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Biological effects of CDP-choline loaded long circulating liposomes on rat cerebral post-ischemic reperfusion

Massimo Fresta*, Giovanni Puglisi

Istituto di Chimica Farmaceutica e Tossicologica, Facoltà di Farmacia, Università di Catania, Viale A. Doria 6, I-95125 Catania, Italy

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Abstract

CDP-choline (CDPc) was encapsulated in unilamellar long circulating liposomes to improve biological effectiveness. The liposomal colloidal suspension was prepared by extrusion of multilamellar vesicles through polycarbonate filters. Unilamellar liposomes with a mean size of 50 nm and a polydispersity index of 0.01 were obtained. CDPc encapsulation capacity was 28 ml/mmol. Unilamellar liposome suspension presented a certain stability both in phosphate buffer and in serum, 25% of CDPc was released after 24 h. The colloidal properties and the presence of ganglioside G_{M1} in liposome composition ensured a long-circulation of the carrier after i.v. administration. The therapeutic effects of CDPc-loaded unilamellar liposomes compared to the free drug were evaluated by an experimental model of ischemia and reperfusion, performed with Wistar rats (320–350 g). The liposomal formulation improved the survival rate of rats subjected to ischemia and reperfusion by approximately 66%, compared to free CDPc. A clinical application may be proposed.

Keywords: CDP-choline; Unilamellar liposomes; Drug release; Liposome body distribution; Experimental ischemia model; Biological effectiveness

1. Introduction

Delivery of hydrophilic drugs to the mammalian brain is limited by the blood-brain barrier (BBB) (Brightman, 1977; Neuwelt and Barnett, 1991). A number of drug delivery strategies have been proposed to improve drug delivery, and particularly of antitumor agents, to the brain: (i) physical bypass, i.e. intrathecal or intratumoral drug administration (Nierenberg et al., 1991); (ii) temporary disruption of the BBB (Newelt et al., 1981); (iii) enhancement of the transcellular drug passage (Greig, 1987; Friden et al., 1991). Many of these potential therapeutic strategies present several drawbacks. In fact, intrathecal administration of drugs may fail to deliver the active compound to the deep cortex, whereas the administration of the drug directly to the site of action is normally associated with a certain neurotoxicity caused by high drug concentrations in normal brain tissues (Nierenberg et al., 1991). The hazard of severe neurotoxicity was also reported

* Corresponding author. Fax: +39 95 222239.

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in the BBB disruption technique (Baster and Lipschutz, 1988), despite a considerable number of studies reporting tollerance in animals and humans (Greig, 1987; Friden et al., 1991; Baster and Lipschutz, 1988; Greig et al., 1987).

Other means of enhancing the BBB permeation, improving transcellular drug passage, are represented by the use of lipophilic prodrugs or macromolecule-drug conjugates. Although these two methods of drug delivery potentially produce less neurotoxicity, they are able to deliver only a limited amount of the active compound, owing to the systemic distribution of the drug-carrier system (Friden et al., 1991). As reported (Laham et al., 1987), some drug delivery systems, e.g. microspheres and liposomes, can improve drug delivery to the brain across the BBB.

In this study, liposome formulation was used to deliver CDP-choline (CDPc) to the brain. This drug is normally used in the treatment of a number of cerebral diseases in which neuronal reorganization is required (Arienti et al., 1979). However, it was reported (Agut et al., 1983) that only 0.25% of the administered dose reaches the brain. For this reason, the liposome formulation could represent a valid device to improve drug delivery to cerebral tissue.

In a recent paper (Fresta et al., 1994), we investigated the possibility of using liposomes as a cerebral delivery system for CDPc in the treatment of ischemia. The encouraging results prompted us to extend the investigation with the aim of improving the formulation parameters and, hence, the therapeutic effectiveness. The present study reports the realization of unilamellar liposomes with a mean size of 50 nm, the in vivo biodistribution of the carrier and the evaluation of the biological effects on rat ischemia model.

2. Materials and methods

2.1. Chemicals

Dipalmitoyl-DL-α-phosphatidyl-L-serine (DPPS) and cholesterol (CH) were obtained from Sigma Chemicals Co. (St. Louis, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC) was purchased from Fluka Chemicals Co. (Buchs, Switzerland). Ganglioside G_{M1} 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) (Fresta et al., 1994). Phospholipid phosphorus content was assayed as inorganic phosphate as elsewhere reported (Bartlett, 1959). Fine sephadex G-50 was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). CDPc was kindly provided by Cyanamid-Italia (purity > 99.5%). [^{3}H]Cholesteryl hexadecyl ether and ^{31}Cr are New England Nuclear products. Double distilled pyrogen-free water was used. All other materials and solvents were of analytical grade.

2.2. Liposome preparation

The aqueous drug solution was prepared just before liposome preparation. CDPc (2g) was solubilized in 50 mL of phosphate buffer while adjusting 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. The final CDPc concentration (40 mg/mL) was determined at pH 1 by spectrophotometric analysis at 279 nm (Uvikon 860, Kontron instruments, Zurich, Switzerland).

The desired amount of lipids was weighed in a round-bottomed flask and dissolved in chloroform in the presence of 40 g of glass beads (2–3 mm mean size) (Carlo Erba, Italy). The use of chloroform-methanol mixtures was avoided because phase separation of cholesterol frequently occurrs during evaporation. The organic solvent was then removed at 30°C on a rotating evaporator under nitrogen stream and stored overnight under high vacuum (Edwards high vacuum pump mod. Serial E 2M8 42810). A thin film of dry lipid was deposited on the inner wall of the flask and along the glass bead surface. Large multilamellar vesicles were prepared under an atmosphere of nitrogen by hydrating (vortex mixing) the dry lipid film with the CDPc isotonic phosphate buffer, maintaining a temperature of 50°C throughout the process. Lipid concentrations of 50 mg·ml^{-1} were routinely employed.

The frozen and thawed multilamellar vesicles were obtained by freezing the MLVs in liquid nitrogen and thawing the samples in a warmed water bath. Eight freeze-thaw cycles were performed. The resulting liposomal suspension was extruded by means of a stainless steel extrusion device (Lipex Biomembranes, Vancuver, B.C.) equipped with 10-ml water-jacketed 'thermobarrel' connected to a thermostat, which allowed extrusion at 50°C. The liposomal suspensions were equilibrated at the selected temperature (20 min). Extrusion was carried out through two (stacked) polycarbonate filters (25 mm diameter; Nucleopore Corp., Pleasanton, CA, USA) at nitrogen pressures up to 5600 kPa. To achieve unilamellar vesicles, the extrusion procedure consisted of ten passages of the multilamellar liposome suspensions through 400-nm filters, followed by another cycle of ten passages through 200-nm polycarbonate filters and then again through 50-nm filters. The lipid recovery after the extrusion procedure was higher than 94%. Empty liposomes were prepared in the same way except that isotonic phosphate buffer was used instead of drug solution. The liposomal suspension (DPPL/DPPS/CH 7:4:7 molar ratio) contained 8 mol % of ganglioside G_{M1}.

2.3. Entrapment of CDPc

Gel-permeation chromatography was performed to separate free CDPc from liposome suspension. One milliliter of the liposome suspension was loaded into a fine Sephadex G-50 column (50 x 1.5 cm), pre-equilibrated with phosphate buffer. Liposomes were eluted in the void volume, whereas the free drug was retarded by the gel. In order to obtain the best experimental conditions, the osmolarity of the pH 7.4 phosphate buffer was corrected with NaCl to reach isotonicity with the trapped solution. The turbidity of the suspension and the concentration of untrapped CDPc were measured at 600 nm and 270 nm, respectively. A mixture of methylene chloride/methanol (2:1 v/v) was added to 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 CDPc content was then evaluated by HPLC analysis (Puglisi et al., 1992) from the linear regression of external CDPc standards relating peak-area and concentration. The amount of CDPc entrapped in liposomes was expressed as encapsulation capacity (EC = mL/mmol) (Benita et al., 1984).

2.4. CDPc release from liposomes

After the separation of the untrapped drug, unilamellar liposomes were made up to 5 mL with pH 7.4 isotonic phosphate buffer or with serum. The final phospholipid concentration was 35 mg/mL. The liposomal suspension was dialyzed with a Spectrapor/por 2 membrane MWCO 12-14000 (Spectrum, Los Angeles, California). The dialysis bag was put into a receiver compartment containing 150 mL of the 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.: mod. BTU 6) equipped with a Variomag electronic stirring system (submersible stirrers). CDPc was determined by HPLC analysis at set time intervals.

2.5. Physico-chemical characterization

Photon correlation spectroscopy was used to determine the vesicle size (Ostrowsky, 1993; Fresta et al., 1995). The morphological characterization was carried out by freeze-fracture electron microscopy, adopting the propane-jet technique (Müller et al., 1980). The cryofixed samples were fractured at −165°C and platinum/carbon replicas were examined in a Philips EM 301 electron microscope at 100 kV. ^{31}P-Nuclear magnetic resonance was employed to provide an indication of the lamellarity of the liposomal samples. ^{31}P-NMR spectra were performed on a GN500 MHz spectrometer operating at 202.45 MHz. Ninety-degree pulses were used with a pulse delay of 250 ms and an acquisition time of 205 ms. The average number of bilayers is calculated as previously reported (Fresta et al., 1995).

2.6. In vivo biodistribution of liposomes

Liposomes labeled with [^{3}H]cholesteryl hexadecyl ether were injected into the tail veins of Wistar rats (weighing 270 g) per group. At different times after injection, mice were anesthetized, bled via the retro-orbital sinus and killed by cervical dislocation. Major organs were dissected and two samples of each organ, blood, liver and spleen were weighed into glass vials and dissolved in tissue solubilizer. For scintillation counting, a 0.1 g portion of tissue was added to 2 ml of soluen-350 (Packard) and stood at 55°C for 24 h before adding 100 μl of hydrogen peroxide. Subsequently, 10 ml Hionic-Fluor (Packard) scintillation cocktail was added. Samples were left for at least 24 h prior to counting in a Packard scintillation counter. Data were expressed as % injected dose in organ or blood.

The blood content in various organs was taken into account by introducing a correction factor. This value was calculated by estimating the distribution of ^{51}Cr-labeled erythrocytes 30 min after i.v. injection, as reported in the literature (Lin et al., 1991). The values of correction factors for various organs resulted relatively low (less than 1%). A value approaching 6% was found for liver. The total blood weight was assumed to be 7.3% of the body weight (Wu et al., 1981).

2.7. Evaluation of CDPc biological effectiveness

The experimental ischemia model was performed by bilateral clamping of the common carotid arteries of Wistar rats (weighing 320–350 g) for 20 min, after which the blood flow was definitively restored. Rats were anesthetized by an i.p. injection of ethyl urethane (1.2 g/kg body weight). The animals were divided into 8 groups of 20 animals. Two groups were treated with the liposomal formulation containing CDPc, two groups with the free drug, and other two groups with saline. Two groups were sham-operated (control groups). A dose of 20 mg/kg CDPc (free or entrapped) was administered by injection into the tail vein 1h before ischemia, immediately after and thereafter once a day for 6 days. Ischemia was evaluated by determining lactate levels. In this case, the cerebral tissue was homogenized in 20 mM glycyl-glycyne buffer (pH 10) containing 70 mM glutammate. The homogenate was deproteinized by 4% $HClO_4$ (w/v) (final concentration). The amount of lactate was evaluated spectrophotometrically determining the formation of NADH at 340 nm (Noll, 1984).

3. Results and discussion

We have extensively studied the possibility of delivering CDPc with liposomes. In particular, physico-chemical studies (La Rosa et al., 1992a; Puglisi et al., 1992) evidenced that the incapsulation of this drug is mainly at the level of the aqueous compartment of the vesicular structures. Depending on the phospholipid composition, CDPc may also be located on liposome surface by means of an adsorption phenomenon mediated by hydrogen-bonds occurring between CDPc and the phospholipid head groups. In fact, negatively charged phospholipids are able to form strong hydrogen bonds and electrostatic interactions with this drug, altering the thermotropic parameters of the various liposome formulations (La Rosa et al., 1992a; Puglisi et al., 1992). Among the various negatively charged phospholipids, only mixtures presenting DPPS in their composition showed fusogenic properties (La Rosa et al., 1992b), which might be of great importance in liposome-cell interactions.

For these reasons, together with the preliminary biological experiments (Fresta et al., 1994), the lipid composition used for liposome preparation throughout this work was DPPC/DPPS/CH (7:4:7 molar ratio). In fact, this liposome composition evidenced both the highest drug-encapsulation parameters, whether as multilamellar vesicles or reverse-phase evaporation vesicles (EC = 5.7 and 15.5, respectively), and the best biological effectiveness in rat post-ischemic reperfusion (Fresta et al., 1993; Fresta et al., 1994).

To improve CDPc encapsulation within liposomes, the freeze and thaw technique was carried out. This method provided a perfect solute distribution equilibrium, ensuring high solute entrapment (Mayer et al., 1985). After ten cycles of

freezing and thawing, the liposome suspension showed an EC value of 28.5 ± 2.7 (n = 6), which was about 5 times higher than that expected (Hope et al., 1986). This result is probably due to the ability of the drug to form hydrogen bonds with DPPS and, hence, to drug-liposome adsorption.

Another important parameter in liposomal drug delivery is the mean size of the colloidal suspension. Thus, extrusion through polycarbonate filters was performed in order to reduce mean liposome size and lamellarity. After the extrusion through filters of different pore size (from 400 to 50 nm), a liposome colloidal suspension with a mean size of 63 nm was obtained. The extrusion procedure achieved not only a reduction in size but also in lamellarity (unilamellar vesicles) and the formation of a highly monodispersed liposome suspension (Fig. 1). Interestingly, the extrusion through polycarbonate filters did not cause any significant variation in the encapsulation capacity of the liposome formulation (EC = 27.9 ± 2.3; n = -6).

The possibility of obtaining 50 nm vesicles with high trapping efficiency is very relevant because

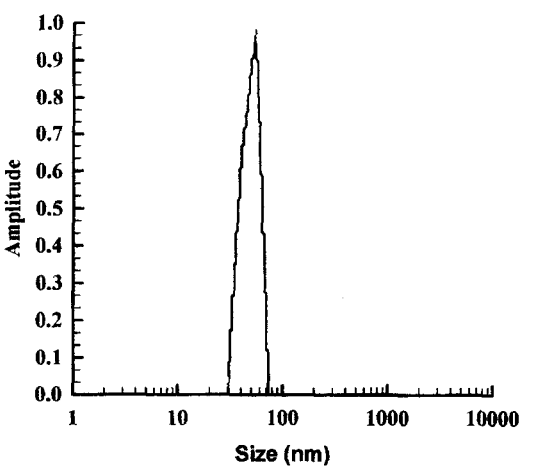

Fig. 1. Size distribution of unilamellar liposomes (DPPC/DPPS/CH 7:4:7 molar ratio) obtained by extrusion through 50 nm polycarbonate filters. The sample was diluted to achieve the most suitable optical density for light-scattering analysis. The measurement was carried out at 21°C with the photomultiplier at 90° to the incident beam (wavelength = 514.5 nm). The distribution function was determined by Laplace inversion transform.

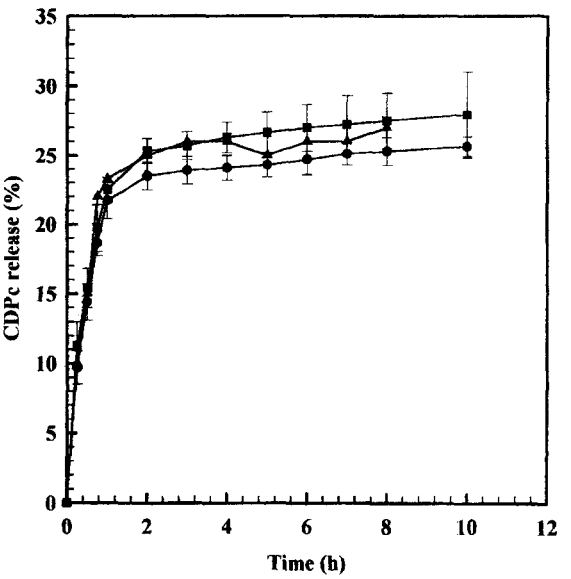

Fig. 2. CDPc release (%) from liposomes made up of DPPC/DPPS/CH (7:4:7 molar ratio). In the experiment carried out in serum, a small amount of sodium azide was added to prevent bacterial growth. The experiments were performed at a temperature of 37 ± 0.2°C. Each value is the average of seven experiments. Symbols: ●, 50 nm unilamellar liposome in pH 7.4 isotonic phosphate buffer; ■, 50 nm unilamellar liposomes in serum; ▲, REV liposomes in serum (data from Fresta et al., 1994).

they might theoretically pass through large fenestrations of the brain blood vessels and at the same time provide a greater carrying capacity than smaller sonicated liposomes. In fact, during an ischemic event there is damage to the BBB leading to the formation of fenestrations (~100 nm in size), with a consequent permeabilization of the barrier which allows the permeation of micro-aggregates (Ito et al., 1992). In order to deliver CDPc efficiently to the cerebral districts, the liposome formulation should present low drug leakage and a body distribution pattern, which ensures the presence of the delivery system in the blood for a long period of time, behaving as a circulating reservoir.

With regard to CDPc release from unilamellar vesicles, the permeability of DPPC-DPPS-CH (7:4:7 molar ratio) liposomes was evaluated by dialysis both in pH 7.4 isotonic phosphate buffer and serum. As shown in Fig. 2, no noticeable difference in CDPc leakage was detected for lipo-

Table 1
In vivo biodistribution at different times after injection via tail veins. The unilamellar liposomes (50 nm; DPPC/DPPS/CH 7:4:7 molar ratio) were prepared either with G_{M1} (8% mol) or without G^{a}_{M1}

Tissue	Without G_{M1}			With G_{M1}		
	3h	12h	24h	3h	12h	24h
Blood	26.7 ± 2.7	6.3 ± 1.4	0.48 ± 0.2	71.4 ± 3.2	46.2 ± 3.4	30.9 ± 2.9
RES[b]	53.7 ± 3.4	60.1 ± 3.6	47.8 ± 2.5	1.8 ± 0.7	8.2 ± 2.3	10.4 ± 2.4
Carcass and Skin[c]	10.2 ± 2.1	10.3 ± 2.5	8.5 ± 2.2	7.9 ± 2.1	19.9 ± 2.5	31.5 ± 3.6

[a]Results were presented as percentage of the injected dose. Each value represents the mean of six animals ± standard deviation.
[b]This compartment represents liver and spleen uptake.
[c]This compartment is constituted of skeletal muscles, bones, skin and appendages.

somes suspended in phosphate buffer or in serum. After a period of drug leakage, a plateau is reached. This behaviour is probably due to a desorption process of CDPc from the outer liposome surface. The good serum stability of our liposome formulation is due to the inclusion of CH and solid-phase phospholipids such as DPPC and DPPS in the lipid matrix. These components have been shown to increase liposome stability in plasma as determined in vitro by the degree of retention of a liposome-encapsulated marker (Senior and Gregoriadis, 1982). Furthermore, the inclusion of ganglioside G_{M1}, or other kinds of gangliosides, confers a negative charge and increased hydrophilicity to the liposome surface, which synergizes with CH to improve liposome stability in plasma (Gabizon and Papahadijopoulos, 1988). The absence of CH resulted in a rapid CDPc leakage (Fresta et al., 1994) owing to the loss of structural integrity in liposomes as a result of phospholipid removal by high density lipoproteins. These findings were similar to those obtained for reverse-phase evaporation vesicles (Fresta et al., 1993). In both cases, we are dealing with unilamellar vesicular systems, even if with different morphological and colloidal characteristics.

Considering that liposomes intravenously injected into animals are rapidly cleared from the blood circulation by uptake of the reticuloendothelial system (RES), primarily in the liver but also in the spleen and in other organs, i.e. lung and bone marrow (Poste, 1983), ganglioside G_{M1} was normaly introduced in the liposome lipid composition. It has recently been reported that liposomes containing ganglioside G_{M1} exhibit a prolonged circulation time in the blood, owing to their reduced affinity with RES in liver and spleen compared to ordinary liposomes (Gabizon and Papahadijopoulos, 1988). Thus, liposomes with longer circulation half-life, which release their contents slowly over a long period in the blood circulation, stand a better chance of penetrating biological barriers. The presence of ganglioside G_{M1}, therefore, may ensure a suitable penetration of the liposome formulation through the blood-brain barrier, particularly if the vesicle suspension presents a mean size less than 100 nm.

The liposomes were labeled with [^{3}H]cholestryl hexadecyl ether and injected i.v. into rats through the tail vein. The lipid vesicle biodistribution was determined up to 24 h after administration. To facilitate a comprehensive analysis of liposome distribution, the tissue distribution data are presented in three parts: (i) blood; (ii) liver and spleen, relating to the total reticuloendothelial system; (iii) carcass and skin, consisting of skeletal muscles, bones, skin and appendages. As reported in Table 1, the incorporation of 8 mol % of ganglioside G_{M1} significantly increased the blood level of liposomes and decreased the reticuloendothelial uptake, compared to the same liposome formulation prepared without the ganglioside G_{M1}. The biodistribution data showed that as the uptake by the RES decreased, there was a concomitant increase in the liposome blood pool and in the fraction of the dose accumulating in the carcass and skin. At 24 h after the labeled lipo-

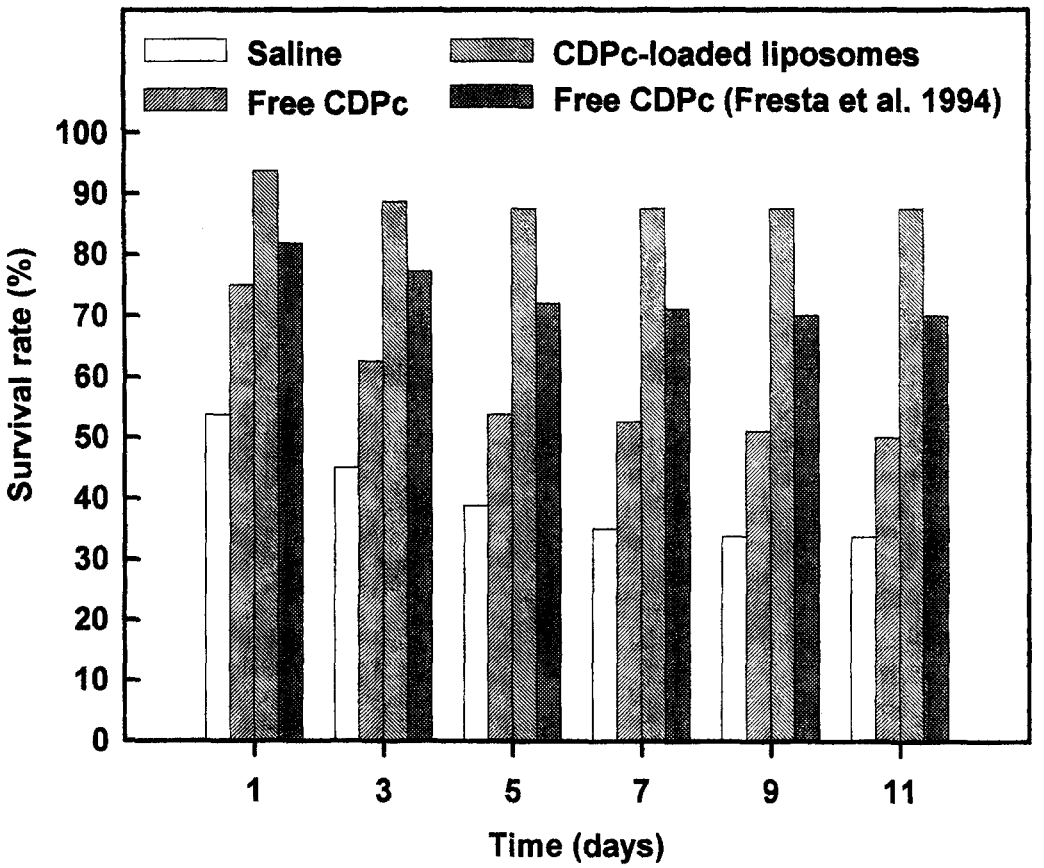

Fig. 3. Survival rate of Wistar rats (320–350 g) following post-ischemic reperfusion as a fuction of time. Animal groups were treated with free, liposome entrapped CDPc or saline. Sham-operated rats (control group) have a survival rate of 100%.

some administration, 31% of the injected vesicles were still present in the blood stream. This value was 65 times higher than that of liposomes prepared without ganglioside G_{M1}, while the dose recovered in the RES decreased by a factor of 4.6, and that in the carcass and skin increased 3.7 times. Thus, the long-ciculating capability of the liposome formulation was provided by the presence of ganglioside G_{M1} which synergizes its action in the presence of rigid lipids (Gabizon and Papahadjopoulos, 1988), further to the improved colloidal properties (mean size 50 nm), which ensure per se low levels of RES uptake. The long-circulating attribute is a very important requisite for liposome formulations used to entrap drugs with target organs or tissues other than RES (liver and spleen), as in the case of CDPc with its therapeutic action at the level of the brain.

The therapeutic effectiveness of liposome-entrapped CDPc compared to the free drug was assayed in Wistar rats (weighing 320–350g) by means of an in vivo experimental model of ischemia, evaluating the survival rate of the rats after ischemia and reperfusion. As shown in Fig. 3, CDPc-loaded unilamellar G_{M1}-liposomes were more effective than the free drug, achieving a 66% higher survival rate than the control dosage-form (isotonic drug solution). Previously, we have reported (Fresta et al., 1994) a more modest 33% improvement in the survival rate when liposomal formulation was used. This difference may be ascribed to various factors: (i) age of male Wistar

rats used in the experiments; (ii) prolonged presence of the formulation in the blood; (iii) improved colloidal characteristics (REV mean size 120 ± 11 nm).

Concerning the first point, 1-month-old Wistar rats (80–100 g) were used in the previous study. In this case, the BBB is not completely formed, allowing permeation of both free and entrapped drug. Using older animals (320–350 g) with a well formed blood-brain barrier, resulted in a greater differenziation in the biological effectiveness of the different drug dosage forms free or liposome entrapped. The real difference between these findings and those previously reported (Fresta et al., 1994) is not represented by the values of the survival rate of the rats treated with the liposome formulation, which are almost similar for both data sets, but in the survival rate values of the animals treated with the free CDPc, ~70% and ~50% for 1 and 7-month-old Wistar rats, respectively. The completely formed blood-brain barrier in the older rats hampered the free passage of the highly hydrophilic drugs through the barrier (CDPc $\log_p$ experimentaly found $= -1.7$), so reduceing the CDPc available to the brain.

Finally, the colloidal characteristic of the liposome formulation, investigated here, can play an important rule both in blood half-life (liposomes as circulating reservoirs) and passage through the fenestration of the BBB caused by the ischemic event.

These results showed the advantage of the liposomal carrier compared to the free CDPc in the treatment of cerebral ischemia. In principle, a large series of cerebrotonic and/or neurotrophic drugs with different physico-chemical properties may be delivered to the brain, considering the great versatility of liposomes in entrapping drugs. Another important aspect that arose in this study was the characteristics of the liposome formulation, i.e. phospholipid composition, mean size of the colloidal suspension, size distribution and biological fate, which may greatly influence the therapeutic effectiveness. A clinical application may be proposed for CDPc-loaded liposomes.

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