Reduction of Maturation Phenomenon in Cerebral Ischemia with CDP-Choline-Loaded Liposomes[1]

Massimo Fresta[2,3] and Giovanni Puglisi[2]

Received March 3, 1999; accepted September 11, 1999

Purpose. Cerebral ischemia represents a serious therapeutic challenge. We investigated the therapeutic efficacy of CDP-choline-loaded liposomes against cerebral ischemia. The determination of post-ischemic brain recovery by EEG analysis was carried out to evaluate the effect of CDP-choline-loaded liposomes with respect to the free drug on the maturation of ischemic injury.

Methods. Long-circulating unilamellar liposomes were prepared by a freeze and thaw procedure followed by an extrusion through polycarbonate membranes. Wistar rats were ischemized by bilateral clamping of the common carotid arteries. Free or liposomally entrapped drug was administered (20 mg/kg) just after ischemia and thereafter once a day for six days. Post-ischemic survival, neuronal membrane peroxidation and brain recovery (EEG analysis) were evaluated.

Results. The post-ischemic reperfused rats treated with CDP-choline-loaded liposomes showed a higher survival rate than animals treated with the free drug. The delayed cerebral neurodegenerative injury due to an ischemic event, referred to as maturation phenomenon, was substantially reduced with the administration of the liposomal formulation. The liposomal carrier showed a marked protection against lipoperoxidative damage.

Conclusions. Liposomes ensured a rapid recovery of the damaged membranous structure of the neuronal cells, allowing a significant improvement of brain functionality. The reduction of the maturation phenomenon may probably be of particular importance in humans, where a fundamental problem is the quality of life after an ischemic event.

KEY WORDS: cerebral ischemia; ischemic maturation; CDP-choline-loaded liposomes; brain lipoperoxidation; liposomal brain delivery.

INTRODUCTION

The supply of oxygen and glucose by the cerebral blood circulation is very important for the biological activity and the survival of neurones (1). The block, even local, of the cerebral blood flow causes primary damage in the brain area involved in the ischemic event and secondary damage in the surrounding area (penumbra zone), where there is only a partial reduction of the energy supply (2,3). After the re-establishment of the blood circulation and brain reperfusion a slow progressive ischemic injury has been reported regarding various portions of the brain, even areas unaffected directly by ischemia (4,5). This delayed development of pathological changes in various biochemical and physiological parameters of ischemic injury is referred to as maturation phenomenon (6). A direct relationship between the intensity of the ischemic event and the rate of maturation of the consequent brain injury has been observed (7). The maturation of the post-ischemic neurodegeneration may be reduced with drugs and/or drug delivery systems which may reach the brain and exert their therapeutic action. Thus, sick-but-not-dead neurones may still be recovered (8).

Colloidal carriers have presented interesting potentialities as a brain drug delivery system (9–11). The therapeutic advantages of the treatment of a cerebral ischemia model on Wistar rats with liposome colloidal suspensions containing CDP-choline have been reported (12–14). In particular, CDP-choline is a therapeutic agent widely used in the treatment of Parkinsonism, extrapyramidal diseases and consciousness disorders in brain injury. CDP-choline contributes to the repair of the membranous structures of brain cells that have been broken down by cerebrovascular damage (15). CDP-choline-loaded liposomes have been able to improve the therapeutic effectiveness of rat cerebral post-ischemic reperfusion in comparison to the free drug, that is, increasing the survival rate of ischemized rats and the cerebral drug delivery, and protecting the brain against peroxidative damage (13).

In the present paper, the effect of CDP-choline-loaded liposomes on the maturation of ischemic injury is presented and discussed. The therapeutic effectiveness of liposomes was determined evaluating brain recovery by electroencephalographic (EEG) analysis. Blood-brain barrier (BBB) damage elicited by an ischemic event was also studied.

MATERIALS AND METHODS

Chemicals

The lipids 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 (DPPC) from Fluka Chemicals Co. (Buchs, Switzerland) and ganglioside G_{M1} from Boehringer Mannheim. Before each experiment, the phospholipid purity (>99%) was checked by two-dimensional TLC on silica gel plates (E. Merck, Darmstadt, Germany) (16). Phospholipid phosphorus content was assayed as inorganic phosphate as reported by Bartlett (17), while the phospholipid concentrations were determined using the method of Rouser (18). CDP-choline was kindly provided by Cyanamid-Italy. The drug purity was greater than 99.5% by HPLC. Inorganic salts were of analytical grade (BDH laboratories Supplies, Poole, UK). Double distilled pyrogen-free water was used throughout. All other materials and solvents were of analytical grade.

Liposome Preparation

The aqueous drug solution was prepared just before liposome preparation. CDP-choline (2 g) was solubilized in 50 ml of phosphate buffer (pH 7.4) while adjusting the ionic strength

[1] Part of this research was presented at the Fourth European Symposium on Controlled Release, April 3–5, 1996, Noordwijk aan Zee, The Netherlands.

[2] Department of Pharmaceutical Sciences, University of Catania, Viale Andrea Doria 6, I-95125 Catania, Italy.

[3] To whom correspondence should be addressed. (e-mail: fresta@mbox.unict.it)

ABBREVIATIONS: EEG, electroencephalographic; BBB, blood-brain barrier; DPPS, dipalmitoyl-DL-α-phosphatidyl-L-serine; CH, cholesterol; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; G_{M1}, ganglioside G_{M1}; MLVs, multilamellar vesicles; SUVETs, small unilamellar vesicles by extrusion technique; PCS, photon correlation spectroscopy; TNW, total number of waves; TAW, total amplitude of waves.

0724-8741/99/1200-1843$16.00/0

of the solution with NaCl to 300 mOsm at 37°C. The final drug concentration was 40 mg/ml.

Multilamellar liposomes (MLVs) were prepared under nitrogen by hydrating (vortex mixing) the dry lipid film with the CDP-choline solution at a temperature of 50°C, followed by eight cycles of freezing in liquid nitrogen and thawing at 55°C. The liposome lipid composition was DPPC-DPPS-CH (7:4:7 molar ratio) with ganglioside G_{M1} 8% mol.

Small unilamellar vesicles with high drug capture (SUVETs) were obtained by extrusion at 50°C, through two (stacked) polycarbonate filters mounted on a stainless steel extrusion device (Lipex Biomembranes, Vancouver, B.C.). The extrusion procedure consisted of ten passages of MLVs through 400 nm filters, followed by another cycle of ten passages through 200 nm filters and then through 50 nm filters. The recovery of SUVETs as lipid material after the extrusion procedure was higher than 94%. The final lipid concentration was 50 mg/ml. The untrapped CDP-choline was removed from SUVETs by gel-permeation chromatography on a fine Sephadex G-50 column (50 × 1.5 cm). To evaluate the loading capacity of liposomes, the chromatographic fractions containing CDP-choline-loaded liposomes (50 μl) were solubilized in methanol and assayed by HPLC analysis with a Hewlett-Packard mod. 1050 (Cernusco S/N, Milano, Italy) (12). Results are expressed as drug content (percentage of CDP-choline in the liposome formulation) and encapsulation capacity (μl/μmol), as reported elsewhere (19). The drug recovery as untrapped and encapsulated material was always greater than 97.5% of the amount added.

Physico-Chemical Characterization of Liposomes

Photon correlation spectroscopy (PCS) was used to determine the vesicle size (Zetamaster, Malvern Instruments Ltd, Sparing Lane South, Worcs, England). The experiments were carried out using a solid state laser as light source. This laser is a nominal 4.5 mW laser diode with a maximum output of 5 mW at 670 nm. The PCS measurements were carried out at a scattering angle of 90°. The correlation functions were performed by a Malvern PCS sub-micron particle analyser and a third-order cumulant fitting with a dilation of 1.20 to obtain the mean diameter and polydispersity. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively (20). The following parameters were used for experiments: medium refractive index 1.330, medium viscosity 1.0 and a dielectric constant of 79. The samples were suitably diluted with filtered water (Sartorius membrane filters 0.22 μm, Göttingen, Germany) to avoid multiscattering phenomena and placed in a quartz couvette. Thirty measurements per sample were performed.

The morphological characterization was carried out by freeze-fracture electron microscopy, using the propane-jet technique (21). The samples were fractured at −165°C and platinum/carbon replicas were observed in a Philips EM 301 electron microscope at 100 kV. ^{31}P-NMR was employed to provide the liposome lamellarity (13).

Induction of Cerebral Ischemia

Young (weighing 80–100 g) and adult (weighing 320–350 g) male Wistar rats were anesthetized by an i.p. injection of ethyl urethane (1.2 g/kg body weight) and made ischemic by bilateral clamping of the common carotid arteries for 5, 15 and 30 min, after which the blood flow was definitively restored. For each experiment concerning the different duration of the ischemic event, the animals were divided into groups of 15 animals. Two groups (a total of 30 animals) were treated with the liposomal formulation containing CDP-choline, two groups with liposome alone, two groups with the free drug, and two groups with saline. Two groups were simply sham-operated and not ischemized (control groups). The liposome formulation, containing a dose of 20 mg/kg CDP-choline, or the same dose of the free drug, was administered by injection into the tail vein immediately after ischemia and thereafter once a day for 6 days.

Rats investigated for brain lipid peroxidation were treated with CDP-choline (alone or entrapped in liposomes) immediately after ischemia and then killed by decapitation after 1 h of reperfusion. Ischemic damage was evaluated by determining lactate levels (22), while brain lipid peroxidation was estimated by measuring the levels of conjugated diene.

In vivo experiments on male Wistar rats adhered to the "Principles of Laboratory Animal Care" (23).

Determination of Lactate Levels

In order to determine the lactate levels, the cerebral tissue was homogenized in 20 mM glycil-glycine buffer (pH 10) containing 70 mM glutamate. The homogenate was deproteinized by 4% $HClO_4$ (w/v) (final concentration). The amount of lactate was determined spectrophotometrically following the formation of NADH at 340 nm using Noll's method (24).

Analysis of Brain Lipid Peroxidation

The determination of conjugated dienes was carried out by homogenizing the cerebral tissue in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA. The lipid component was extracted using a chloroform-methanol (2:1 v/v) mixture. After solvent evaporation, the dried material was resuspended in cyclohexane and assayed at 234 nm. The results are expressed as μmoles lipohydroperoxide/mg lipid by employing ε_M 2.52 × 10^4 M^{-1} cm^{-1} (25). To avoid lipid peroxidation all procedures were carried out under pure nitrogen.

Blood-Brain Barrier Permeability

Following 5 min, 15 min or 30 min ischemia, six male Wistar rats were sacrificed at different time after post-ischemic reperfusion. The evaluation of the BBB damage due to the ischemic injury was performed by injecting i.v. 0.1 ml/100 g of 2% Evans blue dye into the male Wistar rats. This treatment was carried out either immediately before restoration of blood flow or 5 h before sacrifice at 1 week after restoration of blood flow. The animals were sacrificed with transcardiac perfusion of 10% buffered paraformaldehyde solution. The brain was cut into two coronal pieces at the level of the optic chiasma and infundibulum. The permeability of the BBB to the dye was evaluated by visual inspection. The number of male Wistar rats showing BBB permeability to Evans blue dye, expressed by the presence of more than one blue-coloured area with mean diameters of 2 mm or more, was counted.

EEG Experiments

Ischemic maturation phenomenon was monitored by EEG. This is a non-invasive and accurate method to evaluate brain

functionality and neuronal damage recovery (26). Silver electrodes were placed epidurally at the bilateral parieto-occipital cerebral hemisphere. The reference electrodes were placed on both ears. For each rat, monopolar EEGs were bilaterally recorded prior to and immediately after the re-establishment of the blood flow circulation, and thereafter at determined time intervals. The recorded EEG, 1 min in the entire frequency zone (0.5–30 Hz), was analyzed according to a computerized wave-form recognition method (27), which allowed the determination of the total number of waves (TNW). The values of the number of waves in each frequency zone multiplied by the mean amplitude in the same frequency zone were summed for the whole frequency zone (0.3–30 Hz). This value is referred to as the total amplitude of waves (TAW) (28). These two parameters (TNW and TAW) suitably describe EEG traces, thus they can be used to evaluate brain functionality (26,28).

RESULTS AND DISCUSSION

The liposome formulation used in this work consisted of DPPC-DPPS-CH (7:4:7 molar ratio). The extrusion procedure through 50 nm polycarbonate filters was carried out in order to obtain a liposome suspension with improved colloidal properties and narrow size distribution. The final liposome suspension consisted of unilamellar vesicles (Fig. 1) with a diameter of 49.3 ± 3.1 nm and a polydispersity index of 0.01. The encapsulation capacity and the drug content value of this liposome colloidal suspension was 27.9 ± 2.3 μl/μmol lipids and 39.7 ± 4.3%, respectively.

G_{M1} was introduced into the liposome lipid composition at 8 mol% to achieve a liposomal colloidal suspension with long-circulating properties (29). In fact, as previously reported (13), this liposome formulation presented long-circulation properties due to the small vesicle size and the highly hydrophilic surface of liposome bilayers determined by the presence of G_{M1}, that is, 34% of the administered liposomal dose was still present in the blood circulation after 24 h.

The therapeutic effectiveness of the liposome formulation was assessed evaluating the survival rate of ischemized Wistar rats. The experimental ischemia model, carried out on Wistar rats (bilateral clamping of common carotid arteries), reproduces many aspects present in human ischemic events (3).

In order to ascertain the best experimental conditions, ischemia experiments were carried out both on young (80–100 g) and adult (350–400 g) male Wistar rats, as well as administering the drug in a set of experiments both before and after ischemia, and finally a further set only after the ischemic process. Figure 2 shows that young Wistar rats ischemized for 15 min and then reperfused presented a greater survival rate than adult Wistar rats (350–400 g). This result may be due to two factors: i) a reduction in adult Wistar rats of the effectiveness of all physiological processes acting against secondary ischemic damage (30); ii) the BBB of young animals is not completely formed thus allowing an easier penetration of CDP-choline. A slight difference in survival rate (Fig. 2) was observed between rat groups treated with CDP-choline: i) both before and after ischemia, and ii) the group treated only after the restoration of the blood flow. These findings show, according to literature (31,32), that CDP-choline principally acts at the level of the secondary ischemic damage rather than on the neuronal area directly damaged by an oligemic phenomenon.

The effectiveness of CDP-choline is greater in animals with a submaximal ischemic injury (relatively short ischemic

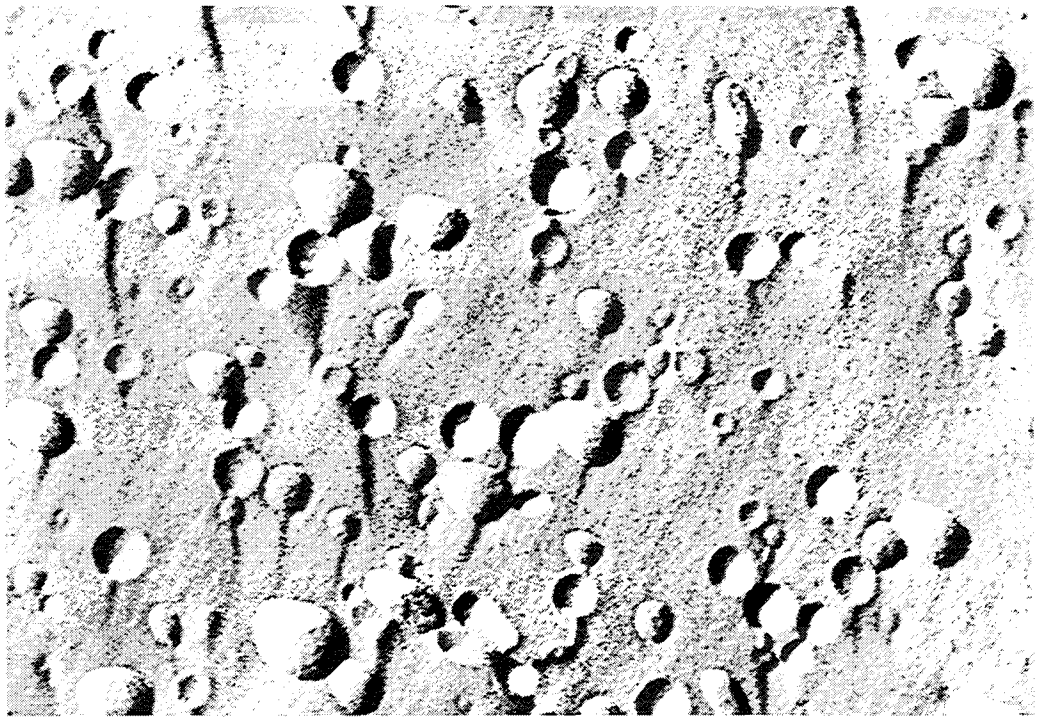

Fig. 1. Freeze-fracture micrograph of colloidal liposome suspension made up of DPPC-DPPS-CH (7:4:7 molar ratio) achieved by extrusion through 50 nm polycarbonate membranes. The lipid concentration was 50 mg/ml. Magnification × 100,000.

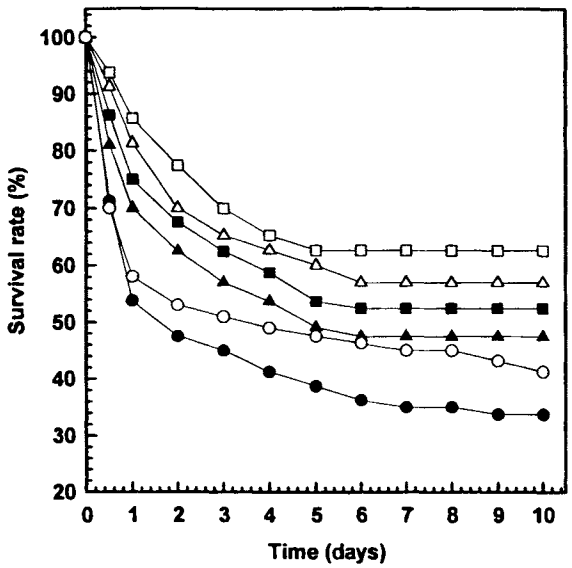

Fig. 2. Survival of the total number of young (80–100 g; hollow symbols) or adult (350–400 g; filled symbols) male Wistar rats made ischemic for 15 min and reperfused. Male Wistar rats (two groups for each experiment and for a total of 30 animals) were treated with saline (○, ●), with CDP-choline (20 mg/kg) both before and after ischemia (□, ■) or with the drug only after restoration of blood flow (△, ▲). Sham-operated animals (control group) presented a survival rate of 100%. Statistical log-rank analysis provided an error probability less than 1% for each survival curve.

event), than in animals suffering maximal ischemic injury produced by a long ischemia (15). For these reasons, adult Wistar rats (350–400 g) were employed to evaluate the influence of ischemia duration (5, 15 or 30 min) on survival of animals treated with free or liposome entrapped CDP-choline, which was administered only after the restoration of cerebral blood flow. As shown in Table I, the liposome formulation ensured a better response than the free drug. In particular, increasing the duration of the ischemic event a reduction of the survival of the rats treated both with CDP-choline and with saline was observed. This trend was probably due to the greater brain injury elicited by the longer duration of ischemia. In the case of the drug-liposome formulation, the survival rate was less influenced ranging from 80 to 97 per cent. The CDP-choline-loaded liposomes achieved an improvement of the survival rate of ischemized and reperfused rats in comparison with the free drug of 45%, 53% and 100% when the duration of ischemia was 5 min, 15 min and 30 min, respectively.

The better biological response of the liposome formulation compared to the free CDP-choline, with an increasing duration time of the ischemic event, could be due to the long-circulating behaviour (presence of ganglioside G_{M1}) of the colloidal suspension, which may act as a circulating reservoir capable of favouring a slow drug entrance into the brain through the BBB, which becomes more permeable as the ischemic damage increases (Table II).

The hyperpermeabilization of the BBB following an ischemic event is triggered by the formation of fenestrations with a mean size of ~100 nm (33–34). As reported in Table II, the incidence and the extent of BBB damage is related to the duration of the ischemic event. In the case of animals ischemized for 5 min, no evidence of BBB ischemic damage was obtained up to 6 h post-ischemic reperfusion. Only after 12 h of reperfusion was evidence of BBB damage seen, but with a low incidence. After a week of post-ischemic reperfusion no BBB damage was observed. Increasing the duration of ischemia, an increase both of the incidence of the number of animals and of the rate of appearance of BBB damage was observed. In fact, in the case of rats ischemized for 15 min an incidence of 6/6 was observed after 24 h of post-ischemic reperfusion; while, in the case of rats ischemized for 30 min, an incidence of 100% was achieved just after 6 h of post-ischemic reperfusion. The ischemic duration influenced not only the incidence of the number of animals presenting BBB damage but also the extent of the damage. Namely, prolonging ischemia from 5 min to 30 min, both an increase of the number of cerebral areas coloured in blue and a widening of these area were observed. In any case, after 24 h of post-ischemic reperfusion a gradual regression of BBB damage was observed, i.e. an incidence of 16.6% and 33.3% for rats ischemized for 15 min or 30 min, respectively. These findings may partially explain the therapeutic efficacy of CDP-choline-loaded long-circulating liposomes, that is, a greater BBB permeability can ensure a more effective drug carrier entrance into brain. In fact, liposome colloidal formulation ensured a greater entrance of CDP-choline (22% of the administered dose) than the free drug (2% of the administered dose) (13).

The improvement of the CDP-choline therapeutic action with regard to ischemia may also be due to a phenomenon of passive targeting caused by the presence of DPPS in the liposome lipid composition. In fact, this phospholipid family is a constituent of the cerebral tissue, carrying out particular metabolic functions in cerebral activity (35).

The liposome formulation was able to improve not only the survival rate of post-ischemic reperfused rats but also the

Table I. Percentage of Survival (after 8 days) of the Total Number of Male Wistar Rats Submitted to an Ischemic Event of Different Duration[a]

Experiment[b]	Duration of the ischemic event		
	5 min	15 min	30 min
Saline	50.0	36.6	23.3
Free CDP-choline	66.7	56.7	40.0
Liposome alone	53.3	43.3	33.3
Liposome with drug	96.7	86.7	80.0

[a] The various rat groups (a total of 30 animals for each experiment) were treated immediately after the ischemic event and thereafter once a day for six days.
[b] The control group (sham-operated) showed a survival of 100%.

Table II. Incidence of the Number of Male Wistar Rats Presenting Damage at the Level of BBB After an Ischemic Event of Different Duration[a]

Ischemia	Post-ischemic reperfusion					
	5 min	1 h	6 h	12 h	24 h	1 week
5 min	0/6	0/6	0/6	1/6	1/6	0/6
15 min	0/6	1/6	2/6	4/6	6/6	1/6
30 min	0/6	3/6	6/6	6/6	6/6	2/6

[a] The incidence of BBB damage with regard to control group is nil, since the BBB is totally impermeable to Evans blue dye.

brain lipid peroxidation. In fact, as reported in Table III, the rats treated with the liposome formulation containing CDP-choline presented the lowest levels of lipohydroperoxide, which indicates a noticeable action against the neuronal lipoperoxidative damage caused by the post-ischemic reperfusion (36). Similarly to survival data, peroxidative damage was ischemia-time-dependent. In the case of long ischemia duration, the damage at the level of lipid neuronal membranes was very severe. It is noteworthy that CDP-choline-loaded liposomes, irrespective of ischemic event duration, were able to achieve lipohydroperoxide levels close to control values. This result may be due to the fact that liposomes containing CDP-choline efficiently antagonize the post-ischemic peroxidative injury, hampering and/or limiting a cascade of events which lead to the development of an irreversible secondary damage. Therefore, our findings show the capacity of the drug-loaded liposomes to repair the neuronal membranes and, hence, to ensure the activation of the functional reorganization of the brain (37). The CDP-choline biological action may come from a synergistic effect between the phospholipid components of the liposomal delivery system and the encapsulated CDP-choline. Thus, the neuronal cells can employ the liposomal phospholipids as a substrate to repair and reactivate the biological membranes.

The maturation phenomenon of the post-ischemic injury following temporary ischemia seems to be directly related to the intensity of ischemia, that is, a less intense ischemic insult results in a lesser and slower development of the lesions. This maturation phenomenon is probably elicited by a later development of small foci of infarction which inter-connect to form larger ones (5). In fact, it was shown (38,39) that neuron vulnerability with regard to ischemic events is determined by a massive entrance into neuronal cells of Ca^{++} ions, which trigger membrane receptor activation mediated by excitatory aminoacids, i.e., aspartate and glutammate released in intersynaptic spaces following ischemia. Both these neurotransmitters and Ca^{++} ions elicit activation of a second intracellular messenger and of Ca-dependent enzymes which cause loss of structural functionality of neuronal membranes leading to an irreversible degenerative process and finally cellular death, even after restoration of blood flow (7). In addition, the formation of oxygen reactive species, which are formed during post-ischemic reperfusion, elicits peroxidation of both membrane lipids and proteic systems contributing to the post-ischemic damage (40). All these mechanisms are responsible for the ischemic maturation phenomenon, which can be monitored by means of EEG analysis.

Table III. Levels of Lipohydroperoxide (μmol/mg lipid) as an Index of Brain Lipoperoxidation in Male Wistar Rat Cerebral Cortex Following Post-Ischemic Reperfusion

Experiment	Duration of the ischemic event		
	5 min	15 min	30 min
Control (Sham-operated)	23.7 ± 6.8	—	—
Saline[a]	93.4 ± 5.2	122.1 ± 9.6	151.7 ± 10.3
Free CDP-choline[b]	47.1 ± 3.5	83.1 ± 4.1	99.2 ± 4.6
Liposome alone[a]	63.2 ± 4.7	95.4 ± 6.3	107.6 ± 5.9
Liposome with drug[b,c]	18.9 ± 1.1	27.5 ± 2.4	34.2 ± 3.3

Note: Each value is the average of five animals ± SD.
[a] $P < 0.005$ (vs control).
[b] $P < 0.001$ (vs saline).
[c] $P < 0.005$ (vs free CDP-choline).

The recording of an EEG during the ischemic event showed a gradual reduction of both TNW and TAW values. After the restoration of the cerebral blood flow, TNW and TAW values recovered rapidly up to 3 h post-ischemia depending on the duration of the ischemia. Namely, the shorter the ischemic event, the more intense the recovery (Figs. 3 and 4). In particular, in the case of untreated rats ischemized for 30 min no evident recovery of the TNW and TAW values to control values was observed (Figs. 3 and 4).

After the first period (~3 h), the recovery was more gradual for the next 2 h (maximum recovery), followed by a plateau until 6–8 h after restoration of blood flow. When the rats were treated with CDP-choline-loaded liposomes the recovery was more rapid than with the free drug. Moreover, the recovery of TAW was also significantly greater ($P < 0.01$) and closer to the control for the group treated with the liposome formulation with respect to the free drug or saline treated groups. In particular, a total recovery of the 5 min ischemized rats treated with the liposome formulation was observed (Fig. 4).

The values of the TAW of the Wistar rats treated with saline or the free drug dropped to lower levels after 48 h post-ischemia. This trend was more evident for the untreated rats (saline group). The decrease in recovery for the liposome-treated rats was almost negligible and depended on the intensity of ischemia. In fact, in the case of 5 min ischemia a 100% recovery compared with the control group was achieved and maintained up to 48 h post-ischemia; whereas, a slight reduction was observed in the case of 30 min ischemized rats.

The typical profile of TAW of post-ischemic reperfused rats was a first period of cerebral recovery (up to ~8 h) and a second phase of neuronal degeneration might reflect an initial period characterized by the recovery of the energy metabolism, followed by a drop in brain activity coinciding with the maturation of histopathological neuronal injury (41). Depending on the duration of ischemia, the maturation phenomenon was noticeably reduced in the case of liposome treated rats, whereas

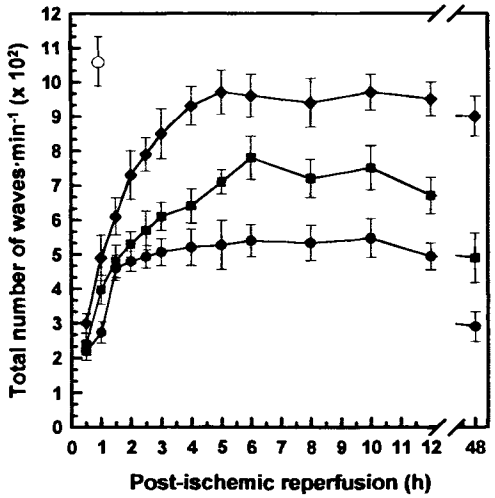

Fig. 3. Values of the total number of waves (TNW) in EEGs recorded for 1 min in the entire frequency zone (0.5–30 Hz) after restoration of blood flow. The male Wistar rats were submitted to an ischemic event with a duration of 15 min. Each point is the mean value ± SD of 7 experiments. The rats were treated with saline (●), free (■) or liposomally entrapped (♦) CDP-choline; (□) control.

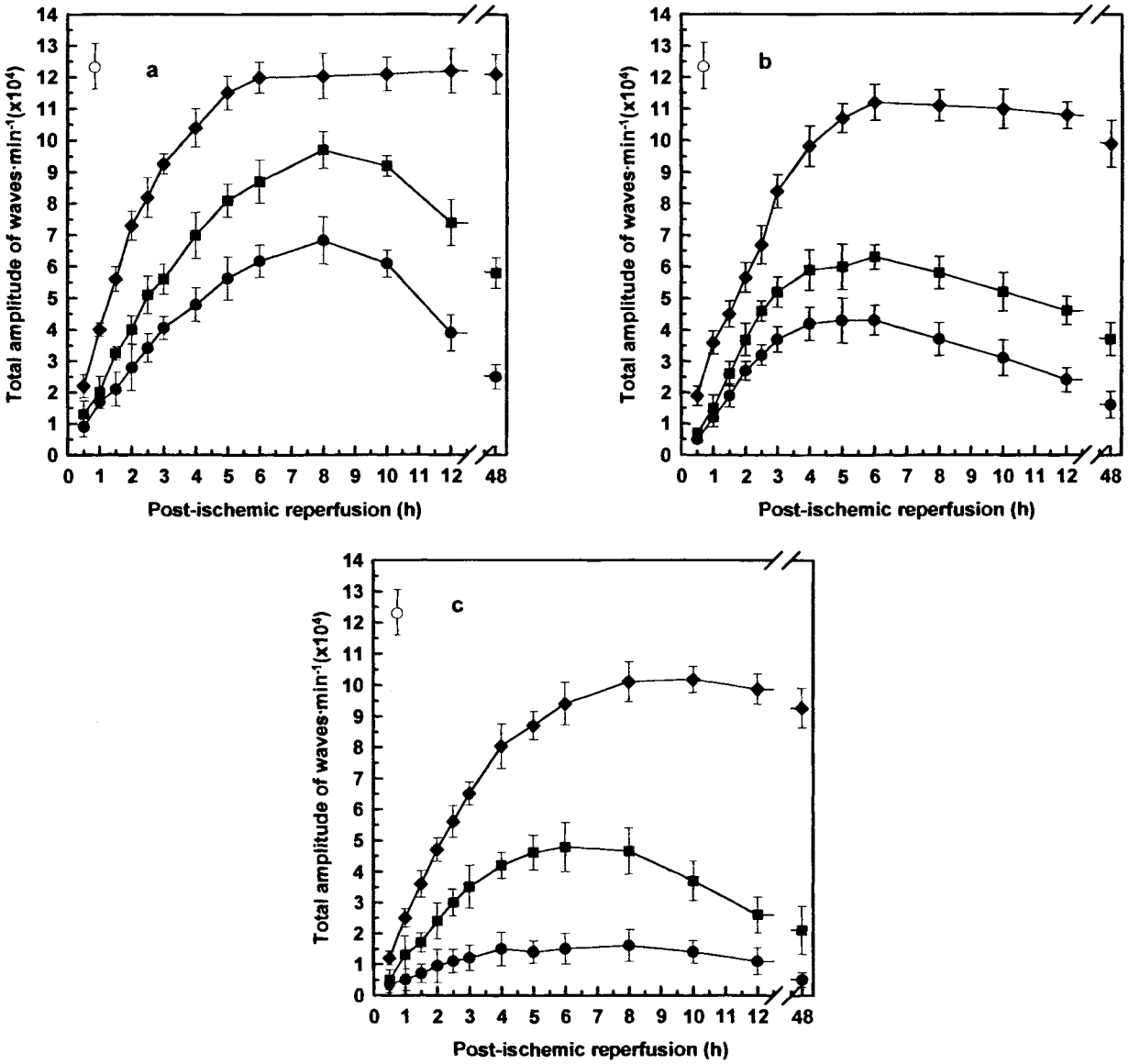

Fig. 4. Values of the total amplitude of waves (TAW) in EEGs recorded for 1 min in the entire frequency zone (0.5–30 Hz) after restoration of blood flow. The male Wistar rats were submitted to an ischemic event with a duration of 5 min (a), 15 min (b) and 30 min (c). Each point is the mean value ± SD of 7 experiments. The rats were treated with saline (●), free (■) or liposomally entrapped (♦) CDPcholine; (○) control.

it was evident for saline or free drug treated animals. In particular, following 30 min ischemia, the EEG of saline treated rats did not recover after the restoration of the blood flow showing a large and homogenous (not focal) infarction area. These findings were due to severe cerebral damage in which an extensive neuronal death elicited a massive energy failure (39). Thus, in this animal group, the maturation phenomenon could not be observed after restoration of blood flow.

The delay of the cerebral damage is partially elicited by a membrane perturbation deriving from the activation of an intracellular message transmitter enzyme system (7). Another important factor that seriously compromises the intracellular homeostasis is the loss of the functional integrity of the cell membrane elicited by degeneration processes induced by the ischemic event. When the cell membrane was not able to ensure its own function as an osmotic barrier, neurons died. Furthermore, free radicals, causing the lipid peroxidation following post-ischemic reperfusion, might also promote the membrane perturbation leading to post-ischemic neuronal damage. As reported in Table III, the lipoperoxidation damage is substantially reduced by the liposomes containing CDP-choline. The possibility of reducing and/or rapidly repairing the membranous structures of neuronal cells may lead to a noticeable reduction of the delayed cerebral degeneration, as in the case of CDP-choline-loaded liposome treated rats (Fig. 4).

In conclusion, the liposome formulation was able to both improve the survival of ischemized rats and to almost completely recovery the neuronal functionality, particularly of the neurons present in the border zone directly damaged by the oxygen free radicals and by high levels of excitatory amino

acids. The significant reduction of the maturation phenomenon may probably be of particular importance in humans, where, besides survival, a fundamental problem is the quality of life after an ischemic event, which is seriously compromised by the maturation of post-ischemic cerebral damage.

ACKNOWLEDGMENTS

This research was supported by Italian MURST.

REFERENCES

1. W. Kuschinsky. Physiology of cerebral blood flow and metabolism. *Arzneim. Forsch.* **41**:284–287 (1991).
2. A. Baethmann, L. Schürer, A. Unterberg, W. Wahl, F. Staub, and O. Kempski. Mediatorsubstanzen des hirnodems bei der zerebralen ischemie. *Arzneim. Forsch.* **41**:310–315 (1991).
3. B. K. Siesjö and M. L. Smith. The biochemical basis of ischemic brain lesion. *Arzneim. Forsch.* **41**:288–292 (1991).
4. J. Araki, H. Kato, and K. Kogure. Selective neuronal vulnerability following transient cerebral ischemia in the gerbil; distribution and time course. *Acta Neurol. Scand.* **80**:548–553 (1989).
5. S. Nakano, K. Kogure, and H. Fujikura. Ischemia-induced slowly progressive neuronal damage in the rat brain. *Neuroscience* **38**:115–124 (1990).
6. T. Kirino. Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res.* **239**:57–69 (1982).
7. K. Kogure, J. Tanaka, and T. Araki. The mechanism of ischemia-induced brain cell injury. The membrane theory. *Neurochem. Pathol.* **9**:145–170 (1988).
8. K. Yamada, E. Kohmura, A. Kinoshita, J. Taguchi, T. Sakaguchi, K. Tsuruzono, and T. Hayakawa. Ischemic neuronal injury modified by basic fibroblast growth factor. In U. Ito, T. Kirino, T. Kuroiwa, and I. Klatzo (eds.), *Maturation Phenomenon in Cerebral Ischemia*, Springer-Verlag, Berlin, 1992, pp. 139–149.
9. A. Laham, N. Claperon, J. J. Durussel, E. Fattal, J. Delattre, F. Puisieux, P. Couvreur, and P. Rossignol. Liposomally-entrapped ATP: improved efficiency against experimental brain ischemia in the rat. *Life Sci.* **40**:2011–2016 (1987).
10. Z. A. Tökes, A. Kulcsar St. Peteri, and J. A. Todd. Availability of liposome content to the nervous system. Liposome and the blood brain barrier. *Brain Res.* **188**:282–286 (1980).
11. J. Kreuter, R. N. Alyautdin, D. A. Kharkevich, and A. A. Ivanov. Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles). *Brain Res.* **674**:171–174 (1995).
12. M. Fresta, G. Puglisi, C. Di Giacomo, and A. Russo. Liposomes as in vivo carrier for CDP-choline: effects on rat cerebral post-ischemic reperfusion. *J. Pharm. Pharmacol.* **46**:974–981 (1994).
13. M. Fresta, E. Wehrli, and G. Puglisi. Enhanced therapeutic effect of citidine-5'-diphosphate choline when associated with G_{M1} containing small liposomes as demonstrated in a rat ischemia model. *Pharm. Res.* **12**:1769–1774 (1995).
14. M. Fresta and G. Puglisi. Survival rate improvement in a rat ischemia model by long circulating liposomes containing cytidine-5'-diphosphate choline. *Life Sci.* **61**:1227–1235 (1997).
15. J. Aronowski, R. Strong, and J. C. Grotta. Citicoline for treatment of experimental focal ischemia: histologic and behavioral outcome. *Neurol. Res.* **18**:570–574 (1996).
16. G. Puglisi, M. Fresta, G. Mazzone, P. M. Furneri, and G. Tempera. Formulation parameters of fluoroquinolones-loaded liposomes and in vitro antimicrobial activity. *Int. J. Pharm.* **118**:65–76 (1995).
17. G. R. Bartlett. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234**:466–468 (1959).
18. G. Rouser, S. Fleisher, and A. Yamamoto. Two dimensional thin layer chromathographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5:494–496 (1970).
19. S. Benita, P. A. Poly, F. Puisieux, and J. Delattre. Radiopaque liposomes: effect of formulation conditions on encapsulation efficiency. *J. Pharm. Sci.* **73**:1751–1754 (1984).
20. B. Chu. *Laser Light Scattering*, Academic Press, New York, 1974.
21. M. Fresta, G. Cavallaro, G. Giammona, E. Wehrli, and G. Puglisi. Preparation and characterization of polyethyl-2-cyanoacrylate nanocapsules containing antiepiletic drugs. *Biomaterials* **17**:751–758 (1996).
22. B. B. Mrsulja, Y. Ueki, and W. D. Lust. Regional metabolic profiles in early stages of ischemia in the gerbil. *Metab. Brain Dis.* **1**:205–220 (1986).
23. Principles of Laboratory Animal Care. NIH publication #85-23, revised 1985.
24. F. Noll. *Methods of Enzymatic analysis*, Verlag Chemie, Weinheim, Germany, 1984.
25. R. O. Recknagel and E. A. Glende. Spectrophotometric determination of lipid conjugated dienes. In L. Packer (ed.), *Methods in Enzymology*, Vol. 105, Academic Press, New York, 1984, pp. 331–337.
26. U. Ito, T. Yamaguchi, H. Tomita, O. Tone, T. Shishido, H. Hayashi, and M. Yoshida. Maturation of ischemic injuries observed in mongolian gerbils: introductory remarks. In U. Ito, T. Kirino, T. Kuroiwa, and I. Klatzo (eds.), *Maturation Phenomenon in Cerebral Ischemia*, Springer-Verlag, Berlin, 1992, pp. 1–13.
27. K. Yamamoto. Basic activity of the healthy adult EEG by the computerized wave-form recognition method. In N. Yamaguchi and K. Fujisawa (eds.), *Recent Advances in EEG and EMG Data Processing*, Elsevier, Amsterdam, 1981, pp. 363–368.
28. U. Ito, K. Ohno, M. Matsuura, M. Seida, S. Tomida, S. Yamazaki, and Y. Inaba. A study on EEG and rCBF during ischemia and after restoration of blood flow. In R. F. Spetzler, L. P. Carter, V. R. Selman, and N. A. Martin (eds.), *Cerebral Revascularization for Stroke*, Thieme, Stuttgart, 1985, pp. 173–180.
29. M. Fresta and G. Puglisi. Biological effect of CDP-choline-loaded long circulating liposomes on rat cerebral post-ischemic reperfusion. *Int. J. Pham.* **134**:89–97 (1996).
30. L. Packer. Free radical scavengers and antioxidants in prophylaxy and treatment of brain disease. In L. Packer, L. Prilipko, and Y. Christen (eds.), *Free Radical in the Brain*, Springer-Verlag, Berlin, 1992, pp. 1–20.
31. M. Kakihana, N. Fukuda, M. Suno, and A. Nagaoka. Effects of CDP-choline on neurologic deficits and cerebral glucose metabolism in a rat model of cerebral ischemia. *Stroke* **19**:217–222 (1988).
32. G. Hamdorf, J. Cervos-Navarro, and R. Muller. Increase of survival time in experimental hypoxia by cytidine diphosphate choline. *Arzneim. Forsch.* **42**:421–424 (1992).
33. H. Karibe, G. J. Zarow, S. H. Graham, and P. R. Weinstein. Mild intraischemic hypothermia reduces postischemic hyperperfusion, delayed postischemic hypoperfusion, blood-brain barrier disruption, brain edema, and neuronal damage volume after temporary focal cerebral ischemia in rats. *J. Cereb. Blood. Flow Metab.* **14**:620–627 (1994).
34. M. Shinnou, M. Ueno, H. Sakamoto, and M. Ide. Blood-brain barrier damage in reperfusion following ischemia in the hippocampus of the Mongolian gerbil brain. *Acta Neurol. Scand.* **98**:406–411 (1998).
35. K. P. Wheeler and R. Whittam. ATPase activity in the sodium pump needs phosphatidylserine. *Nature* **225**:449–450 (1970).
36. A. Vanella, V. Sorrenti, C. Castorina, A. Campisi, C. Di Giacomo, A. Russo, and J. R. Perez-Polo. Lipid peroxidation in rat cerebral cortex during post-ischemic reperfusion: effect of exogenous antioxidants and Ca^{++}-antagonist drugs. *Int. J. Dev. Neurosci.* **10**:75–80 (1992).
37. J. J. Secades and G. Frontera. CDP-choline: pharmacological and clinical review. *Methods Find. Exp. Clin. Pharmacol.* **17**:2–54 (1995).
38. J. K. Desphande, B. K. Siesjö, and T. Wieloch. Calcium accumulation and neuronal damage in the rat hippocampus following cerebral ischemia. *J. Cereb. Blood Flow Met.* **7**:89–95 (1987).
39. A. B. McDermott and N. Dale. Receptor, ion channels and synaptic potentials underlying the integrative actions of excitatory amino acids. *Trends Neurosci.* **10**:280–284 (1987).
40. M. Hayashi and T. F. Slater. Inhibitory effects of Ebselen on lipid peroxidation in rat liver microsomes. *Free Radic. Res. Commun.* **2**:179–185 (1986).
41. H. Arai, J. V. Passoneau, and W. D. Lust. Energy metabolism in delayed neuronal death of CA 1 neurons of the hippocampus following transient ischemia in the gerbil. *Metab. Brain Dis.* **1**:263–278 (1986).