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Thrombolysis and Neuroprotection in Cerebral Ischemia

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Abstract
Stroke is a major cause of death and disability worldwide. The resulting burden on society grows with the increase in the incidence of stroke. The term brain attack was introduced to describe the acute presentation of stroke and emphasize the need for urgent action to remedy the situation. Though a large number of therapeutic agents, like thrombolytics, NMDA receptor antagonists, calcium channel blockers and antioxidants, have been used or are being evaluated, there is still a large gap between the benefits of these agents and the properties of an ideal drug for stroke. So far, only thrombolysis with rtPA within a 3-hour time window has been shown to improve the outcome of patients with ischemic stroke. Understanding the mechanisms of injury and neuroprotection in these diseases is important to target news sites for treating ischemia. Better evaluation of the drugs and increased similarity between the results of animal experimentation and in the clinical setting requires critical assessment of the selection of animal models and the parameters to be evaluated. Our laboratory has employed a rat embolic stroke model to investigate the combination of rtPA with citicoline as compared to monotherapy alone and investigated whether neuroprotection should be provided before or after thrombolysis in order to achieve a greater reduction of ischemic brain damage.

Physiopathologic Cerebral Ischemia

The significant advances in our understanding of the physiopathological mechanisms of cerebral ischemia are leading to a considerable development of drugs that, at various levels, can block or modify the chain of biochemical processes set off by the hypoperfusion of the cerebral parenchyma.

The ischemic cascade starts within seconds to minutes of loss of perfusion. Protein synthesis initially ceases as the ischemic neuron attempts to conserve its rapidly waning energy store. Membrane ion-transport systems fail, and the neuron becomes depolarized. Membrane depolarization results in calcium influx, which in turn causes

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the release of stored neurotransmitters like glutamate, the major excitatory neurotransmitter in the brain. This release worsens the cellular insult by further increasing intracellular calcium and depolarizing other metabolically compromised neurons. The massive calcium influx stimulates several enzymes, which become unregulated and may provoke the destruction of cellular homeostatic mechanisms. Free radical formation and nitric oxide synthesis contribute to neuronal damage. Hours to days after a stroke, the ischemic territory activates specific genes and the consequent cytokine and cell adhesion molecule formation stimulates local inflammation and may further impair microcirculatory blood flow. Last, the activation of apoptotic genes may promote programmed cell death in the surviving neurons.

Ischemic Penumbra

It appears that intervention must occur very early for any substantial portion of brain tissue to be preserved. The penumbral region is fundamentally salvageable and is therefore the most important target of therapy for acute stroke. If the target of acute stroke therapy is the ischemia core, where the neurons most severely affected by oxygen starvation die rapidly, only fast and effective reperfusion strategies can reverse the blockage of the blood supply and potentially increase the flow above the critical threshold, before the cells are irreversibly damaged [1]. Bordering the core of the ischemia is the penumbra zone [2] where blood flow gradually drops below the functional threshold but is still sufficient to maintain morphological integrity for a certain time, but this depends on the degree of the residual perfusion [1]. This penumbra zone is usually considered the most promising target for acute stroke therapy because the therapeutic window can last several hours [3] and because these areas can be revealed by functional neuroimaging modalities [4]. Again the penumbra would benefit mainly from sufficient reperfusion before irreversible cell damage has occurred, but additional neuroprotective agents targeted at various steps in the pathobiochemical cascade could help, or might even be necessary, to prevent or mitigate secondary ischemic cell damage. The rate of progression of the penumbra from reversible to irreversible ischemic injury depends on many variables and may be accelerated in the presence of poor collateral circulation, hyperglycemia, and other exacerbating factors [4]. If reversible ischemia is not present at the time of treatment, then neuroprotective therapy cannot be expected to work.

Therapeutic Window

This therapeutic window of opportunity, specifically the time between the occurrence of the stroke and the time that treatment is initiated, has, until recently, often been assumed to differ in animals and humans. That is, there is the view that damage develops more slowly in human brains and that a short time window in a rat model did not preclude giving the drug after a longer interval between stroke and administration in humans. A good example of this is the clinical investigation of NMDA antagonist. Despite substantial evidence that these compounds only provide protection when given shortly after (60–90 min) the ischemic insult [5], they have nevertheless been administered to stroke patients up to 6 h after stroke onset [6]. The predominant reason is probably practicality because it is difficult to get patients to hospital and diagnosed within 90 min of stroke onset whereas 6 h is a reasonable time frame for presentation and treatment. Indeed, the problems of carrying out a clinical trial within a short time frame are substantial. However, the success of the tissue plasminogen activator (tPA) trial with a 3-hour time window [7] shows that such studies are possible. It is noteworthy that tPA is also effective in animal stroke models within the same time frame [8], supporting the idea that animals and humans may be similar in the time their window of opportunity is open. Most investigated compounds act on the early events in the neurodegenerative cascade. Consequently, one can extrapolate that these drugs ought to be given rapidly after the ischemic insult if they are to be of any value. If, as we believe, the time window of neurodegenerative events is similar in experimental animals and humans, then we must use one of two approaches: (1) administer the drug very soon after the stroke – an approach that is practically very difficult, or (2) develop a compound acting on a later part of the ischemic cascade that can be given some time after the ischemic insult, indicating its practicality for clinical practice.

Development of Acute Stroke Therapies

The two most important therapeutic approaches in acute cerebral ischemia consist of improving cerebral blood flow by early reperfusion and blocking the biochemical and metabolic changes at the ischemic cascade level. Most likely, the effective time windows for these treatments are different: rather short for effective reperfusion, probably because of the hemorrhagic complica-

tions associated with late reperfusion of ischemic brain tissue, and later for neuroprotection, and particularly prolonged in the anti-inflammatory and antiapoptotic approaches. Reperfusion induced by thrombolysis has been shown to be effective when initiated within 3 h of symptom onset [7]. In contrast, neuroprotective strategies have been disappointing clinically so far and have not improved stroke outcome [9–11], although significant reductions of infarct size were demonstrated in animal models with the use of strategies to antagonize the various steps in the excitotoxic cascade [9, 12], and inhibit free radical toxicity [13, 14], harmful secondary inflammatory mechanisms [15] and attenuate cell death due to apoptosis [16, 17]. The discrepancy between animal models results and clinical efficacy of the neuroprotective drugs is probably due to the limits of animal models in reflecting complex clinical stroke.

Animals Models of Cerebral Ischemia

The efficiency of various neuroprotective strategies is well documented in animal experiments but has thus far given disappointing results in ischemic stroke. The different causes of discrepancies between the animal models and clinical studies depend on both the drugs studied and the design of the experimental model and clinical study [18, 19].

Neuroanatomical, pathophysiological and metabolic differences exist between the rat, the animal most often used in preclinical studies of neuroprotective therapies, and humans, and these differences may help explain why the results of experimental studies are generally more favorable.

The objective of an experimental animal model is to achieve homogeneous and reproducible lesions with a minimum of variability, so as to maximize reliability and results.

The most appropriate model must be chosen when designing the experimental investigation. There are various models of focal cerebral ischemia [18], although the ones most frequently used at present are the model of middle cerebral artery ligation after craniotomy [20, 21], the middle cerebral artery intraluminal occlusion model, inserting a filament via the internal carotid artery [22] and the model of occlusion with autologous blood clot emboli [23, 24] (fig. 1a). The first produces very homogeneous cortical lesions but is traumatic and not very physiological, and is the furthest removed from clinic. It is very useful in pathophysiological studies of ischemia thanks to its regular results and can be very useful in the study of neuroprotective agents for demonstrating a certain effect on the lesion, but its results are hard to reproduce in clinical trials for all the reasons mentioned above. On the other hand, the intraluminal occlusion models, particularly the embolic method, are more similar to the cerebral infarction produced by arterial emboli in humans, and produce to very extensive lesions of widely varying size which affect basal ganglia and the cortex and cause high mortality. This model is very attractive to study neuroprotectors, particularly in combination with pharmacological thrombolysis, but it has the inconvenience of being much less cost-effective due to the variability of the resulting lesions and high mortality.

Thrombolysis for Ischemic Stroke

Once we have produced a stroke we then experiment with methods to prevent or protect against its effect. One method is thrombolysis to restore cerebral blood flow.

The aim of thrombolytic therapy is to lyse an occluding thrombus or embolus and reduce the volume of irreversibly damaged cerebral tissue. However, a major complication of thrombolysis in stroke is cerebral hemorrhage, which would offset any beneficial effects.

Restoration of cerebral blood flow after an acute vascular occlusion may be achieved by the administration of thrombolytic agents. Reperfusion plays an important role in the pathophysiology of cerebral ischemia. tPA and streptokinase are of effective in acute ischemic stroke and are the most extensively studied agents for thrombolysis in stroke. However, the results of streptokinase and tPA studies are not directly comparable. The mechanisms of action of the two agents differ substantially, and the prolonged and nonspecific systemic lytic effects of streptokinase may have contributed to the high risk of hemorrhage.

The specific choice of thrombolytic drug to treat acute stroke depends on several pharmacokinetic factors. The timing of thrombolysis is of paramount importance. Ischemic brain tissue may be salvageable if reperfusion occurs before the tissue is irreversibly damaged, and moreover, the risk of hemorrhage appears to increase once the ischemic tissue becomes edematous [7, 9]. The time between symptoms onset and initiation of medication and the dose levels of the thrombolytic agents are important determinants for the risk of cerebral hemorrhage. Thrombolysis is an effective therapy for acute stroke, but only one thrombolytic agent, tPA, has proven efficacy and

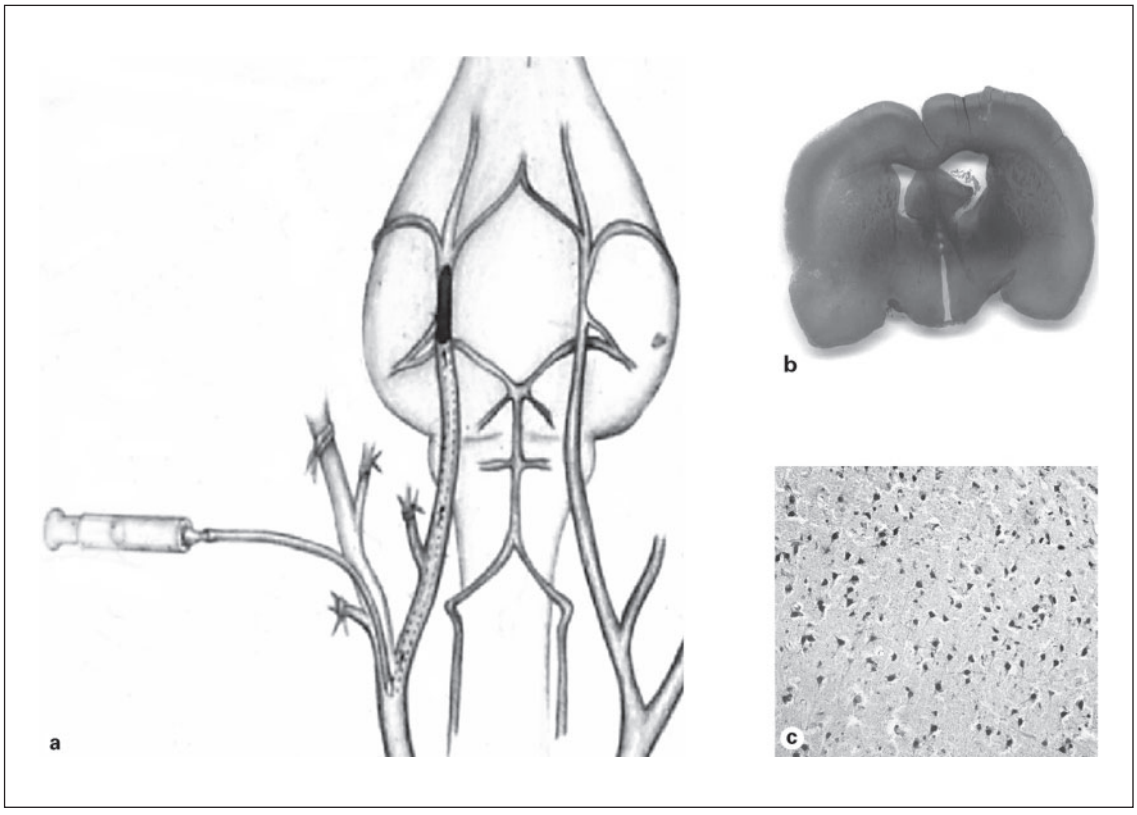

Fig. 1. a Schematic representation of experimental embolization procedure. **b** Infarct volume identified in a coronal section of brain stained with HE. **c** TUNEL-positive cells at the infarct border zone.

safety. Early and rapid assessment is essential because animal and human studies have shown that treatment must begin in the 3 h after stroke onset. A major limitation in thrombolysis for acute ischemic stroke is this restricted time window, and any method that could widen the reperfusion time window would be important. Only a small proportion of acute stroke patients are currently eligible for thrombolysis, mainly because of excessive delay in reaching the presenting at the hospital.

Thrombolysis with intravenous tPA has been demonstrated to be an effective treatment for acute ischemic stroke with unselected subtypes of vasculo-occlusive disease. Unfortunately, there is a substantial risk of cerebral hemorrhage when thrombolytic agents are used in the setting of cerebral ischemia [7, 25, 26]. This risk of hemorrhage is greatest in patients with the most severe neurologic deficits and they have the least chance for a good outcome [25]. Strategies that provide better information regarding the response to thrombolysis may help evaluate patients' identity for intravenous tPA or alternative therapies. Theoretically, patients with smaller and more distal clots represent a subset of patients with a greater probability of benefit from tPA due to less severe deficits at onset, smaller volumes of cerebral ischemia and a greater likelihood of adequate collateral circulation. These patients may also have a lower risk of intracerebral hemorrhage due to their smaller volume of tissue injury [27, 28]. The recommendation for the intravenous administration of rtPA within 3 h of stroke onset in carefully selected patients should not be changed [29, 30]. The evidence is strong that all delays in treating patients should be avoided.

Neuroprotective Therapies

At present, no agent with putative neuroprotective effects can be recommended for the treatment of patients with acute ischemic stroke [29, 30].

Neuroprotective drugs aim to salvage ischemic tissue, limit infarct size, prolong the time window for reperfu-

sion therapy, or minimize postischemic reperfusion injury or inflammation and the risk of hemorrhage. Each step along the ischemic cascade is a potential target for therapeutic intervention. In cerebral ischemia, only thrombolysis has been shown to improve clinical outcome. Neuroprotective therapies have been effective in experimental models of ischemia but, at the moment, there is no definitive evidence of its benefit in the numerous trials carried out in humans, although some subgroups of patients seem to benefit from some of them. The observed lack of efficacy from these drugs may be due to delays in the initiation of treatment, inadequate dose, inadequate penetration, adverse effects, or insufficient matching of the mode of action of the drug to the mechanism of brain injury [31, 32]. Active neuroprotection in acute stroke should include control of blood pressure within certain limits, antipyretic therapy, maintenance of blood glucose, and early feeding and fluid replacement. Manipulation of blood pressure in acute stroke may improve outcome. The design of new clinical trials with neuroprotective drugs requires adequate preclinical assessment and the use of the new magnetic resonance techniques to the select patients and assess the efficacy of the treatment. Some drugs (citicoline, clomethiazole, piracetam and ebselen) have shown a certain degree of clinical efficacy, limited to subgroups of patients, and with a narrow therapeutic window, longer lasting in the case of citicoline [33]. The ECCO 2000 study [34] involved 899 patients at 125 centers who, within 24 h of ischemic hemispheric stroke onset, were randomly assigned to receive either oral citicoline (1,000 mg twice daily) or placebo for 6 weeks. The primary outcome measure, a 7-point or greater improvement in the National Institutes of Health stroke scale score, was achieved by almost the same proportion of patients in both groups (52% citicoline, 51% placebo), suggesting no benefit of citicoline. However, approximately 5% more patients treated with citicoline had excellent outcomes (modified Rankin score </= 1) than those receiving placebo. In this study the results were not conclusive but a positive neuroprotective tendency of citicoline was evidenced. Citicoline is the only putative neuroprotectant that has shown partial positive results in all randomized, double-blind individual trials and that has demonstrated efficacy in the predefined primary end-point of a meta-analysis. The treatment with oral citicoline within the first 24 h after symptom onset in patients with moderate to severe stroke increases the probability of complete recovery at 3 months [35]. Recently, the results of SAINT-1 study have been communicated. This study included more than 1,600 subjects, outcome on the mRS was significantly improved by NXY-059 (Lees et al. for the SAINT-1 Study Group: Preliminary results of the SAINT-1 Trial presented at the 14th European Stroke Conference, Bologna, May 2005). However, one should wait for the final publication to evaluate the data and usefulness of drugs.

Effective neuroprotection may require polytherapy that combines drugs with different mechanisms of action, perhaps administered at different poststroke intervals, to maximize efficacy and/or extend the window for reperfusion, minimize reperfusion injury or hemorrhage, or inhibit delayed cell death [36–38]. Furthermore, because the failure of several neuroprotective trials has been attributed to dose-limiting toxicity [39], combination therapy may permit lower doses of each agent and minimize adverse effects.

Combination of Thrombolysis and Neuroprotection

Combined thrombolysis-neuroprotective approaches have shown promise in animal studies and are beginning to be investigated in clinical trials. The addition of neuroprotective medication may enhance effectivity of thrombolysis and reduces the incidence of hemorrhages. Synergistic effects have been demonstrated in animals when thrombolysis is combined with citicoline [40], an AMPA antagonist [41], and an NMDA antagonist [42]. Administration of antileukocytic adhesion antibodies has been shown to extend the therapeutic window for thrombolysis [43].

Animal models suggest that the combination of low doses of intra-arterial urokinase with a neuroprotective agent, topiramate, may benefit ischemic stroke treatment by improving neurologic recovery, attenuating infarction size, and reducing the risk of cerebral hemorrhage [44]. In a model of focal cerebral ischemia, citicoline may offer significant protection that may be further enhanced with the addition of urokinase. In other experimental studies, the administration of eliprodil, a neuroprotective agent which blocks both the modulatory polyamine site of the NMDA receptor and neuronal voltage-sensitive calcium channels or a thrombolytic agent (rtPA) have similarly reduced the volume of brain damage and the neurological deficit. Combined cytoprotective therapy and thrombolysis markedly improved the degree of neuroprotection and may, thus, represent a valuable approach for the treatment of stroke in humans [45].

Various experimental animal models studies show that the combination of thrombolysis with neuroprotectors (citicoline, MK-801, tirilazad, NBQX, anti-CD18) produces beneficial effects superior to those obtained with monotherapy [40, 42, 43, 46, 47]. However, clinical trials combining therapy (lubeluzole, clomethiazole) [48, 49] did not show efficacy. Combining neuroprotective drugs such as lubeluzole simultaneously with rtPA is feasible and safe. The efficacy of this strategy, using potentially more effective neuroprotective agents, should be evaluated in an adequately powered clinical trial [48]. In a pilot study, there were no safety concerns related to the combination of tPA and clomethiazole. The combination proved effective even though many patients received clomethiazole several hours after thrombolysis; future studies must require prompt administration of the neuroprotector either before or during administration of the thrombolytic. Patients with major strokes may be able to benefit from the combination tPA and clomethiazole [49]. Recently, the results of SAINT-1 study show data where the combination alteplase and NXY-059 reduced the risk of any hemorrhagic transformation and of symptomatic intracranial hemorrhage (Lees et al. for the SAINT-1 Study Group: Preliminary results of the SAINT-1 Trial presented at the 14th European Stroke Conference, Bologna, May 2005). Benefits of combined therapy are already being demonstrated in this study, but, once more, only final results would allow further conclusions for better understanding of mechanisms of this combination and to demonstrate its usefulness.

Our Experience

As above mentioned, we chose citicoline due to its proven clinical efficacy [35]. Citicoline has been demonstrated to be beneficial in several models of cerebral ischemia. The good results with citicoline are probably the result of its mechanism of action providing a neuroprotective effect against both early and delayed ischemic damage, since it inhibits different steps of the ischemic cascade simultaneously and protects the targets (membranes, nucleus, nucleic acids...). Citicoline stabilizes and repairs the membrane [50], favors the synthesis of phosphatidylcholine, nucleic acids, proteins, acetylcholine and other neurotransmitters, inhibits free fatty acid release [51] and protects against apoptosis [52]. Citicoline is used in our study for these reasons.

The treatment regimen, which, would theoretically allow us to reduce the extent and seriousness of cerebral infarction to the maximum, would be the combination of thrombolysis to restore blood flow, as soon as possible together with effective neuroprotection to the inhibit the injury-causing mediators produced by ischemia- reperfusion. To obtain the desired efficacy of combined therapy, the most effective administration regimen must be identified. Two possibilities are proposed in general: (1) administer the neuroprotector before reperfusion to delay progression to irreversible infarction in the penumbra zone and prolong the therapeutic window for thrombolysis, or (2) administer the neuroprotector once reperfusion has been carried out so as to improve neuroprotector penetration in the penumbra zone, and its protective action against injuries due to ischemia-perfusion.

Experimental studies have demonstrated the superiority of combining thrombolysis with different neuroprotectors [47, 53]. Specially, the association of citicoline with rtPA [40] and urokinase [54], with the first citicoline dose given before or simultaneous to thrombolysis, produces a greater reduction in the brain lesion than when either drug was used alone in animal models of ischemic stroke. However, to our knowledge, no evaluation of administering citicoline once reperfusion has occurred has yet been published. With the objective of investigating whether neuroprotection should be provided before reperfusion or once it is ensured, we have compared the effect of rtPA (5 mg/kg i.v.) with citicoline at low (250 mg/24 h for 3 days by the intraperitoneal route) [55] or high (1,000 mg/24 h for 3 days by the subcutaneous route) [56] doses and the combination of both treatments by two routes [57], giving citicoline before or after rtPA in a rat embolic stroke model. This experimental rat model can be useful in preclinical studies of thrombolytics and neuroprotectors. The study has considered a combination of clinical (reduction of mortality and neurological scale score), morphological (infarct volume and TUNEL) (fig. 1b, c) and biochemical markers (IL-6, TNF-α) of ischemic damage. The model also has associated high mortality rates due to the seriousness of the cerebral damage it produces (table 1). Global mortalities do not differ irrespective of whether citicoline is given before or after rtPA. Mortality due to brain damage was decreased with reperfusion and even more in the groups with a combined treatment, particularly when citicoline was administered after thrombolysis. Citicoline as a monotherapy, if anything, has an equally high mortality as rtPA monotherapy. The high scores obtained on the neurological scale illustrate the seriousness of the brain damage, but outcome was more favorable when reperfusion occurred and when the neuroprotector was associated to thrombolysis.

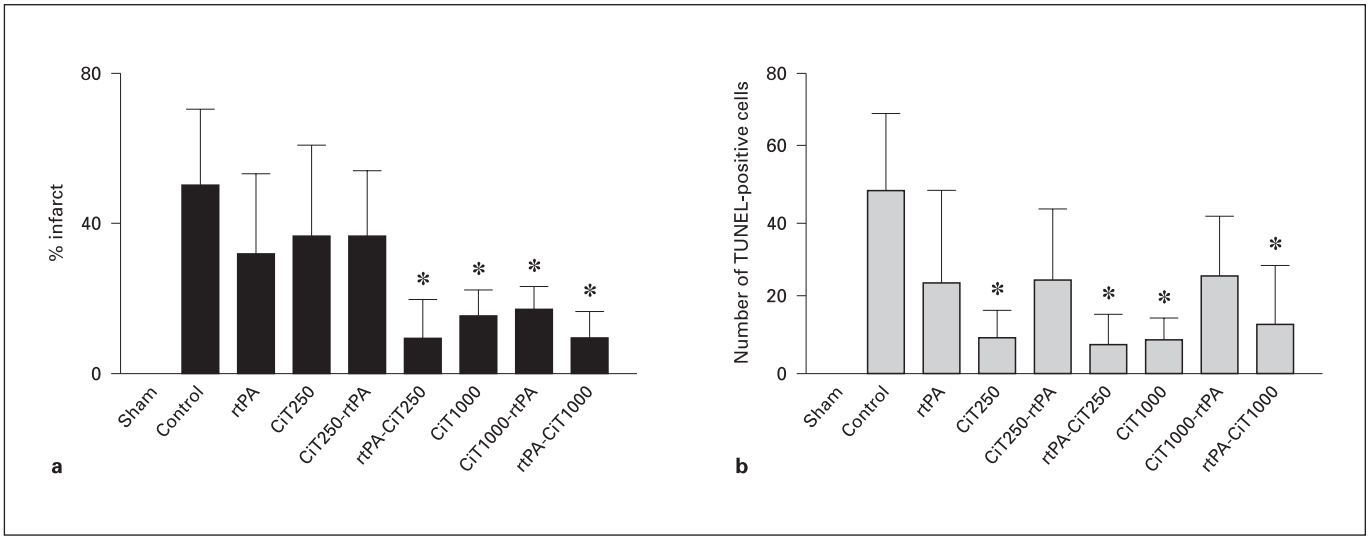

Fig. 2. a Infarct volume in each treatment group expressed as a percentage of the embolized hemisphere. Data are means ± SD. * Significant difference compared with controls (Mann-Whitney test $p < 0.05$). **b** Number of deaths and percentage are shown. * Significant difference ($p < 0.05$ χ^2 test). The groups of treatment were: Sham, Control, rtPA = recombinant tissue plasminogen activator; CiT = citicoline; CiT-rtPA = combination of citicoline before rtPA; rtPA-CiT = combination of citicoline after rtPA.

Table 1. Mortality rates and causes of mortality (number of deaths and percentage are shown)

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)
CiT250	27	23 (85.19)	0 (0)	20 (74.7)	3 (11.11)
CiT250-rtPA	12	8 (66.67)	3 (25)	4 (33.33)	1 (8.33)
rtPA-CiT250	13	9 (69.23)	5 (38.46)	4 (30.77)	0 (0)
CiT1000	16	12 (75)	0 (0)	9 (56.25)	3 (18.75)
CiT1000-rtPA	10	6 (60)	2 (20)	4 (40)	0 (0)
rtPA-CiT1000	18	14 (77.78)	3 (16.67)	11 (61.11)	0 (0)

Figures in parentheses are percentage.

Lower doses of citicoline as a monotherapy failed to reduce lesion size significantly, but our study observed that higher doses of citicoline produced a greater reduction of brain damage than did low doses (unpubl. data). When citicoline was used in combination after rtPA therapy, there was a significant reduction in the ischemic lesion (fig. 2a). This would support the hypothesis that combined neuroprotection after thrombolysis can optimize results. Our results suggest that reperfusion enhances the supply of neuroprotector to the penumbra zone, thus increasing its inhibition of the ischemic cascade and reperfusion injury. A significant benefit of any treatment in regards to reduction of neuronal death (TUNEL) (fig. 2b) was observed when citicoline was administered after rtPA, but isolated reperfusion did not reduce cell death, probably because it failed to inhibit the mechanisms of delayed neuronal death. In summary, the combination of citicoline after reperfusion with rtPA appears to be the optimal treatment. Citicoline at a high dose is most efficacious and might be superior to thrombolysis as mono-

therapy, without the associated risk of hemorrhage (unpubl. data). Low dose of citicoline or rtPA when given alone did not significantly reduce ischemic damage.

The final consideration that most agents claimed to be neuroprotective in animal models has failed in human trials. The human data from the failed trials indicate that the neuroprotective agents were administered long after the successful administration times in animal models. In contrast, thrombolytic therapy has been reported as beneficial in animal and human stroke.

Optimization of therapeutic treatments might involve a complex series of interventions. Disruption of the ischemic cascade of events at multiple levels is likely to be more effective than disruption at any single point. A cocktail of drugs could be administered within the first few hours of illness.

With the use of multiple neuroprotective therapies, each agent or approach could be given or applied either simultaneously or in rapid succession, allowing each agent to work on different ischemic injury mechanisms. Multiple drug therapy and the use of lower doses of individual agents in the mixture thus potentially reduce side effects. The combination of neuroprotection and tPA markedly improved the degree of neuroprotection and opens a route for future studies on the management of acute ischemic stroke. The possible additive or synergistic effects of these drugs should be investigated in future leading studies.

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