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Serial MRI study of the enhanced therapeutic effects of liposome-encapsulated citicoline in cerebral ischemia

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ABSTRACT

Liposome encapsulation of active principles enhances their bioavailability to the brain. We investigated whether encapsulation of citicoline in liposomes increases its therapeutic effects in ischemia, performing a longitudinal MRI study of lesion volumes and edema in an animal model of stroke. Nineteen rats were submitted to permanent occlusion of the middle cerebral artery and treated with: (1) saline, (2) intraperitoneal citicoline (500 mg/kg), (3) intravenous citicoline (48 mg/kg), and (4) intravenous liposome-encapsulated citicoline (48 mg/kg). Lesion volumes were measured by MRI at days 0, 1, 3 and 7 following surgery. Encapsulation in liposomes increased the therapeutic effects of citicoline, as reflected by a 32% reduction of the infarct sizes at day 7, in contrast with controls where infarct sizes at day 7 increased by 39%, respect to values at day 0. Intravenously injected citicoline reduced infarct sizes by 9% while intraperitoneal citicoline resulted in an increase of infarct sizes by 10%. A slight (not significant) reduction of edema formation was observed for animals treated with citicoline, in all of its delivery forms. Liposome-encapsulated citicoline causes a noticeable reduction in lesion volumes as compared to free citicoline (either i.p. or i.v.) at days 1, 3 and 7 following permanent stroke.

1. Introduction

Ischemic stroke is a major cause of death and incapacity in developed countries, with an increasing incidence because of the progressive aging of the population in such countries. So far, the only treatment that has proven efficiency in clinical practice is thrombolysis, though it presents serious safety-related restrictions that limit its use to a reduced fraction of patients (<3%)(Kleindorfer et al., 2009). For the large number of patients where acute recanalization is not used, there is a need for neuroprotective and neuroreparative strategies to contain brain damage, and boost brain repair after the onset of ischemia. There is a hot debate on the stroke community about strategies to be followed to treat this disease. While neuroreparative therapies are under development, and seem to be far from being translated into the clinics, most neuroprotective strategies with impressive results in preclinical research have failed in the clinical setting, for different reasons(Dyken, 2010). In this regard, some authors claim that research should not be focused just on the development of new treatments for stroke, but must also consider how to deliver those agents efficiently to the stroke-stricken brain(Adibhatla et al., 2005; Pardridge, 2002).

A good example of this disjunctive is citicoline (CDP-choline, cytidine-5′-diphosphocholine) an essential intermediate in the synthesis of phosphatidylcholine (a major brain phospholipid)(Adibhatla and Hatcher, 2005), citicoline is believed to interfere in cell membrane damage, providing a benefit for disorders of the central nervous system, including stroke(Adibhatla and Hatcher, 2005). After the demonstration of efficiency in the preclinical field (Clark, 2009; Hurtado et al., 2005), citicoline has also been tested in several clinical trials with unclear outcome, and its usefulness for the treatment of stroke is under discussion(Adibhatla and Hatcher, 2005; Adibhatla et al., 2005; Clark, 2009; Davalos et al., 2002). One of the main reasons for the controversy on the efficiency of citicloine in the clinics is the different ways of administration used for the drug (oral versus intravenous), especially considering that only 0.2–2.0% of the administered citicoline ends up in the brain parenchyma, depending on the administration route(Adibhatla and Hatcher, 2005; Adibhatla et al., 2005; Clark, 2009; Fresta and Puglisi, 1996, 1997, 1999; Fresta et al., 1995; Puglisi et al., 1992). This fact is highly influenced by the polar nature of citicoline, which hampers the crossing of the drug through the blood–brain barrier (BBB). Therefore, the use of alternative ways of administration for citicoline, to increase its bioavailability in

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the brain parenchyma, would potentially enhance the therapeutic effects of this drug for the treatment of stroke.

We have performed a serial MRI study on an animal model of cerebral ischemia, comparing the therapeutic effects of liposome-encapsulated citicoline versus the free form of the drug, administered by two different routes (i.p. versus i.v.). Our intention was to investigate whether this well-known pharmacological approach to facilitate the trespassing of hydrophilic compounds through the BBB could become a feasible way to increase the therapeutic effects of citicoline in the clinics, bringing some light to the existing doubts of its possibilities for the treatment of stroke.

2. Materials and methods

2.1. Animal management

Nineteen (19) male Sprague–Dawley rats (Harlan) weighting 261 ± 12 g were kept at controlled conditions of temperature ($22 \pm 1\,^{\circ}C$) and humidity ($60 \pm 5\%$), with a 12/12 h light/dark cycle, and granting free access to food and water. One animal died during surgery and two died within the first 24 h after surgery. For surgery and MRI rats were anesthetized with sevoflurane (3% in 70% N_2O and 30% O_2). Rectal temperature was monitored and maintained at $37 \pm 1\,^{\circ}C$ with a feedback controlled heating system (1025 system, SA Instruments, NY, USA). Animals were sacrificed under deep anesthesia (8% sevoflurane). All procedures were performed under EU regulations (European Communities Council Directive of 24 November 1986 – 86/609/EEC), with the approval of our institution's ethics committee.

2.2. Middle cerebral artery occlusion

Permanent occlusion of the left middle cerebral artery (MCA) was performed by suture of the artery following the method of Shigeno et al. (1985). In brief, an incision was practiced along the temporal muscle of the rat. A small (3 mm) hole was drilled in the exposed skull and the MCA was proximally exposed, where the artery bifurcates in its frontal and parietal branches, and was carefully retracted from the brain using a Sinskey manipulation hook attached to a micromanipulator (both from World Precision Instruments Inc., Fl, USA). Then, the MCA was sutured with a 10-0 Ethilon (polyamide 6) surgical suture (Ethicon Inc., NJ, USA), the hook was retracted letting the artery to lay back over the brain, and the absence of blood flow was visually confirmed under the microscope. Finally, the temporal muscle was gently relocated over the skull and the skin of the animal was sutured. Brain surgery was performed after permanent ligation of the ipsilateral carotid artery.

2.3. Treatments and experimental groups

Citicoline (generous gift from Ferrer Internacional S.A., Spain) was solved in saline to prepare two stock solutions of different concentration (125 mg/ml, for i.p. injections, and 2 mg/ml, for i.v. injections). One hundred nm-sized DSPC:Cholesterol:PEG-DSPE (0.62/0.33/0.05 molar ratio) liposomes containing citicoline were prepared at the Photophysics and Photochemistry Laboratory of the University of Santiago de Compostela (Spain). Liposomes were obtained by the well-known method of lipid film rehydration using a mixture of methanol and $CHCl_3$ as organic solvent and HBS (Hepes buffer solution) 20 mM as rehydrating solution containing citicoline at a concentration of 40 mg/ml. After rehydration, liposomes were extruded at $60\,^{\circ}C$ 11 times through a 200 nm polycarbonate membrane, followed by another 11 extrusions through a polycarbonate membrane of 100 nm using a miniextruder from Avanti Lipids Inc., Alabama. Resulting liposomes were ultra-centrifuged twice (11,000 rpm, $4\,^{\circ}C$, 16 h) to separate free from encapsulated citicoline. Proper size of resulting liposomes was determined with a Zetasizer DLS system from Malvern Instruments Ltd (UK). The exact content of citicoline in the liposomes (2 mg/ml, 8%) was certified by fluorescence methods developed and performed at the laboratory of origin. Citicoline solutions and liposomes were used within 2 days from their preparation and kept protected from light at $4\,^{\circ}C$ until use.

Treatments where applied either intravenously (i.v.), injecting 1 ml of treatment in the jugular vein, or intraperitoneally (i.p.), injecting 1 ml of treatment in the abdomen of the animals. Rats were randomly assigned to the following groups: (1) control: receiving i.v. injections of saline at $t = 30$ min, 6 h, 12 h, 18 h, 24 h and 30 h post ischemia ($n = 4$); (2) citicoline IP: receiving one i.p. injection of citicoline in saline (1 ml, 125 mg/ml, total dose of 500 mg/kg) at $t = 30$ min post ischemia ($n = 4$); (3) citicoline IV: receiving i.v. injections of citicoline in saline (1 ml, 2 mg/ml, total dose of 48 mg/kg) at $t = 30$ min, 6 h, 12 h, 18 h, 24 h and 30 h post ischemia ($n = 4$), and (4) liposomes: receiving i.v. injections liposomes in saline (1 ml, 2 mg/ml of citicoline, total dose of 48 mg/kg) at $t = 30$ min, 6 h, 12 h, 18 h, 24 h and 30 h post ischemia ($n = 4$). The 500 mg/kg dose was chosen as the minimum one that has shown a therapeutic effect on the rat after i.p. administration(Hurtado et al., 2005). The 1/10th dose of 48 mg/kg of citicoline injected i.v. (either free of in liposomes) was adopted according to Adibhatla et al., who calculated that a dose ranging 7–40 mg/kg is equivalent to that administered to patients in the clinical setting (Adibhatla et al., 2005).

2.4. MR imaging

MRI explorations were performed 30 min and 1, 3 and 7 days following surgery. MR images were acquired at 9.4 T on a horizontal bore MR system Bruker Biospec USR94/20 (Bruker Bioespin, Ettlingen, Germany), and equipped with gradient coils of 440 mT/m and a transmitting RF "birdcage" coil and a receiving surface (4-channels) coil, working at 400 MHz. T2 weighted images (T2w) were obtained from a series of multi-slice multi-echo images (train of 16 echoes) acquired with a spin-echo sequence, using the following parameters: field-of-view: 19.2 mm × 19.2 mm; matrix size: 192 × 192 pixels (in-plane resolution of 100 μm); 14 consecutive coronal slices of 1 mm thickness, covering the whole brain from the rhinal fissure to the cerebellum; echo time: 9 ms (16 echoes); repetition time: 3000 ms; spectral bandwidth: 60,000 Hz. Diffusion weighted images (DWI) and apparent diffusion coefficient (ADC) maps were obtained using the Stejskal–Tanner model (Stejskal and Tanner, 1965) by acquiring a set of 3 DW images with the following parameters: diffusion-weighted echo-planar-imaging (DW-EPI) sequence; field-of-view: 28.8 mm × 28.8 mm; matrix size: 256 × 192 points, zero-filled to 256 × 256 points (in-plane resolution of 112.5 μm); echo time: 30 ms; repetition time: 7000 ms; 3*b* values of 0, 400 and 1000 s/mm^2; spectral bandwidth: 100.000 Hz.

2.5. Image analysis

All images were processed with custom-made applications for Image J (Rasband, 1997–2009). ADC maps were constructed by pixel-wise fitting of the sets of 3 DW Images to the Stejskal–Tanner equation (Stejskal and Tanner, 1965), using the Levenberg–Marquardt algorithm.

2.6. Statistical analysis

Data is presented as mean ± standard deviation. For the comparison of each individual group versus the control group, and between liposome-encapsulated group and free citicoline (i.p. and i.v.) groups, in an individual basis, a two-tailed Student's *t*-tests

was used. For the comparison including all groups at a time, an one-way ANOVA test has been used. Differences at the level of $p < 0.05$ were considered statistically significant. Statistical analysis was performed with SPSS V16.0.

3. Results

All animals surviving MCAo surgery presented ischemic lesions visible as hypo-intense regions on apparent diffusion coefficient (ADC) maps, and as hyper-intense regions on T2-weighted (T2w) MR images. For T2w imaging, lesions do not become apparent until 6–7 h of evolution from the onset of ischemia, therefore acute imaging (<30 min) was performed using DWI. Evolution of the lesion on MR images is presented in Fig. 1, for a representative animal of each of the studied groups.

Lesion volumes estimated from DW images, acquired prior to the application of treatments ($t < 30$ min), show no significant differences in mean lesion volumes for all studied groups (one-way ANOVA, $p = 0.831$): control group = $166 \pm 16\ mm^3$; citicoline IP = $175 \pm 41\ mm^3$; citicoline IV = $162 \pm 66\ mm^3$; liposomes = $197 \pm 59\ mm^3$, giving a mean lesion volume of $176 \pm 48\ mm^3$ for all studied animals. This value corresponds to 11.5% of total brain volume (mean brain volume measured for all animals $1529 \pm 50\ mm^3$), which is a typical value for this experimental model. Lesions cover mainly the cortical areas of the affected hemisphere and extension into the ipsilateral caudate-putamen was not rare.

After the occlusion of the MCA, the mean value of the apparent diffusion coefficient of healthy tissue $(0.80 \pm 0.05) \times 10^{-3}\ mm^2\ s^{-1}$ dropped to $(0.55 \pm 0.04) \times 10^{-3}\ mm^2\ s^{-1}$ in the lesion (decrease of 31%), helping us to delineate the affected regions and calculate lesion volumes from ADC maps like those presented in Fig. 1. These values are in agreement with existing literature(Bardutzky et al., 2005).

Mean value of the transversal relaxation time (T2) obtained from T2-maps constructed from T2-weighted images (not shown) was $T2 = 47.0 \pm 1.5$ ms for healthy tissue for all studied animals, with no significant differences among studied groups (one-way ANOVA, $p = 0.23$). For the lesion, obtained T2 values were: control group: $T2_{(day\,1)} = 66.5 \pm 7.4$ ms, $T2_{(day\,3)} = 67.6 \pm 2.6$ ms and $T2_{(day\,7)} = 63.9 \pm 3.5$ ms; citicoline IP: $T2_{(day\,1)} = 73.4 \pm 3.3$ ms, $T2_{(day\,3)} = 76.7 \pm 3.6$ ms and $T2_{(day\,7)} = 67.9 \pm 2.7$ ms; citicoline IV: $T2_{(day\,1)} = 73.6 \pm 3.0$ ms, $T2_{(day\,3)} = 67.3 \pm 3.7$ ms and $T2_{(day\,7)} = 63.7 \pm 1.6$ ms; liposomes: $T2_{(day\,1)} = 72.9 \pm 4.3$ ms, $T2_{(day\,3)} = 70.6 \pm 7.4$ ms and $T2_{(day\,7)} = 64.23 \pm 3.8$ ms. Differences for T2 values among groups were not significant for each of the studied days (one-way ANOVA, $p = 0.174$ at day 1, $p = 0.123$ at day 3 and $p = 0.210$ at 7 days), although differences on T2 contrast between healthy and ischemic tissue allowed a good delineation of the ischemic lesion.

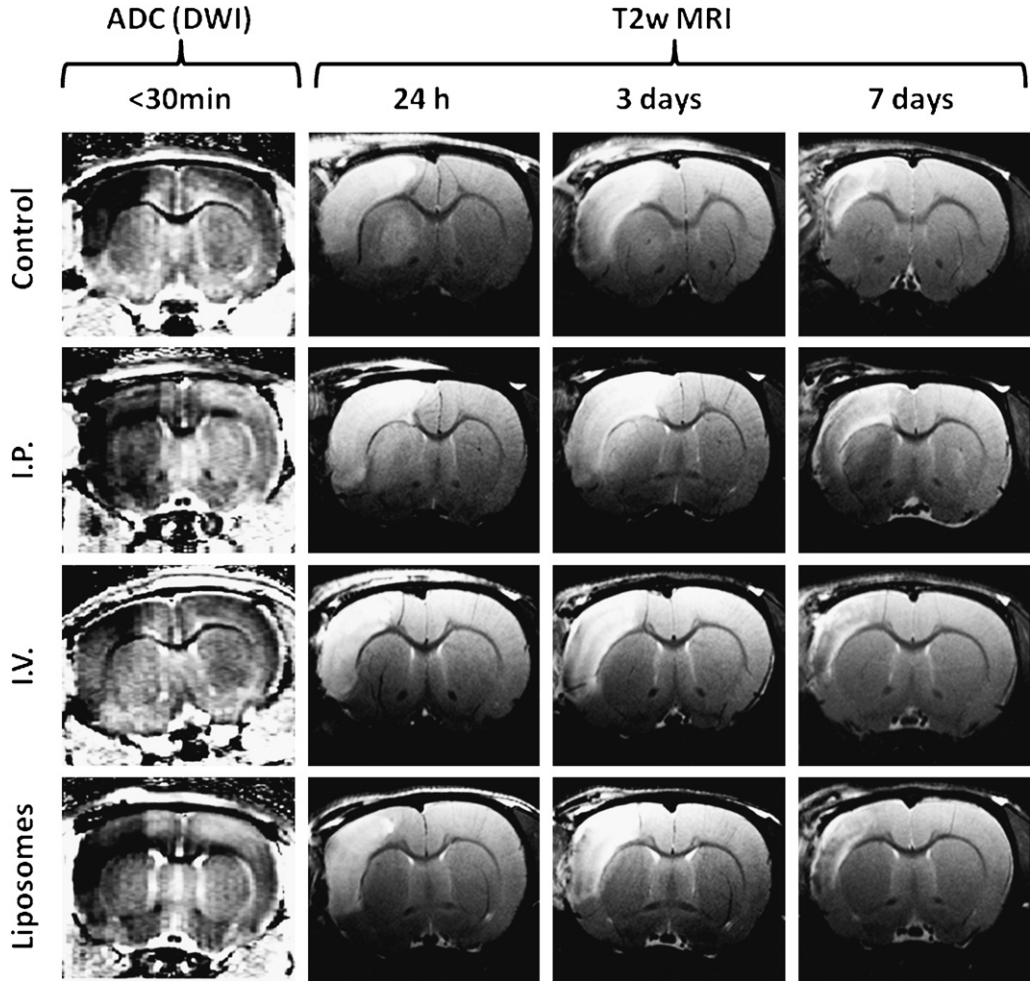

Fig. 1. Left column: apparent diffusion coefficient (ADC) maps, obtained from DWI MR images acquired within 30 min from the permanent occlusion of the MCA. Columns to the right: T2-weighted MR images acquired at days 1, 3 and 7 following MCA occlusion. Images show the evolution of the ischemic lesion and edema formation (evident from the displacement of the midline of the brain), for the four studied groups of animals; top row: controls (treated with saline); 2nd row: intraperitoneal injection of 500 mg/kg of free citicoline (I.P.); 3rd row: intravenous injection 48 mg/kg of free citicoline (I.V.); bottom row: intravenous injection of 48 mg/kg of citicoline encapsulated in liposomes (Liposomes).

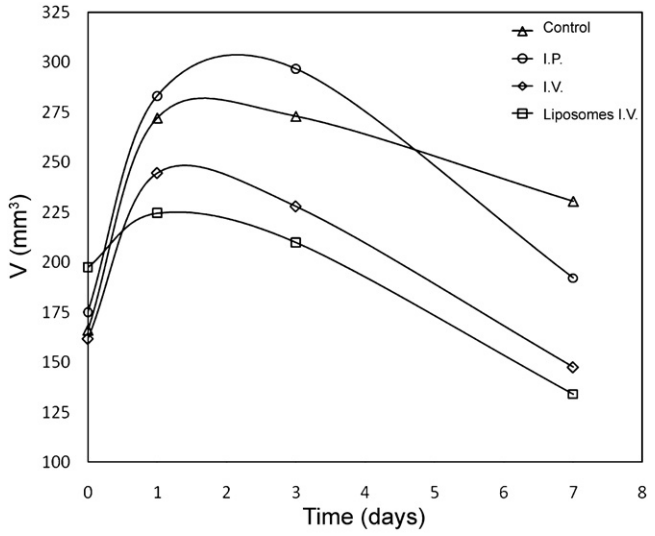

Fig. 2. Evolution of lesion volumes (in mm^3) for the studied groups of animals (values correspond to the mean value of the groups). Control: treated with saline; (I.P.): intraperitoneal injection of 500 mg/kg of free citicoline; (I.V.): intravenous injection 48 mg/kg of free citicoline; (Liposomes I.V.): intravenous injection of 48 mg/kg of citicoline encapsulated in liposomes.

Lesion volumes estimated from T2 images varied in different way for the studied groups, as it is shown on Fig. 2. Volumes reach their maxima around days 2 and 3 for all groups, when edema formation is higher (Fig. 3). It is important to mention that lesion volumes presented in Fig. 2 are corrected for edema formation using the following equation: $V_{lesion}(corrected) = V_{lesion}(measured) \times (V_c/V_i)$, where V_c and V_i are the volumes of the whole contralateral and ipsilateral hemispheres, respectively.

Absolute lesion volumes presented in Fig. 2 were treated to calculate relative changes in lesion volumes (Fig. 4), to take into account the (not significant) differences of the mean lesion volumes between groups, prior to treatment (as determined from DW images).

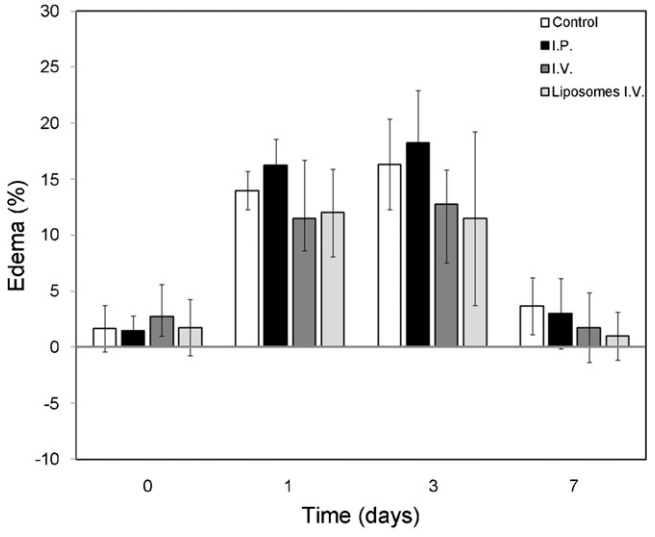

Fig. 3. Evolution of edema calculated from DWI and T2-w MRI images. Edema $(\%) = 100 \times [V_{ipsi} - V_{contra}]/V_{contra}$, where V_{ipsi} is the volume of the whole ipsilesional brain hemisphere and V_{contra} is the volume of the whole contralesional brain hemisphere. Groups: control: treated with saline; I.P.: intraperitoneal injection of 500 mg/kg of free citicoline; I.V.: intravenous injection 48 mg/kg of free citicoline; Liposomes I.V.: intravenous injection of 48 mg/kg of citicoline encapsulated in liposomes.

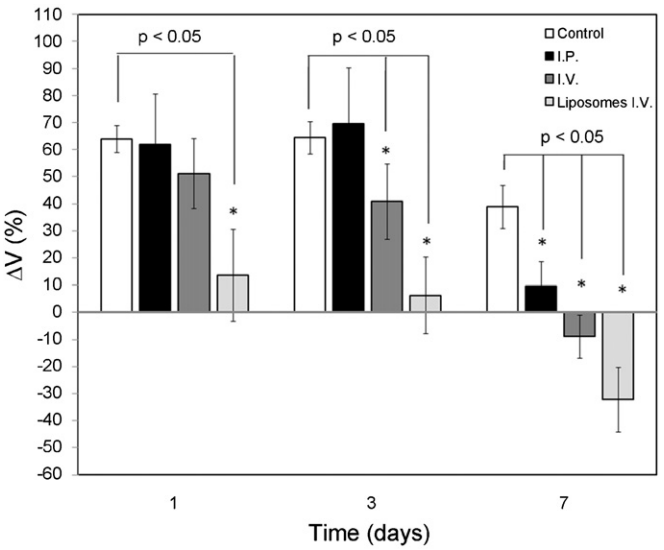

Fig. 4. Increment of lesion volumes from the initial values (calculated from DWI images). $\Delta V = 100 \times [V_{(T2w,ti)} - V_{(DWI,t<30\,min)}]/V_{(DWI,t<30\,min)}$, where $V_{(T2w,ti)}$ is the volume of the lesion calculated from T2-weighted images at $t_i = 1$, 3 and 7 days, and $V_{(DWI,t<30\,min)}$ is the volume measured from ADC maps at $t < 30$ min. Groups: control: treated with saline; I.P.: intraperitoneal injection of 500 mg/kg of free citicoline; I.V.: intravenous injection 48 mg/kg of free citicoline; Liposomes I.V.: intravenous injection of 48 mg/kg of citicoline encapsulated in liposomes.

As seen in Fig. 4, infarct volumes for the control group tend to increase up to 40% from baseline at the end of the observation period, peaking at days 2–3 ($\Delta V_{(day\,1)} = +64\%$; $\Delta V_{(day\,3)} = +65\%$; $\Delta V_{(day\,7)} = +39\%$). For the group that received an i.p. injection of free citicoline, values were not significantly different, except for the lesion volume at day 7 ($\Delta V_{(day\,1)} = +62\%$; $\Delta V_{(day\,3)} = +69\%$; $\Delta V_{(day\,7)} = +10\%$). For the group that received i.v. injections of free citicoline, infarct volumes were significantly smaller respect to controls, starting from day 3 ($\Delta V_{(day\,1)} = +52\%$; $\Delta V_{(day\,3)} = +41\%$; $\Delta V_{(day\,7)} = -9\%$). Finally, for the group of animals treated with liposome-encapsulated citicoline, infarct volumes were significantly smaller already from the first day, respect to controls ($\Delta V_{(day\,1)} = +14\%$; $\Delta V_{(day\,3)} = +6\%$; $\Delta V_{(day\,7)} = -32\%$). Differences at day 3 between groups I.V. and liposomes were significantly different, and differences at day 7 between groups I.P. and I.V., between groups I.P. and liposomes, and between groups I.V. and liposomes, were all significantly different on the *t*-test. Differences for all groups where significant at days 3 and 7 but not at day 1 (one-way ANOVA, $p = 0.351$ at day 1, $p = 0.018$ at day 3 and $p = 0.003$ at 7 days).

Regarding to edema formation (Fig. 3), values obtained for groups I.V. (3%, 12%, 13% and 2% at days 0, 1, 3 and 7 respectively) and liposomes (2%, 12%, 12% and 1%) were slightly inferior to those obtained for controls (2%, 14%, 16% and 4%) and group I.P. (1%, 16%, 18% and 3%), although such differences were never significant (one-way ANOVA, $p = 0.118$ at day 0, $p = 0.437$ at day 1, $p = 0.222$ at day 3 and $p = 0.147$ at 7 days).

4. Discussion

In this study we have confirmed that exogenous administered citicoline presents a beneficial effect in animal models of stroke, in agreement with other preclinical studies that found that citicoline reduces infarct sizes, decreases neurological deficits, reduce glutamate-related damage, improves neuronal survival, preserves levels of phosphatidylcholine, reduces inflammatory mediators and improves neuronal plasticity (see recent reviews of Adibhatla and Hatcher, 2005; Clark, 2009 for a complete set of references).

We have also found that therapeutic effects of citicoline for the treatment of acute stroke are higher when the drug is administered intravenously, in comparison to the intraperitoneal route. I.v. injections yield significantly smaller lesion volumes than controls starting from day 3 after administration, even using a 10-fold smaller dose, as compared to i.p. injections. Intraperitoneally administered citicoline also reduces lesion volumes respect to controls, but in our study differences only become significant at later stages (day 7). This result is in line with the current discussion on the benefits of citicoline in humans. As we have already mentioned, while an improvement of neurological and global function of treated patients has been found in European and Japanese clinical trials, where citicoline was administered i.v., results are not so convincing in US clinical trials, where oral administration was used(Adibhatla and Hatcher, 2005; Clark, 2009). Our results reinforce the idea that the origin of this controversy may be in the different route of administration used for the drug. It is believed that when citicoline is exogenously administered, as sodium salt, it is hydrolysed into choline and cytidine to be re-synthesised later in the brain (Clark, 2009), since passing of the drug as a whole through the BBB is seriously hampered by its polar nature. Thus it has been measured that only 0.2–2.0% of the administered citicoline ends up in the brain parenchyma, depending on the administration route(Adibhatla and Hatcher, 2005; Adibhatla et al., 2005; Clark, 2009; Fresta and Puglisi, 1996, 1997, 1999; Fresta et al., 1995; Puglisi et al., 1992).

Regarding edema formation, we have seen that animals treated with citicoline present a tendency to develop less edema than controls, although differences are very small and not significant. This result is in agreement with other authors who found only a slight effect of citicoline in edema formation in a model of traumatic brain injury (Baskaya et al., 2000), and stroke (Schabitz et al., 1996) but only when a high dose of citicoline was used. Thus, Tazaki et al. have suggested to use co-treatments for the reduction of edema together with the application of citicoline, indicating no effect of this drug on edema formation, in the clinics (Tazaki et al., 1988).

This is not the first report that shows that encapsulation of citicoline in liposomes enhances its therapeutic effects for the treatment of stroke. Fresta and co-workers(Fresta and Puglisi, 1996, 1997, 1999; Fresta et al., 1994, 1995; Puglisi et al., 1992) have used a different sort of liposomes to demonstrate that animals' survival is increased when liposome-encapsulated citicoline is injected to ischemic rats, as compared to free citicoline. Nevertheless, their study is based on a model of temporal (20 min) global ischemia, which pathophyisology is different from focal ischemia, and the assessment of efficacy based on survival rates may reflect other therapeutic parameters than those strictly related to the action of the drug in the brain.

Adibhatla et al. (2005) have also used liposome-encapsulated citicoline on a model of focal transient ischemia, invasively studying its effects on infarct sizes, 24 h after ischemia. These authors obtained similar results to ours, but their data is restricted to a single point after stroke onset (24 h, when edema and brain damage by apoptosis are still under development). Furthermore, they use a transient model with reperfusion after 1 h of occlusion, and it could be considered that our permanent model may be more relevant for the clinical situation, since most patients treated with citicoline do not receive recanalization treatment, while only 1/4th of untreated patients experience acute spontaneous recanalization (Rha and Saver, 2007).

A strong point in favour of our study is that we have been able to determine stroke outcome in vivo, analyzing the time profile of the evolution of the disease after treatment, during its acute and sub-acute phases. This allows us to have a clearer vision of the therapeutic effects of citicoline, in regard to its administration form. In fact, in agreement with Adibhatla et al. (2005) we have found no significant differences between free citicoline injected i.p. or i.v. at 24 h post ischemia, but differences on lesion outcome between both administration forms become quite significant at 3 and 7 days, in our longitudinal study. Furthermore, we show that despite the different degree of achievement, all forms of administration for citicoline exhibit a therapeutic effect 7 days post ischemia (reflected in a reduction of lesion volumes on MR images), but changes on the administration route (specially stabilization and protection of citicoline in liposomes) highly enhanced those effects, and speeds up the action of this drug.

5. Conclusions

In conclusion, animals treated with citicoline i.v. show lesion volumes significantly smaller than controls and animals treated intraperitoneally. Encapsulation of citicoline in liposomes further enhances the therapeutic effect of this drug for the treatment of stroke, and drug effects are significantly higher starting at earlier stages, as compared to the drug administered in its free state. The administration route in which citicoline is used highly influences its therapeutic effects. We believe that these results have important implications for the use of citicoline in the clinics, opening a new opportunity for this drug in the treatment of stroke.

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