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Liposomes as In-vivo Carriers for Citicoline: Effects on Rat Cerebral Post-ischaemic Reperfusion#

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Abstract—Citicoline is a therapeutic agent widely used in the treatment of brain injury, for example in cerebrovascular disease or traumatic accidents. Unfortunately, the strong polar nature of this drug prevents it crossing the blood-brain barrier. In this paper, the possibility of efficiently trapping citicoline in liposomes to improve its therapeutic effects is reported. The citicoline-encapsulation efficiency, drug leakage and size analysis of various liposome systems were studied. The real therapeutic effectiveness of these citicoline liposome formulations was evaluated by biological assay. The effects of free and liposome encapsulated citicoline on survival rate of ischaemic reperfused male Wistar rats (80–100 g) were investigated. Of the phospholipid mixtures used in citicoline liposome formulation the best in terms of delivery and therapeutic effects was 1,2-dipalmitoyl-sn-glycero-phosphocholine: dipalmitoyl-DL-α-phosphatidyl-L-serine: cholesterol (7:4:7 molar ratio). This phospholipid mixture was also assayed for brain conjugated diene levels in rats, since this parameter is an index of lipid peroxidation in rat cerebral cortex during post-ischaemic reperfusion. A citicoline-loaded phospholipid mixture has produced an increase in rat survival rate of about 24% and a reduction in diene levels of 60%, compared to the free drug.

The activity, and survival, of neurons depends on oxygen and glucose supplied by the circulation of the blood (Kuschinsky 1991). The block, even local, of the cerebral ematic flow causes primary damage in the ischaemic area and secondary damage in the surrounding area, where there is only a partial reduction of the energy supply (Baethmann et al 1991; Siesjö & Smith 1991). The prevention or reduction of this secondary damage by means of pharmacological agents has long been studied, despite discouraging results and insufficient information regarding the mechanisms and the endogenous agents involved in neuronal ischaemic damage (Krieglstein & Peruche 1991).

Recently, many in-vivo experiments have offered some theoretical basis for a therapeutic strategy. It has been suggested that a large depolarization of nerve cells with an abnormal extracellular accumulation of endogen neurotransmitters results in a loss of energy substrates and a consequent intracellular calcium overload, thus initiating a cascade leading to irreversible neuronal damage (Greenberg et al 1991; McCulloch 1991).

Direct experimental evidence for this massive extracellular accumulation of neurotransmitters was obtained in animal models of clinical ischaemia (Benveniste et al 1984; Hillered et al 1990). It has been demonstrated that similar phenomena also occur after cerebral traumatic damage, where part of the nerve tissue, although not mechanically damaged, is in an unstable metabolic state and therefore susceptible to secondary damage (Gordon et al 1990; Katayama et al 1990; Palmer et al 1990; Panter et al 1990).

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It has been possible to identify a common denominator in the differing conditions of nerve cell damage: the accumulation of endogenous neurotransmitters, which become toxic if present, even briefly, in a high concentration (Manev et al 1990).

Several studies have allowed the identification of some new molecules which could reduce neuronal damage after an ischaemic event, but their therapeutic use is limited by undesirable side-effects (Ginsberg et al 1991; Hogan et al 1991; McCulloch 1991), since they are not sufficiently selective in reducing the pathological cellular events alone.

Citicoline (Cytidine diphosphate choline) is widely used in brain injury due to the absence of serious side-effects. This drug has also proved to be selectively effective on cerebral blood circulation and metabolism (Arienti et al 1979), without provoking marked variations in blood pressure and pulse rate. The advantage of citicoline in the treatment of brain ischaemia is due to its action on both the infarction and the necrosis site, and also on the ischaemic penumbra zone. While the enhancement of blood-brain circulation ensures the removal of excitatory amino acids (EAAs), responsible for neurodegeneration in the ischaemic border zone, the increased metabolism could contribute to the repair of brain cell membranes, thereby accelerating their functional reorganization (Yasuhara & Naito 1974; Shimamoto & Aramaki 1975).

Unfortunately, citicoline does not readily cross the blood-brain barrier (Agut et al 1983) because of its strong polar nature. To improve citicoline therapeutic effects in the central nervous system, we have previously (La Rosa et al 1992a,b; Puglisi et al 1992; Fresta et al 1993a) studied the possibility of delivering the drug with liposomes. Moreover, the lipid component of the liposome systems could have an active role in the therapeutic effects of such a formulation.

The aim of this work was to optimize the citicoline-loaded liposome formulations and to evaluate their biological effectiveness in an experimental in-vivo model of partial cerebral ischaemia.

Materials and Methods

Chemicals

Dipalmitoyl-DL-α-phosphatidyl-L-serine (PS) and cholesterol were obtained from Sigma Chemicals Co. (St Louis, USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (PC), 1,2-dipalmitoyl-sn-glycero-phosphate disodium salt (PA), 1,2-dipalmitoyl-sn-glycero-phosphoethanolamine (PE), dihexadecyl hydrogen phosphate (DP) and sodium dodecyl sulphate (SDS) were purchased from Fluka Chemicals Co. (Buchs, Switzerland). Ganglioside GM1 was a Boehringer Mannheim product. Before each experiment, the phospholipid purity (>99%) was controlled by two-dimensional TLC on silica gel plates (E. Merck, Darmstadt, Germany), loaded with solutions of the lipids in chloroform : methanol (3 : 1 v/v) and developed first with chloroform : methanol : 5 M ammonium hydroxide (60 : 3 : 5) and then with chloroform : methanol : acetic acid : water (12 : 60 : 8 : 2·5) and stained with molybdophosphoric acid, ninhydrin, or Dragendorff's reagent, depending on the lipid type. Phospholipid phosphorous content was assayed as inorganic phosphate, as described elsewhere (Bartlett 1959).

A fine Sephadex G-50 column was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Inorganic salts: sodium chloride, sodium hydroxide and potassium dihydrogen orthophosphate were of analytical grade (BDH Laboratory Supplies, Poole, UK). Citicoline was kindly provided by Cyanamid-Italia. The purity was greater than 99·5% by HPLC. Double distilled pyrogen-free water was used. All other materials and solvents were of analytical grade.

Liposome preparation

The aqueous drug solution was prepared just before liposome preparation. For 50 mL of drug solution 2 g citicoline was added; then phosphate buffer was added to adjust the ionic strength of the solution to 300 mOsm at 37°C (Osmomat 030 cryoscopic osmometer, Gonotec, Berlin, Germany). The final pH was 7·4 (Beckman model pHI 40 pH-meter). The final citicoline concentration (40 mg mL^{-1}) was determined at pH 1 by spectrophotometric analysis at 279 nm (Uvikon 860, Kontron instruments, Zurich, Switzerland).

Two different preparation procedures were performed to trap citicoline into liposomes, giving either multilamellar vesicles (MLVs) (Amselem et al 1990) or reversed-phase evaporated vesicles (REVs) (Szoka & Papahadjopoulos 1978).

For MLV preparation, the lipid components were dissolved in chloroform : methanol (7 : 3) in a 100-mL round-bottomed flask containing 40 g glass beads (2–3 mm mean size; Farmitalia Carlo Erba, Milano, Italy). The beads increase the surface area of the dried lipid film, thereby improving the lipid contact with the aqueous solution containing the drug. In this way the hydration and dispersion of the lipids was facilitated. The final volume of lipid solution was adjusted to 40 mL with chloroform. The organic phase was dried under reduced pressure at 25°C using a rotary evaporator. The possible residual organic solvent was eliminated by connecting the flask to a lyophilizer (Edwards Freeze Dryer Modulyo, equipped with an Edwards high vacuum pump model Serial E 2M8 42810) overnight. The hydration step was carried out by adding the citicoline isotonic phosphate buffer solution to the dried material to give a final lipid concentration of 30 mg mL^{-1}. The MLVs were prepared by mechanical stirring of the dried lipids with the drug solution for 4 h. The aqueous dispersion was heated at 60°C. The liposome suspension was then sonicated (15 min) to reduce the mean size.

For REV preparation, the required amount of lipids was weighted into a round-bottomed flask and dissolved in chloroform : methanol (3 : 1). The organic phase was dried under reduced pressure to deposit a thin film of lipids on the inner wall of the flask, by high vacuum storage overnight. This film was dissolved in ethyl ether and citicoline aqueous solution was then added. The buffer-lipid mixture was emulsified at 10°C for 20 min under a nitrogen atmosphere in a Bransonic model 2200 bath sonifier. Any residual organic solvent present in the emulsion was removed by rotary evaporation under reduced pressure at room temperature (21°C). The resulting viscous gel was resuspended in an isotonic pH 7·4 phosphate buffer. The final turbid, white liposome suspension had a lipid concentration of 30 mg mL^{-1}.

Empty liposomes were prepared in the same way except that isotonic phosphate buffer was used instead of drug solution; every liposomal formulation contained an amount of 6 mol% of ganglioside GM1.

Citicoline encapsulation efficiency

To evaluate the loading capacity of the various liposomal systems, free citicoline was removed from the suspension by a gel-filtration technique. One millilitre of the liposome suspension was loaded into a fine Sephadex G-50 column (50 × 1·5 cm), pre-equilibrated with phosphate buffer. Liposomes were eluted in the void volume, whereas the free drug was retarded by the gel. To obtain the best experimental conditions, the osmolarity of the pH 7·4 phosphate buffer was adjusted with NaCl to obtain isotonicity with the trapped solution. The absorption of each fraction was measured at 600 and 270 nm to estimate the turbidity of the suspension and the concentration of untrapped citicoline.

Given that in-vivo experiments were to be performed, the separation of the free drug needed a less time-consuming technique. Considering the dilution of liposomal systems, submitted to gel-permeation chromatography, an ultrafiltration procedure is necessary. Therefore, a centrifugation technique was performed for the biological assay. Thus, MLVs and REVs were centrifuged at 10 000 and 20 000 rev min^{-1}, respectively. The centrifugations were performed at 10°C with a Beckman model J2-21 centrifuge, equipped with a Beckman JA-20.1 fixed angle rotor. The supernatant was spectrophotometrically assayed for free citicoline. The experimental data obtained with this procedure were similar to those obtained by gel filtration.

After the separation of the untrapped drug, the amount of citicoline encapsulated in the liposomes was determined.

A mixture of methylene chloride : methanol (2 : 1) was added to the liposomes to destroy the phospholipid bilayer structures. This solution was poured into a 10-mL round-bottomed flask and made up to 10 mL with methanol. The citicoline content was then evaluated by HPLC analysis. The apparatus consisted of a Hewlett-Packard HPLC model HP 1090 M equipped with a variable wavelength UV detector (DAD HP). The separation was performed on a Bondapack C18 column (10 μm, 300 mm × 3·9 mm i.d.; Waters Associates Milford, MA, USA) with a flow rate of 1·5 mL min^{-1}. The mobile phase consisting of 0·1% ammonium dihydrogen orthophosphate : methanol : acetonitrile : 10% tetrabutylammonium hydroxide (89·11 : 9·9 : 4·9 : 4·9), was filtered through 0·2 μm microporous PTFE membrane filters (Millipore, USA) and degassed by ultrasonication before use. HPLC analysis was carried out at room temperature (21°C). The UV detection was performed at 280 nm. Results were calculated from the linear regression of external citicoline standards relating peak-area and concentration.

The recovery of citicoline as untrapped and encapsulated material was always greater than 97·5% of the amount added.

The amount of citicoline entrapped in liposomes was expressed as encapsulation capacity or drug content, as reported elsewhere (Benita et al 1984).

Citicoline release from liposomes

After the separation of the untrapped drug by centrifugation, the liposome pellet was made up to 5 mL with pH 7·4 isotonic phosphate buffer or with serum. The final phospholipid concentration was 35 mg mL^{-1}. The liposomal suspension was dialysed with a Spectrapor/por 2 membrane MWCO 12-14000 (Spectrum, Medical Industries, Inc., Los Angeles, CA). The dialysis bag was put into a receiver compartment containing 150 mL medium. In the experiments involving serum, a small amount of sodium azide was added to prevent bacterial growth. The release experiments were carried out at 37°C in a thermostatic bath (I.S.Co.: model BTU 6) equipped with a Variomag electronic stirring system (submersible stirrers). At predetermined time intervals, 2-mL samples were taken from the receiver compartment and citicoline was determined by HPLC analysis. As each sample was taken, it was replaced by an equal volume of the dialysis medium.

Sizing of liposomes

A light-scattering method was used for liposome size analysis. The experiments were carried out using as light source a He-Ne Spectra-Physic mod. 120 laser, having a power of 7 mW. The PC 8 Malvern holding sample cell was kept at 20°C by an Haake F3-R and equipped with a Microcontrol precise mechanical goniometer and an optical system Melles-Griot f.150. The photomultipliers were Hamamazu R 1333 and RCA 8852. Two methods were performed in light scattering analysis: photon correlation spectroscopy (PCS) and elastic light scattering (ELS). The PCS measurements were carried out at a scattering angle of 20° and 40°. The correlation functions were performed by a Malvern 4700C sub-micron particle analyser and a cumulant fitting (Chu 1974; Berne & Pecora 1976) was used to obtain the mean diameter and polydispersity. In the ELS method, the intensity scattered by the samples was measured at different observation angles (from 20° to 140°) and the Rayleigh-Debye fitting (Chu 1974; Berne & Pecora 1976) was performed to obtain the mean diameter. The results were in agreement with those obtained by PCS.

Induction of cerebral ischaemia

Male Wistar rats, 80–100 g, were subdivided into groups of 60 animals: two groups for each citicoline liposome formulation, two groups for free citicoline, two groups of saline-treated rats and two groups of sham-operated animals (control groups). Each liposome formulation, containing a dose of 20 mg kg^{-1} citicoline, or the same dose of free citicoline was administered by intramuscular injection 1 h before ischaemia, immediately after and thereafter once a day for five days. The animals, anaesthetized by an intraperitoneal injection of ethyl urethane (1·2 g kg^{-1}), were made ischaemic by bilateral clamping of the common carotid arteries for 20 min after which the blood flow was definitively restored in each group studied for survival rate.

Rats investigated for lipid peroxidation were treated with citicoline, either alone or trapped in liposomes, 1 h before ischaemia and then killed by decapitation after 20 min reperfusion. Ischaemia was evaluated by measuring lactate levels, while lipid peroxidation was estimated by measuring the levels of conjugated dienes.

Lactate determination

The tissue was homogenized in 20 mM glycyl-glycine buffer, pH 10, containing 70 mM glutamate for the determination of

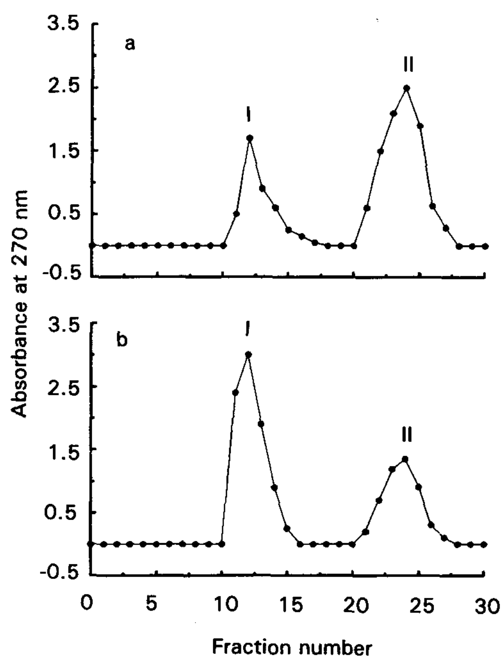

FIG. 1. Gel-permeation chromatograms of a liposomal suspension (PC : PS : cholesterol, 7 : 4 : 7) containing citicoline (a = MLVs; b = REVs) on a Sephadex G-50 column (length 50 cm; diam. 1·5 cm). The mobile phase flow rate was of 720 μL min^{-1}. Each fraction was 3·6 mL. Peak I is the elution of citicoline-loaded liposomes, peak II is the elution of free drug. Similar elution profiles were obtained for the different phospholipid mixtures. The variations of the entrapped/free citicoline ratios were less than 15%.

Table 1. Citicoline encapsulation efficiency parameters of liposomes composed of different lipid mixtures and prepared with MLV or REV methods.

Liposome composition	MLVs		REVs	
	Encapsulation efficiency ($mL\,mmol^{-1}$)	Drug content (%)	Encapsulation efficiency ($mL\,mmol^{-1}$)	Drug content (%)
PC : PS : cholesterol (7 : 4 : 7)	5·7 ± 0·4	15·8	15·5 ± 0·8	45·6
PC : PA : cholesterol (7 : 4 : 7)	4·3 ± 0·6	11·3	15·2 ± 1·1	44·1
PC : DP : cholesterol (7 : 4 : 7)	4·1 ± 0·3	10·6	15·0 ± 0·9	42·1
PC : PE : cholesterol (7 : 4 : 7)	3·6 ± 0·7	9·7	14·7 ± 0·8	39·7

Each value is the average of six experiments.

the lactate levels. The homogenate was deproteinated by 4% $HClO_4$ (final concentration). The amount of lactate was determined spectrophotometrically following NADH formation at 340 nm using Noll's method (Noll 1984).

Lipid peroxidation assay

For the determination of conjugated dienes, the tissue was homogenized in 50 mM phosphate buffer, pH 7·4, containing 1 mM EDTA. Lipids were extracted using chloroform : methanol (2 : 1); the extract was evaporated to dryness under a stream of nitrogen at room temperature (21°C) and then redissolved in cyclohexane. Cyclohexane solution was assayed at 234 nm; the results were expressed as μmol lipohydroperoxide (mg protein)$^{-1}$ or (mg lipid)$^{-1}$ using an ε_M value of $2{\cdot}52 \times 10^4\,M^{-1}\,cm^{-1}$ (Recknagel & Glende 1984). Proteins were determined by the method of Lowry et al (1951). Lipids were determined by the Henry's method (1964).

Results and Discussion

In this research we have investigated various phospholipid mixtures as in-vivo carriers for citicoline.

To evaluate the encapsulation efficiency of liposomes prepared with the two different methods, the free citicoline was separated from drug-loaded liposomes by gel permeation. The elution profile of liposomes containing citicoline, obtained after chromatography on a Sephadex G-50 column, is shown in Fig. 1. The absorbing material eluted in two well-separated peaks, the first being the elution of liposomes with trapped citicoline (fractions 10–15), and the second reflecting free citicoline (fractions 20–27). The presence of liposomes in the first peak was revealed by UV absorbance at 600 nm. To determine the encapsulation capacity and drug content of the various liposomal systems, both free and trapped citicoline were determined spectrophotometrically at 270 nm, after adding methanol to the aqueous suspension to destroy the liposomal structure. In this way any interference related to the scattering of the colloidal suspension was avoided.

As shown in Table 1, the encapsulation efficiency parameters for both MLVs and REVs were higher than those expected. In fact, as reported elsewhere (Hope et al 1986), the encapsulated aqueous volume of a vesicle ranged from 0·5 to 20 $\mu L\,\mu mol^{-1}$, depending on the liposome type. Considering the size of our liposomes (Table 2), we expected encapsulation capacities of about 1 for MLVs and 7 for REVs, rather than the 4·5 and 15 mean values obtained, respectively. These differences were probably due to the ability of citicoline to form hydrogen bonds with the hydrophilic heads of charged phospholipids, whereas no interaction occurs with cholesterol or PC (Puglisi et al 1992; Fresta et al 1993a).

For this reason, we deduce that citicoline was not only inserted into the aqueous core of the liposome structure, but also strongly absorbed on the surface of the phospholipid bilayers. The phospholipid mixture PC : PS : cholesterol (7 : 4 : 7 molar ratio) showed the best encapsulation efficiency parameters as MLVs.

The differences in encapsulation capacity and drug content values between MLVs and REVs were due to the greater amount of aqueous solution captured by REVs, whose preparation gave uni- or oligolamellar vesicles and allowed a better interaction between the drug and charged phospholipids. Differences among the various MLVs phospholipid mixtures (Table 1) could be ascribed to the different liposome size (Table 2) as well as drug-phospholipid interactions. No difference in encapsulation efficiency

Table 2. Mean diameter and polydispersity index values of MLV and REV liposomes composed of different lipid mixtures.

Liposome composition	MLVs		REVs	
	Mean diameter (nm)	Polydispersity index (nm)	Mean diameter (nm)	Polydispersity index (nm)
PC : PS : cholesterol (7 : 4 : 7)	390 ± 13	1·8	120 ± 11	0·1
PC : PA : cholesterol (7 : 4 : 7)	340 ± 19	2·7	135 ± 15	0·3
PC : DP : cholesterol (7 : 4 : 7)	350 ± 15	3·1	110 ± 12	0·1
PC : PE : cholesterol (7 : 4 : 7)	325 ± 17	2·1	115 ± 10	0·4

Each value is the average of six experiments.

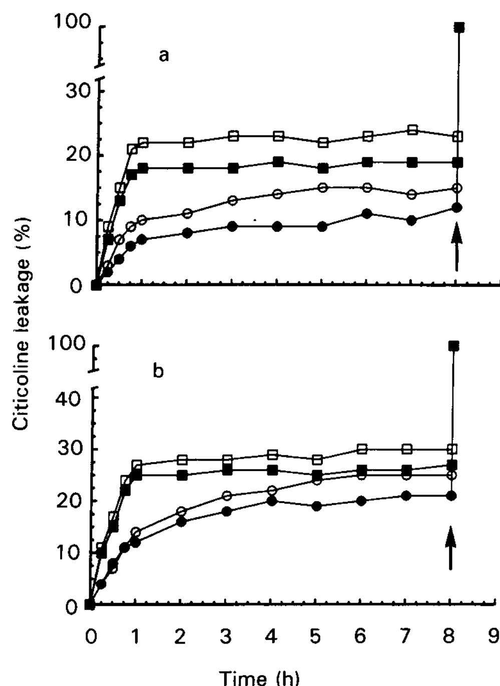

FIG. 2. Citicoline leakage from liposomes in pH 7·4 isotonic phosphate buffer (a) or in serum (b). In serum, a small amount of sodium azide was added to prevent bacterial growth. The experiments were carried out at a constant temperature of 37 ± 0·2°C. ● MLVs (PC:PS:cholesterol, 7:4:7), ○ MLVs (PC: PA:cholesterol, 7:4:7), ■ REVs (PC:PS:cholesterol, 7:4:7), □ REVs (PC:PA:cholesterol, 7:4:7). The arrows indicate the time at which sodium dodecyl sulphate was added, collapsing the liposomes. Each value is the average of five experiments.

was observed for REVs; this liposome type consisted principally of an unilamellar vesicle and thus the slight differences were triggered by different interactions between citicoline and the charged phospholipids.

The permeability of various kinds of liposomes to trapped citicoline was evaluated by dialysis both in pH 7·4 isotonic phosphate buffer and serum. As shown in Fig. 2, no significant difference in citicoline release was observed among the various phospholipid mixtures either in pH 7·4 isotonic phosphate buffer or serum, due to the presence in the liposome structure of cholesterol, which ensures the integrity of the liposome membrane, and also reduces the temperature-dependent permeability. Different release profiles were observed between MLVs and REVs. We observed a biphasic leakage of citicoline: rapid drug release in the first period followed by a more gradual drug loss. It is possible to attribute the rapid citicoline leakage to residual drug from manufacturing and handling coupled to a desorption phenomenon of citicoline from the outer bilayer of the liposome structure. This behaviour is more evident for REVs, which have a higher surface area/volume ratio compared with multi- or oligolamellar vesicles. The more gradual release of the drug was due to normal permeation through the liposome lamellae.

The presence of cholesterol is very important in liposome formulations used as biological delivery devices. As reported in Fig. 3, liposomes without cholesterol were very permeable to citicoline in serum, while no significant difference was observed among liposomes prepared in the presence or absence of cholesterol in pH 7·4 phosphate buffer. The

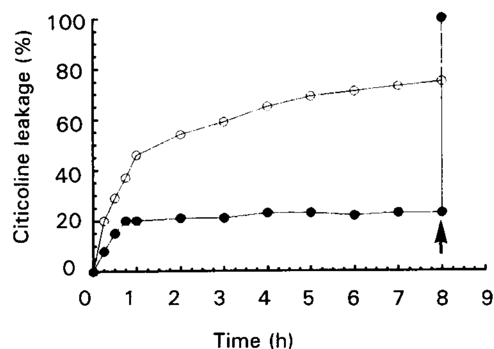

FIG. 3. pH 7·4 phosphate buffer (●) or serum (○) citicoline leakage from REVs (PC:PS, 3·5:1 molar ratio) prepared in the absence of cholesterol. In the liposomes without cholesterol the molar ratio between neutral and charged phospholipids was kept constant to leave the lipid behaviour and the drug-phospholipid interactions unchanged. The arrow indicates the time at which sodium dodecyl sulphate was added, collapsing the liposomes. Each value is the average of five experiments.

physiological fate of liposome-trapped drugs has been studied by several authors (Kirby et al 1980; Tall 1980; Allen 1981; Widder et al 1982) who point out that, after intravenous injection, there was an almost immediate release of drug into the blood circulation. The rate at which this occurred was much higher than that expected from a simple solute diffusion through intact bilayers. This phenomenon has been ascribed in part to the loss of structural integrity in liposomes as a result of the phospholipid removal by high density lipoproteins. It has been shown that liposome serum stability could be enhanced by adjusting their cholesterol content. Thus, the inclusion of cholesterol in the liposome reduces the rate of leakage of trapped drugs only and does not appear to significantly alter liposome tissue distribution.

The storage stability of the phospholipid mixtures was evaluated by storing liposomes at 4°C for three months in a very dense colloidal suspension to reduce the volume of water in which the drug could diffuse. At predetermined intervals, 200 μL of the liposome colloidal suspension were made up to 1 mL with 0·9% NaCl solution and then submitted to gel-permeation on a Sephadex G-50 column to determine the percentage of leakage, by means of relative integration of the peaks of free and trapped citicoline. The total recovery of citicoline was always >98%. Considering that under these conditions, the liposome structural integrity was guaranteed (very low levels of lyso-phospholipids were formed) (Fresta et al 1993b), the liposome presented an elevated retention of the trapped drug. After a period of citicoline leakage, a plateau was reached, ranging from 75 to 85% of retained drug for the various liposomes (Fig. 4). No particular difference in storage stability was observed between MLVs and REVs (data not reported). The good retention of citicoline, compared with other drugs (Mayhew et al 1984), was probably due to its strong interaction with the phospholipid head-group by forming hydrogen bonds, which drastically reduce the drug permeability.

The low partition coefficient of citicoline (log P = 1·7) also explains its relative inability to pass through the lipid zone of the liposome bilayers. During the storage period, no appreciable variation of liposome size was detected by light scattering (data not reported).

For the biological assays, we chose REVs, because these

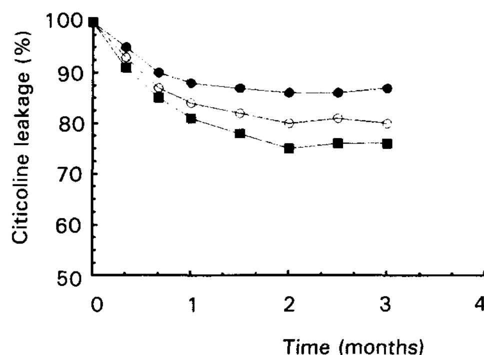

FIG. 4. Citicoline retention from REVs stored at 4°C as concentrated colloidal suspension. Each point was the average of three experiments. ● PC:PS:cholesterol (7:4:7), ○ PC:PA:cholesterol (7:4:7), ■ PC:PE:cholesterol (7:4:7).

showed encapsulation efficiency values higher than MLVs, while offering almost identical storage and serum stability. Another very important factor was smaller size for REVs than for MLVs (mean size 120 vs 350 nm); this, increasing the plasma lifetime of liposomes, improves the possibility of cerebral localization compared with larger liposomes (Juliano & Stamp 1975; Allen & Everest 1983).

To evaluate the therapeutic effectiveness of citicoline-loaded liposomes, male Wistar rats 80–100 g, made ischaemic and reperfused, were treated with the same amount of free or trapped citicoline before and after ischaemia.

The experimental in-vivo model of partial cerebral ischaemia consisted of the surgical bilateral clamping of common carotid arteries. This model mimics most aspects of human ischaemic events. In clinical medicine two types of ischaemia predominate: that associated with cardiac arrest, and that due to stroke (Siesjö & Smith 1991). The first type of ischaemia is characteristically intense and brief. Stroke on the other hand, yields focal ischaemia, since it affects only a part of the brain and usually allows survival even though vessel occlusion is permanent. Stroke, then, causes intense ischaemia only in part of the tissue (the focus), that is surrounded by a border of less intensely ischaemic tissue, often called the penumbra (Siesjö & Smith 1991). The evaluation of survival rate of rats submitted to an ischaemic

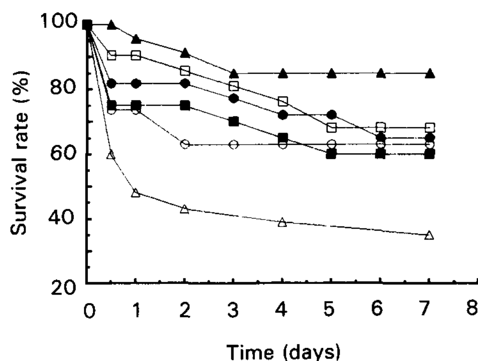

FIG. 5. Survival rate of Wistar rats following cerebral post-ischaemic reperfusion as a function of time. The various animal groups were treated with free citicoline (●) or citicoline trapped in REVs of different lipid composition. ○ PC:PE:cholesterol (7:4:7), ■ PC:DP:cholesterol (7:4:7), □ PC:PA:cholesterol (7:4:7), ▲ PC:PS:cholesterol (7:4:7). △ Untreated ischaemic and reperfused group. Control group (sham-operated rats) had a survival rate of 100%.

process and treated with free and trapped citicoline is a suitable method for determining effectiveness and advantages of the liposomal formulation.

As shown in Fig. 5, no particular difference was observed between free and citicoline-loaded liposomes in improving the survival rate, except for a PC:PS:cholesterol (7:4:7 molar ratio) phospholipid mixture. This lipid system showed an improvement in the survival rate of ischaemic rats of 24% compared with free citicoline. The reason that the liposome formulations (apart from PC:PS:cholesterol) did not present any biological advantages over free citicoline could be ascribed to the fact that in 1-month-old rats the blood-brain barrier is not completely formed. This would allow a better permeation of both the free and liposome-encapsulated drug. The better therapeutic response obtained with PC:PS:cholesterol (7:4:7 molar ratio) could be due to a passive target of the serine component: the cerebral tissue is rich in serine glycerophospholipids, which have a particular metabolic role in brain activity (Wheeler & Whittam 1970).

During an ischaemic event, there is a reduction of oxygen availability leading to energy failure with a consequent reduction in ATP and increase in lactate levels. The rapid fall in cellular ATP causes inactivation of the biological membrane ionic pumps, with intracellular calcium overload. The increase in the intracellular concentration of Ca^{2+} leads to the release of excitatory amino acids (e.g. glutamic and aspartic acid), that cause permanent depolarization of other neurons by the activation of glutamate receptors, most notably of the N-methyl-D-aspartate (NMDA) subtype (McCulloch 1991). In addition, the elevation of Ca^{2+} during ischaemia can trigger several deleterious enzymatic processes in the neurons. Many Ca^{2+}-dependent enzymes such as phospholipases, proteases, nucleases, Na^+/K^+ ATP-ase and adenylate cyclase can be activated, leading to disorganization of neurons and finally to irreversible damage (Greenberg et al 1991). Loss of calcium homeostasis and the massive presence of excitatory amino acids in the synaptic spaces are not the only cause of neuron death. Studies by Vanella et al (1992) have suggested that oxygen free radicals generated during post-ischaemic reperfusion are responsible for damage to cellular constituents, such as the inactivation of membrane-bound enzymes, DNA strand breaks and peroxidation of biomembrane phospholipids. Increased amounts of conjugated dienes, markers of lipid peroxidation, were found in rat cerebral cortex after post-ischaemic reperfusion (Vanella et al 1990), confirming the involvement of partially reduced oxygen species.

To investigate the effectiveness of citicoline-loaded liposomes against neuronal damage due to ischaemia, and to study the possibility of repairing the biological structures and obtaining a functional reorganization, the conjugated diene levels in rat cerebral cortex after post-ischaemic reperfusion were determined. As reported in Table 3, citicoline-loaded liposomes showed the lowest conjugated diene levels. The treatment with liposomal formulation (PC:PS:cholesterol, 7:4:7) induced a marked reduction in lipoperoxidation (60 and 90%) compared with free citicoline-treated and untreated animals, respectively. Evidently, citicoline-loaded liposomes show a greater protection against peroxidative injury during ischaemia-reperfusion than the free drug, which demonstrates a

Table 3. Conjugated diene levels in rat cerebral cortex after post-ischaemic reperfusion.

Experiment	Lipohydroperoxide in protein (mmol (mg protein)$^{-1}$)	Lipohydroperoxide in lipids (mmol (mg lipid)$^{-1}$)
Control (sham-operated)	10 ± 1.5	27 ± 3
Untreated, ischaemic, reperfused	32 ± 5.2^{a}	77.3 ± 10^{a}
Citicoline	7 ± 6.5^{b}	16 ± 3.6^{b}
PC : PS : cholesterol liposomes		
without citicoline	3.8 ± 2^{b}	7.5 ± 2^{b}
with citicoline	$2.5 \pm 0.7^{b,c}$	$2.5 \pm 0.72^{b,c}$

$^{a}P < 0.001$ compared with control, $^{b}P < 0.001$ compared with untreated, ischaemic, reperfused animals, $^{c}P < 0.001$ compared with animals treated with citicoline alone.

therapeutic effect comparable with other drugs (Vanella et al 1990). The lipid mixture without trapped citicoline was also very effective. The increased protection showed by a liposome delivery device trapping citicoline could be due to a synergistic effect between the carrier and the drug. Citicoline increases cerebral blood circulation and metabolism, so allowing a rapid structural reorganization of the nervous system. The cell can employ the substrate readily available as phospholipids coming from the liposome formulation to repair and reactivate the biological membranes. Our data suggest that citicoline-loaded liposomes could ensure an almost complete recovery of the neuronal functionality, especially of the neurons present in the penumbra zone directly damaged by oxygen free radicals and high levels of excitatory amino acids. Most probably, the therapeutic advantage of using citicoline liposome formulation could be due to the fact that they may act as circulating reservoirs which can slowly release the drug that can penetrate from plasma into the brain. The presence of ganglioside in GM1 in the liposome constitution, prolonging the vesicle circulation time as recently demonstrated (Gabizon & Papahadjopoulos 1988; Maruyama et al 1993), could ensure an accumulation of the therapeutic substances (citicoline and phospholipids) by means of the repeated passage of the citicoline-loaded liposomes through the brain blood. Furthermore, during ischaemia a hyperpermeability of the blood-brain barrier could occur (Ito et al 1992). For this reason, the passage of very small lipid vesicles (less than 100 nm) directly through such a compromised vesicular bed could be hypothesizable.

The in-vivo experiments were also carried out with liposome formulations after a storage period of two months. No significant modification in the biological response compared with freshly prepared liposomal vesicles was observed (data not reported).

Considering the pharmaceutical parameters and the biological results, PC : PS : cholesterol (7 : 4 : 7 molar ratio) would appear the best phospholipid mixture as in-vivo carrier for citicoline. In conclusion, our data clearly show the therapeutic effectiveness of the liposomal formulation trapping citicoline, and furthermore suggest the possibility of a clinical application, considering that the optimization and scaling-up of liposomes for clinical use were recently reported (Amselem et al 1990).

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