inhibits free fatty acid release [15,16] and has antiapoptotic effects [17]. Due to these effects, CiT may simultaneously inhibit different steps of the ischemic cascade thus protecting the targets (membranes, nucleus…) against early and delayed mechanisms responsible for ischemic brain injury. The dose was chosen considering its efficacy in earlier studies [29,30] although it has been demonstrated that higher doses of CiT produce a greater reduction of brain damage in experimental strokes [31,32]. We decided to use a suboptimal dose of CiT to more easily evaluate the synergistic effect of the combined treatment with thrombolysis and the potential differences between the two combined therapy regimes. Dosage was administered over 48 h to ensure maintenance of its prolonged effects beyond the acute phase in order to improve neuroprotection.

CiT as monotherapy failed to produce a significant reduction of lesion size although it effectively did so in earlier experimental studies [29,33,34]. As a consequence, mortality due to brain damage was neither reduced nor was clinical outcome improved by CiT. This could be explained by the low dosage and also by the fact that, when administered at low dose and not preceded by reperfusion, it probably reached the ischemic tissue at very low concentrations. However CiT as monotherapy reduced the neuronal death in the striatum indicating some neuroprotective effect of the dose regime used, and also tended to reduce plasma expression of TNF-α.

Intravenous rtPA produced efficacious thrombolysis as demonstrated by MCA recanalization on angiography. The dose of rtPA used in this study is higher than that used in humans. It is known that in rats, rtPA must be given at doses 10 times higher than in humans to have an equal effect [25,35–37]. The dose of 5 mg/kg provides arterial recanalization and is associated with a theoretically lower bleeding risk [30]. Despite MCA recanalization and restoration of blood flow, rtPA as monotherapy produced only a slight and non-significant reduction of infarct volume (Fig. 7) and did not reduce cell death quantified by TUNEL. Some previous experimental studies have shown a reduction of infarct size with thrombolysis as monotherapy [35]. Reperfusion provided by early thrombolysis may inhibit the initial disorders in the ischemic cascade, but as has been previously suggested, it might not be sufficient to inhibit delayed mechanisms of damage such as apoptosis, and furthermore, it may add deleterious effects due to enhancement of injury mechanisms if reperfusion does not occur early enough. We suggest that the failure of rtPA to reduce brain lesion in this study might be explained by the so-called reperfusion damage. Another possible explanation could be the occurrence of distal occlusion of the microvasculature by microthrombi generated after lysis of the main clot provided by rtPA. Our methodology did not allow us to discard this possibility, as angiograms do not have enough spatial resolution. The lack of significance of the slight benefit produced by reperfusion regarding infarct volume may be due in part to the variability of lesion size in this experimental model of focal cerebral ischemia. Despite the slight effect on infarct volume, reperfusion achieved a significant reduction of mortality due to brain damage, although this beneficial effect was masked by an excess of fatal bleeding complications. There was not a significant benefit on clinical outcome associated to reperfusion alone.

Administration of CiT before thrombolysis was not superior to either CiT or rtPA as monotherapies in terms of reducing infarct volume, neuronal death, clinical outcome, mortality, nor plasma levels of inflammatory cytokines; however when it was used in combination after rtPA therapy, there was a significant benefit considering all morphological clinical and biochemical parameters used to evaluate the ischemic lesion. This result would support the hypothesis that combined neuroprotection after thrombolysis is necessary to optimise results of ischemic stroke treatment. Other experimental studies have demonstrated the superiority of combining thrombolysis and different neuroprotectors [37,38]. Specifically, the association of CiT with rtPA [30] and urokinase [34], giving the first CiT dose before or simultaneously to thrombolysis produced a greater reduction in brain lesion than when either one was used separately in animal models of ischemic stroke. However, to our knowledge, no previous evaluation of administering CiT once reperfusion has occurred compared to administration before thrombolysis, has yet been published. The superiority of combining the neuroprotector after thrombolysis could be related to a more effective supply of the neuroprotector to the area of penumbra after reperfusion, thus increasing its inhibition of the ischemic cascade and reperfusion injury.

This experimental rat model can be useful in preclinical studies of thrombolytics and neuroprotectors since it resembles human embolic stroke. However, this particular model produces large lesions affecting the cortex and the basal ganglia that vary in size even though the site of occlusion, duration of ischemia, experimental conditions, metabolic homeostasis and hemodynamics are all constant between groups. The variability of lesions reduce study yield and make it difficult to obtain statistically significant results when considering infarct volume as the unique outcome measure [22]. This variability could be explained in part by inter-individual differences in the efficacy of the collateral leptomeningeal circulation to the area of brain dependent on the occluded MCA, which may account for variable residual blood flow and for a variable amount of viable tissue after MCA occlusion. The striatal lesion in this model is less variable than the cortical lesion, because the striatum is supplied by terminal branches from MCA, while the cortex has more effective collateral circulation [3,4,22]. This might explain why the benefit of treatments in regards to reduction of neuronal death (TUNEL) reaches significance in striatum and not in the cortex. The possibility of distal migration of fragments of clot resulting from thrombolysis with random occlusion of the microvasculature could also account for the variability observed as has been suggested. The difficulties caused by the variability of damage produced may be overcome if a combination of different measurements of damage, rather than lesion size alone, are considered for preclinical evaluation of a therapeutic agent using this model. This study has considered a combination of clinical (reduction of mortality and neurological scale score), morphological (infarct volume and TUNEL) and biochemical (inflammatory cytokines) parameters. It is of particular interest to consider any reduction of mortality due to brain damage as an outcome measure for evaluation of treatments, since the model associates high mortality rates due to the severity of the ischemic lesions it produces. The seriousness of brain damage is illustrated by the high scores obtained on the neurological scale. Nevertheless, the neurological score did not correlate well with infarct volume in this model, again probably due to the variability described.

Inflammatory mechanisms mediated by cytokines have been implicated in the pathophysiology of ischemic cerebral damage as injury mechanisms [39,40]. IL-6 and TNF-α have been shown to increase in plasma, cerebrospinal fluid and brain tissue after focal cerebral ischemia and to correlate with the severity of damage [39,41,42]. In our study, plasma levels of TNF-α and IL-6 increased in both sham-operated animals and those with cerebral infarction. These findings suggest that plasmatic expression of IL-6 and TNF-α could be an unspecific response to tissue damage independent of its aetiology or location and that, after focal cerebral ischemia, they could be considered as biochemical markers of the severity of damage and not indicators of a pathogenic pathway for brain injury. Plasmatic quantification of these cytokines could therefore be used as an outcome measure for treatment evaluation. However, in this work, levels of these cytokines did not correlate with infarct volume nor with neuronal death, once again due to variability of results, and this reduces the yield of cytokine measurements.

In conclusion, the results of our work suggest that, although citicoline and rtPA as monotherapies both produce some reduction of ischemic brain damage, the combination of the two is better. Considering different clinical, morphological and biochemical indicators of the seriousness of the ischemic damage, administration of citicoline after thrombolysis seems to be the most effective treatment as it produces the greatest reduction of infarct size, neuronal death, plasma expression of TNF-α and mortality. The superiority of this combined treatment regime supports the hypothesis that early reperfusion should be followed by effective neuroprotection to inhibit ischemia-reperfusion injury and to better protect the tissue at risk.

Acknowledgments

Supported by grants from Ferrer International S.A. and from the Carlos III Health Institute, Spanish Ministry of Health.

We thank Ferrer International for the supply of citicoline.

We thank C. F. Warren and J. Siegfried for their linguistic assistance.

References

[1] Siesjö BK. Pathophysiology and treatment of focal cerebral ischemia: Part I. Pathophysiology. J Neurosurg 1992;77:169–84.
[2] Siesjö BK. Pathophysiology and treatment of focal cerebral ischemia: Part II. Mechanisms of damage and treatment. J Neurosurg 1992;77:337–54.
[3] García JH, Liu KF, Ho KL. Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex. Stroke 1995;26:636–43.
[4] García JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. Stroke 1995;26:627–35.
[5] Guégan C, Boutlin H, Boudry C, McKenzie ET, Sola B. Apoptotic death in cortical neurons of mice subjected to focal ischemic. C R Acad Sci III 1996;319:879–85.
[6] Benchoua A, Guégan C, Couriaud C, Hosseini H, Sampaio N, Morin D, et al. Specific caspase pathways are activated in two stages of cerebral infarction. J Neurosci 2001;21:7127–34.
[7] Hossmann KA. Viability thresholds and the penumbra of focal ischemia. Ann Neurol 1994;36:557–65.
[8] Baron JC. Perfusion thresholds in human cerebral ischemia: historical perspective and therapeutic implications. Cerebrovasc Dis 2001;11 (suppl 1):2–8.
[9] Martínez-Vila E, Irimia P. Current status and perspectives of neuroprotection in ischemic stroke treatment. Cerebrovasc Dis 2001;11(suppl 1):60–70.
[10] The National Institute of Neurological Disorders, Stroke rtPA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. N Engl J Med 1995;333:1581–7.
[11] Hacke W, Kaste M, Fieschi C, von Kummer R, Davalos A, Meier D, et al. Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Lancet 1998;352:1245–51.

[12] Hacke W, Brott T, Caplan L, Meier D, Fieschi C, von Kummer R, et al. Thrombolysis in acute ischemic stroke: controlled trials and clinical experience. Neurology 1999;53(supp 4):S3–S14.
[13] Hacke W, Donan G, Fieschi C, Kaste M, von Kummer R, Broderick JP, et al. Association of outcome with early stroke treatment: pooled análisis of ATLANTIS, ECASS and NINDS rt-PA stroke trials. Lancet 2004;363:768–74.
[14] Tovarelli G, DeMedio G, Dorman R, Piccinin G, Horrocks L, Porcellati G. Effects of cytidine diphosphate choline (CDP-choline) on ischemia-induced alteration of brain lipid in the gerbil. Neurochem Res 1981;6:821–33.
[15] Weiss GB. Metabolism and actions of CDP-choline as an endogenous compound and administered exogenously as citicoline. Life Sci 1995;56:637–60.
[16] Secades JJ. CDP-choline: updated pharmacological and clinical review. Methods Find Exp Clin Pharmacol 2002;24(suppl 2):1–56.
[17] Krupinski J, Ferrer I, Barrachina M, Secades JJ, Mercadal J, Lozano R. CDP-choline reduces pro-caspase and cleaved caspase-3 expression, nuclear fragmentation, and specific PARP-cleaved products of caspase activation following middle cerebral artery occlusion in the rat. Neuropharmacology 2002;42:846–54.
[18] Clark WM, Weschler LR, Sabounjian LA, Schwiderski UE, For the citicoline Stroke Study Group. A phase III randomized efficacy trial of 2000 mg citicoline in acute ischemic stroke patients. Neurology 2001;57:1595–602.
[19] Clark WM, Williams BJ, Selzer KA, Zweifler RM, Sabounjian LA, Gammans RE. A randomized efficacy trial of citicoline in patients with acute ischemic stroke. Citicoline stroke study group. Stroke 1999;30:2592–7.
[20] Davalos A, Castillo J, Alvarez-Sabin J, Secades JJ, Mercadal J, Lopez S, et al. Oral citicoline in acute ischemic stroke: an individual patient data pooling analysis of clinical trials. Stroke 2002;33:2850–7.
[21] Grotta JC. Acute stroke therapy at the millennium: consummating the marriage between the laboratory and the bedside. The Feinberg lecture. Stroke 1999;30:1722–8.
[22] Alonso de Leciñana M, Díez-Tejedor E, Carceller F. Cerebral ischemia: from animal studies to clinical practice. Should the methods be reviewed? Cerebrovasc Dis 2001;11(suppl 1):20–30.
[23] Gladstone DJ, Black SE, Hakim AM, for the Heart and Stroke Foundation of Ontario Centre of Excellence in Stroke Recovery. Toward wisdom from failure. Lessons from neuroprotective stroke trials and new therapeutic directions. Stroke 2001;33:2123–36.
[24] Overgaard K, Sereghy T, Boysen G, Pedersen H, Hoyer S, Diemer NH. A rat model of reproducible cerebral infarction using thrombotic blood clot emboli. J Cereb Blood Flow Metab 1992;12:484–90.
[25] Alonso de Leciñana M, Díez-Tejedor E, Carceller F, Vega A, Roda JM. Advantages of associate inhibitors of ischemia reperfusion injury to intravenous thrombolysis for treatment of focal cerebral ischemia. Description of an experimental model. Rev Neurol 1996;24:207–13.
[26] Rogers DC, Campbell CA, Stretton JL, Mackay KB. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 1997;28:2060–6.
[27] Gundersen HGJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 1988;96:379–94.
[28] Avendaño C, Roda JM, Carceller F, Díez-Tejedor E. Morphometric study of focal cerebral ischemia in rats: a stereological evaluation. Brain Res 1995;673:83–92.
[29] Sobrado M, López M, Carceller F, García A, Roda JM. Combined nimodipine and citicoline reduce infarct size, attenuate apoptosis and increase bcl-2 expression after focal cerebral ischemia. Neuroscience 2003;118:107–13.
[30] Andersen M, Overgaard K, Meden P, Boysen G. Effects of citicoline combined with thrombolytic therapy in a rat embolic stroke model. Stroke 1999;30:1464–71.
[31] Schäbitz WR, Weber J, Tacaño K, Sandage Jr BW, Locke KW, Fisher M. The effects of prolonged treatment with citicoline in temporary experimental focal ischemia. J Neurol Sci 1996;138:21–5.
[32] Hurtado O, Moro MA, Cardenas A, Sanchez V, Fernandez-Tome P, Leza JC, et al. Neuroprotection afforded by prior citicoline administration in experimental brain ischemia: effects on glutamate transport. Neurobiol Dis 2005;18:336–45.
[33] Aronowski J, Strong R, Grotta JC. Citicoline for treatment of experimental focal ischemia: histologic and behavioral outcome. Neurol Res 1996;18:570–4.
[34] Shuaib A, Yang Y, Li O. Evaluating the efficacy of citicoline in embolic ischemic stroke in rats: neuroprotective effects when used alone or in combination with urokinase. Exp Neurol 2000;161:733–9.
[35] Overgaard K, Sereghy T, Boysen G, Pedersen H, Diemer NH. Reduction of infarct volume and mortality by thrombolysis in a rat embolic stroke model. Stroke 1992;23:1167–74.
[36] Overgaard K. Thrombolytic therapy in experimental embolic stroke. Cerebrovasc Brain Metab Rev 1994;6:257–86.
[37] Overgaard K, Sereghy T, Pedersen H, Boysen G. Neuroprotection with NBQX and thrombolysis with rt-PA in rat embolic stroke model. Neurol Res 1993;15:344–9.
[38] Sereghy T, Overgaard K, Boysen G. Neuroprotection by excitatory aminoacid antagonist augments the benefit of thrombolysis in embolic stroke in rats. Stroke 1993;24:1702–8.
[39] Fassbender K, Rossol S, Kammer T, Daffertshofer M, Wirth S, Dollman M, et al. Proinflammatory cytokines in serum of patients with acute cerebral ischemia: kinetics of secretion and relation to the extent off brain damage and outcome of disease. J Neurol Sci 1994;122:135–9.
[40] Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, et al. Tumor necrosis factor. A mediator of focal ischemic brain injury. Stroke 1997;28:1233–44.
[41] Vila N, Castillo J, Dávalos A, Chamorro A. Proinflammatory cytokines and early neurological worsening in ischemic stroke. Stroke 2000;31:2325–9.
[42] Clark WM. Cytokines and reperfusion injury. Neurology 1997;49 (suppl 4):S10–4.
[43] Sánchez C, Alonso de Leciñana M, Díez-Tejedor E, Carceller F, Vega A, Frade JM. Treatment of embolic cerebral infarct in rats by means on thrombolysis and cytoprotection with U-74389-G. Rev Neurol 1998;27:653–8.