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Polydeoxyribonucleotide (defibrotide) protects against post-ischemic behavioral, electroencephalographic and neuronal damage in the gerbil

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Abstract

The effectiveness of defibrotide, a single-stranded polydeoxyribonucleotide compound, in preventing damage caused by cerebral ischemia was studied. Global ischemia was induced in anesthetized gerbils by bilateral carotid artery occlusion for 10 min. Defibrotide (100 mg/kg) or saline was injected, i.v., immediately after reperfusion. The following parameters were evaluated simultaneously: (1) electroencephalographic (EEG) spectral power, recorded before, during and after the ischemic period; (2) body temperature, monitored with a rectal thermistor probe after reperfusion for 120 min; (3) spontaneous motility, evaluated through a photocell system and quantified in terms of total distance travelled in 30 min, 1 h after recirculation and at periods over 15 days; (4) mnemonic functions assessed by passive avoidance test from 3 to 15 days after ischemia; (5) histological examination, 7 days after reperfusion, counting CA1 hippocampal neuronal cells. The ischemia-induced complete flattening of spectral power was significantly reversed ($P < 0.01$) by post-ischemic treatment with defibrotide between 30 and 90 min after ischemia. A complete recovery of total EEG spectral power was seen in the defibrotide group at 6 h and the saline ischemic group at 1 day. Seven days after bilateral carotid occlusion, there was a significant decrease in spectral power ($-70\% \pm 6$) together with a loss of the number of CA1 cells in the saline ischemic group (-64%). Treatment with defibrotide significantly protected against the decrease in spectral power ($-30\% \pm 7$) and cell loss (-9%). Finally, the number of animals found to be protected against the ischemia-induced flattening was significantly larger for defibrotide-treated gerbils than for saline-treated animals throughout the experiment except for the third day. Body temperature was significantly decreased only at 30 min after reperfusion in both ischemic and sham-operated groups. Defibrotide reduced ischemia-induced hypermotility but only 6 h after the insult. The ischemia-induced impairment of memory was partially reversed within 3 days in the defibrotide-treated animals and fully reversed within 7 days in the defibrotide group and 15 days in the saline group. Our results demonstrate that defibrotide, even when administered after the post-ischemic period, possesses anti-ischemic properties. The mechanism by which defibrotide protects the ischemic reperfused brain is still largely unknown. However, a neuroprotection via adenosine A_1 and A_2 subtype receptor interaction can be put forward.

Keywords: Defibrotide; Cerebral ischemia; EEG (electroencephalographic) spectral power; Memory; Neuronal damage

1. Introduction

Several recent lines of evidence suggest that adenosine may be an endogenous protective agent in cerebral ischemia (Rudolphi et al., 1992a,b). Adenosine has a number of activities that can serve to minimize neuronal degeneration during cerebral insult, such as ischemia, including vasodilatation, reduction of excitatory transmitter release and membrane calcium permeability, inhibition of platelet and neutrophil aggregation (Phillis, 1989; Ongini and Fredholm, 1996). Several studies have indicated that manipulation of central adenosine tone can alter the extent of cerebral ischemic damage, indicating a potential new therapeutic approach to treatment of stroke. In fact, adenosine analogues, such as cyclohexyladenosine, 2-chloroadenosine and *R*-phenylisopropyladenosine (*R*-PIA), decrease brain damage in different types of cerebral ischemia produced in several species even when the agonist is given

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5–15 min after the insult, while antagonists, such as theophylline and caffeine, increase the brain damage (Rudolphi et al., 1992a,b).

Defibrotide is a single-stranded polydeoxyribonucleotide sodium salt, obtained by controlled depolymerization of deoxyribonucleic acid (DNA) of mammalian organs (European Patent, 1991). The active fraction corresponding to about 60 nucleotides with a defined ratio of purine and pyrimidine bases has a molecular weight of approximately 20 kDa. However, the precise chemical structure of the compound is not yet known. It has been shown to selectively bind to adenosine A_1 and A_2 subtype receptors in rat brain synaptosomal preparations (Barone et al., 1992; Palmer and Goa, 1993). Defibrotide has been developed for treatment of a number of peripheral occlusive disorders. It has profibrinolytic, antithrombotic, antiatherosclerotic action and selectively increases circulating prostaglandin E_2 levels.

On this basis, we designed the present series of experiments to investigate during the post-ischemic period (from 30 min to 15/21 days) the effects of defibrotide administered i.v. to gerbils immediately after the recirculation following a transient occlusion (10 min) of common carotid arteries. The gerbil is a useful animal for studying cerebrovascular disease (Hunter et al., 1995) since the circle of Willis connecting the carotids and vertebrobasilar circulation is incomplete (Levine and Sohn, 1969). To obtain a quantitative evaluation of ischemic damage, we simultaneously evaluated the spectral analysis of electroencephalograms of the parieto-occipital cortex, as previously suggested by Araki et al. (1986), with body temperature, locomotor activity and passive avoidance.

Seven days after ischemia, which is the optimal time for demonstrating ischemic delayed neuronal death, we also determined the number of pyramidal neuron changes in the CA1 zone of the hippocampus, which are the most vulnerable to mild ischemia, both in humans (Brierly and Graham, 1984) and experimental animals (Hunter et al., 1995).

2. Materials and methods

2.1. Animals

196 male Mongolian gerbils (*Meriones unguiculatus*), weighing 60–70 g, supplied by Charles River (Calco, Como, Italy) were used. They were housed singly in an air-conditioned room (22 ± 2°C) with a 12-h light/12-h dark illumination cycle and free access to food and water.

The gerbils were allowed to acclimatize themselves to the environment for a period of 1 week prior to surgical implantation of cortical electrodes. Before, during and after ischemia, food and water consumption and body weight were recorded for 21 days.

All gerbils were submitted to EEG electrodes implantation. To avoid further stress each group of animals was submitted only to one test (EEG spectral analysis, locomotor activity and passive avoidance).

Animal care was in accordance with the State regulations governing the care and treatment of laboratory animals.

2.2. Surgical procedure

2.2.1. Chronic EEG electrode implantation

All the gerbils ($n = 196$) were anesthetized with an i.p. injection of chloral hydrate 5% (Sigma) (8 ml/kg), dissolved in saline, and four silver-silver chloride ball electrodes were fixed epidurally, as described in detail elsewhere (Sala et al., 1995), with acrylic dental cement (Palaferm, Kluzer, Wehrheim, Germany) on the right and left of the parieto-occipital cortex, according to the coordinates of a brain atlas (Loskota et al., 1974). The four electrodes, as well as a fifth inserted into the nasal bone and used as ground, were connected to a micro-connector attached to the head of the animals with the same acrylic dental cement. After the chronic implantation of the electrodes, 1 week was allowed for recovery from surgery before the experiments were started.

2.2.2. EEG recording

The gerbils were allowed to acclimatize themselves to a sound-attenuated Faraday chamber for a period of 3 days. Briefly, the micro-connector on the head of the animal was connected to a rotating connector (Air Precision, France) attached to the cage so as to allow the recording of electrophysiological parameters without hampering movement. The rotating connector was connected to a B8P polygraph (Mortara-Rangoni, Casalecchio di Reno, Italy) and an IBM PS72 computer by means of an A/D converter (Cambridge Electronic Design). The signals were filtered with a band-pass filter set at cut-off frequencies of 0.2 and 50 Hz. The filtered signals were not only recorded on paper (low speed: 1.5 mm/s; high speed: 30 mm/s), but also stored in a computer to be digitized and processed for fast Fourier transform spectral analysis (sampling frequencies of 100 Hz, ten 5-s epochs). The power spectra between 0 and 25 Hz (0.2–4.0 Hz δ, 4.2–8.0 θ, 8.2–13.0 α, 13.2–25.0 β) were evaluated using a resolution of 0.2 Hz. Quantitative EEG analysis was expressed as the mean spectral power (μV^2). The electroencephalogram was recorded for 1 h before ischemia, at the 6th min during ischemia and for 1 h after reperfusion. Additional 1 h recordings were made 6 h and 1, 3, 7, 15 and 21 days after reperfusion. Each 1-h spectral power was expressed as the mean of six recordings evaluated for 1 min each, every 10 min. All recordings, except those made during ischemia, were then analyzed as described above.

2.2.3. Cerebral ischemia

The day of the experiment basal EEG recordings were made of animals previously placed in the Faraday cham-

ber. They were then lightly anesthetized with an i.p. injection of pentobarbital (Carlo Sessa, Milan, Italy) (200 mg/kg; 10 ml/kg) and placed in the supine position. The body temperature of the animals was maintained at 37°C throughout surgery with a heating lamp connected to an Indicating Controller YSI 73ATA (Yellow Springs Instruments, Yellow Springs, OH, USA).

A first group of animals ($n = 112$) was submitted to cerebral ischemia: a 2 cm mid-ventral incision was made and both common carotid arteries were exposed and isolated, as described by Chandler et al. (1985), with slight modifications. Briefly, care was taken to separate the carotid arteries from the surrounding tissue and the vagus and sympathetic nerves. A loop of unwaxed dental floss (Johnson&Johnson) was placed around each carotid artery. Each end of the dental floss was inserted into a vinyl catheter (1.1 mm i.d., 2.0 mm o.d., 2 cm length) and clamped with Dieffenbach clips.

The induced ischemia was verified only qualitatively on paper by the complete flattening of the electroencephalogram from 30 s to 10 min after the occlusion. After 10 min, the clips and dental floss were removed and the skin sutured and the wound treated with mercurochrome. Another group of animals (sham-operated, $n = 28$) was treated in the same manner except for clamping. Immediately after recirculation was established, seven sham-operated and 14 ischemic gerbils had EEG recordings made again.

In the remaining gerbils, evaluated for locomotor activity, passive avoidance and body temperature, the induced ischemia was verified only qualitatively on paper.

2.3. Body temperature

The experiments were performed in the sound-attenuated Faraday chamber. Seven controls, seven sham-operated and 14 ischemic gerbils were trained to the temperature measurements for 5 days before thermoregulator reactions were tested as described by Szikszay et al. (1983). Briefly, after a 1-h acclimatization period in the test room, body temperature was monitored with a rectal thermistor probe (PRA-22002-A, Ellab, Roedovre, Denmark) inserted 3 cm into the colon. After a 30-s equilibration period, the temperature was recorded to the nearest 0.1°C on a CTD-85-M Thermometer (Ellab, Roedovre, Denmark). During temperature measurements, gerbils were unrestrained and were held gently by hand at the base of the tail.

Temperature was measured three times (every 10 min) before induction of ischemia (basal) and again at 30, 60, 90 and 120 min after reperfusion.

2.4. Locomotor activity

Seven controls, seven sham-operated and 14 ischemic gerbils were used to assess locomotor activity.

A 'Videomex-2' (Columbus Instruments, Columbus, OH, USA) was adopted to quantify the distance travelled (m) by each single gerbil. The video signals from a TV camera looking down on a operating arena ($43 \times 43 \times 21$ cm) in which the gerbil was free to move were digitized and sent to an Apple II computer for picture analysis and pattern recognition. Locomotor activity was measured every 10 min beginning at 60 min after the end of ischemia for 1 h. Additional measurements were also made 6 h, 1, 3, 7 and 15 days after ischemia.

2.5. Passive avoidance

Seven controls, seven sham-operated and 14 ischemic gerbils were submitted to passive avoidance procedures, to assess reference memory, as suggested by Ader et al. (1972). The apparatus consisted of a box divided by a wall into two compartments of the same size ($24 \times 21 \times 27$ cm) in which the floor had a grid of stainless rods. In the middle of the wall a guillotine door could be lifted manually. One compartment was lit with a 20 W electric bulb, the other compartment was dark. On the first day animals were placed individually in the dark compartment of the apparatus for 2 min; then, they were placed in the lit compartment, the guillotine door was opened and the animals were allowed to enter the dark compartment. The latency to crossing was recorded (max. 60 s). On the second day animals were placed three times in the lit compartment and the latency to crossing the dark compartment was recorded. Immediately after the last entered the dark chamber, the guillotine door was closed and the gerbil received a single 3 s unavoidable scrambled foot shock (2 mA). Retention of the avoidance response was tested 24 h after the shock trial: the latency to re-enter the dark compartment was recorded up to a maximum of 60 s.

Passive avoidance was tested in three different groups of animals 3, 7 and 15 days after ischemia.

2.6. Histology

After a 7-day recovery, three gerbils previously submitted to EEG recording for spectral analysis and three gerbils previously submitted to passive avoidance test, from each group of animals (Sal + isc, defibrotide + isc and sham-op), were chosen for histology.

They were anesthetized with an overdose of chloral hydrate 5% and killed by decapitation. Their brains ($n = 6$ for each group) were carefully removed and placed in 10% buffered formalin. After 1 day of fixation, the brains were processed in graded ethanols and xylol and embedded in paraffin.

Five serial coronal sections (4 μm in thickness) were cut on a microtome from the block at the level of the hippocampus and stained with hematoxylin-eosin. Neurons with normal appearance in the pyramidal cell layer of the CA1 were counted blind (from coded slides) in each section for each group of treated gerbils. The mean ± S.E.M of five consecutive sections was calculated for each

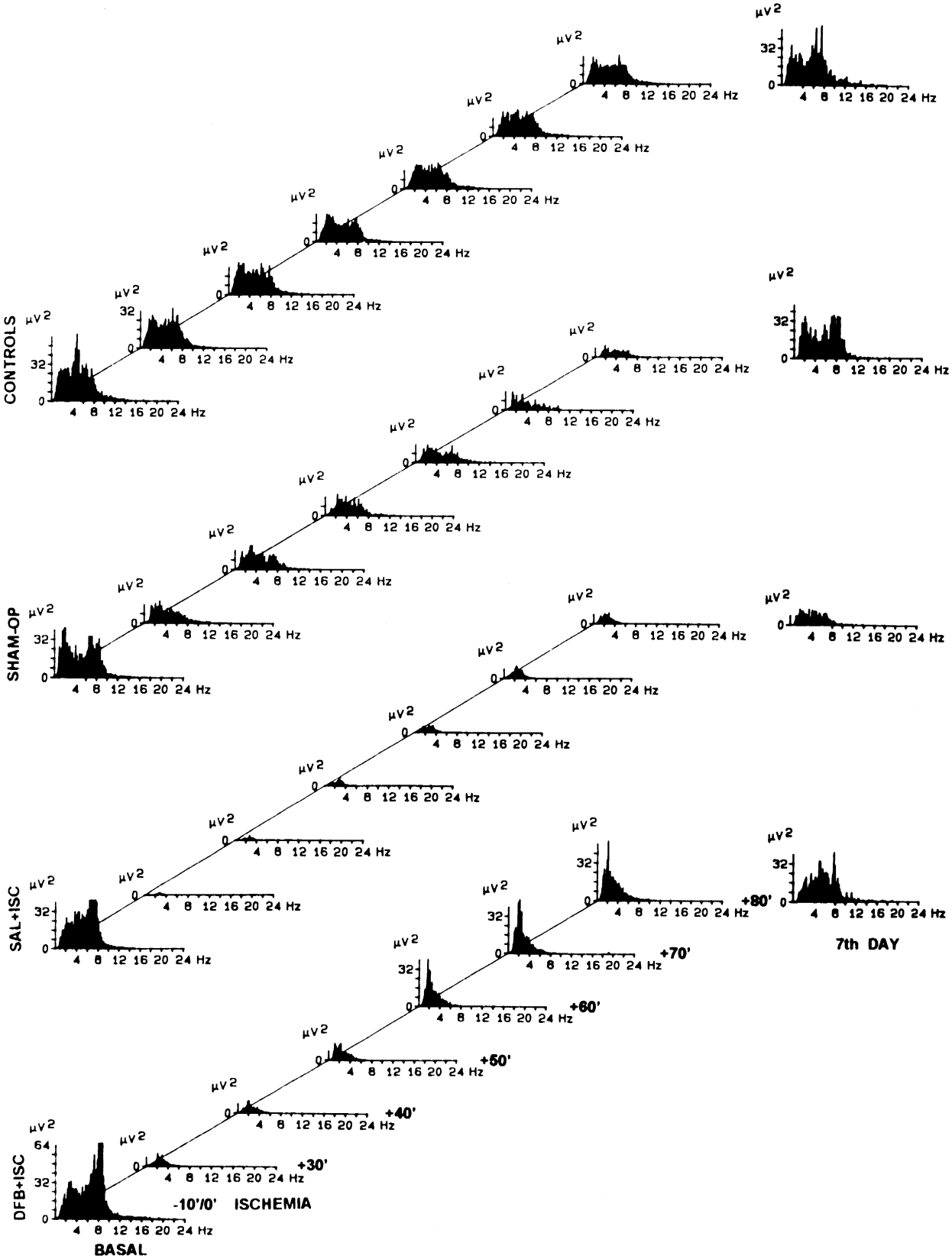

Fig. 1. Spectral power of EEG before and during post-ischemic period, after a 10-min bilateral carotid occlusion in freely moving, awake gerbils treated with defibrotide (DFB) (100 mg/kg, i.v.) and saline (SAL). Each power spectrum was calculated by fast Fourier transform, as the average of seven animals for each group. Treatments were given immediately after the end of ischemia.

hippocampus. Only neurons that were clearly inside the pyramidal somal layer were counted (Nakamura et al., 1993).

2.7. Treatment

The gerbils submitted to ischemia who had flattening of the recording ($n = 84$) were divided into two groups: the first was given immediately after reperfusion an i.v. injection of defibrotide (Crinos, Villaguardia, Como, Italy) (100 mg/kg) in a volume of 0.2 ml/40 g of saline, in the femoral vein previously exposed by an incision of the skin. This dose was chosen on the basis of its ability to be the highest tolerated dose from our previous experiments (unpublished data). The drug solution, dissolved in saline, was freshly prepared prior to use. The second group was given the same volume of saline, by the same route. Sham-operated gerbils were also given saline in the same way.

2.8. Statistical analysis

The data are presented as means ± S.E.M. The means for different groups were compared by one- or two-way analysis of variance (ANOVA) followed by the Tukey test when appropriate. For the temperature data, the results were expressed as the means ± S.E.M. of the °C deviation from the baseline. Total EEG spectral power was expressed as means ± S.E.M. of the percent difference vs. pre-ischemic value. The frequency of animals protected against EEG flattening was calculated for the defibrotide and saline groups for the entire period and analyzed by the χ^2 test.

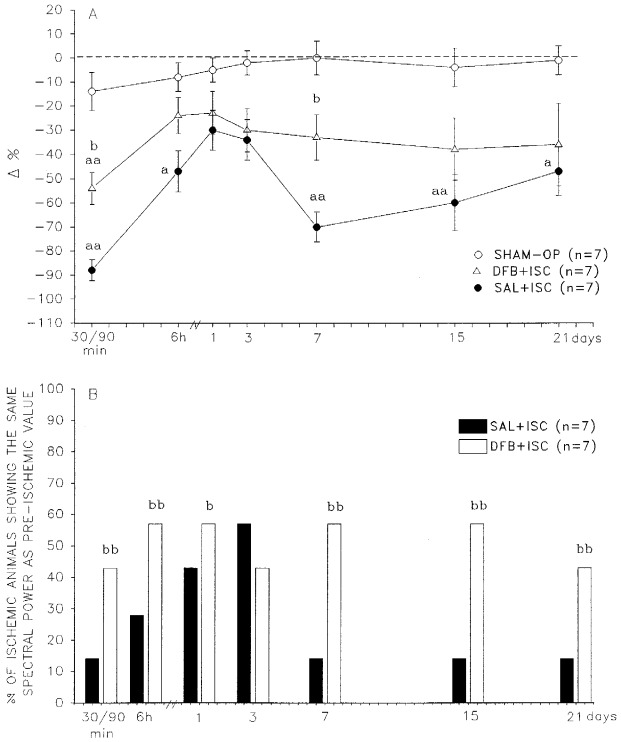

Fig. 2. (A) Cortically derived time-dependent EEG changes, evaluated as difference (Δ%) from pre-ischemic value; mean total spectral power (±S.E.M.) density of freely moving, awake gerbils treated with defibrotide (DFB) (100 mg/kg, i.v.) and saline (SAL), measured during the post-ischemic period (up to 21 days) after a 10-min bilateral carotid occlusion. (-----) = pre-ischemic value. (B) Effects of DFB (100 mg/kg, i.v.) and SAL administration on the number of gerbils protected against EEG flattening after 10 min carotid occlusion. Protected gerbil = each gerbil with the same or greater spectral power as the pre-ischemic value. $^{a}P < 0.05$; $^{aa}P < 0.01$ vs. PRE and SHAM-OP at the same time. $^{b}P < 0.05$; $^{bb}P < 0.001$ vs. SAL + ISC at the same time (ANOVA test).

3. Results

The physiological parameters, such as food and water consumption and body weight, were stable throughout the study in all four groups (data not shown).

A lethality of 7% (2/28 animals) within 21 days was observed in ischemic gerbils treated with defibrotide, while in untreated ischemic gerbils the lethality was 25% (7/28 animals). This difference, even though indicative of protection, was not statistically significant.

3.1. EEG

Between 30 s and 10 min after occlusion 75% of animals presented an EEG flattening. Thus, the remaining animals were discarded from the experiment.

Fig. 1 shows the mean spectral power in ischemic gerbils evaluated during the 30/90 min and 7 days after reperfusion. Spectral power and 1–8 Hz frequency powers of a further non-ischemic group treated with defibrotide showed a similar pattern observed in controls (data not shown). In the control group the mean spectral power remained unchanged throughout the experiment. The slight decrease in EEG activity observed in sham-operated gerbils during the 30/90 min of recording was probably due to the depressive effect of the anesthesia. The 10-min occlusion produced flattened EEG activity in animals treated with saline between 30 and 90 min after basal recording. In the defibrotide-treated group there was also a flattening after 30 min, but to a lesser degree. After 7 days, a decrease was observed only in the saline-ischemic group.

A quantitative analysis of these patterns, systematically analyzed from 30 min to 21 days after recirculation, is given in Fig. 2A, in which significant between-group differences (ANOVA) were observed in terms of percent versus pre-ischemic value of mean total spectral power. Post-hoc comparison (Tukey test) showed that, in comparison with pre-ischemic and sham-operated values, the occlusion had significantly decreased EEG power in saline- and defibrotide-treated animals from 30 to 90 min. However, the mean spectral total power recorded in the defibrotide-treated group was significantly higher than in the saline-treated group for the same time period. Six hours

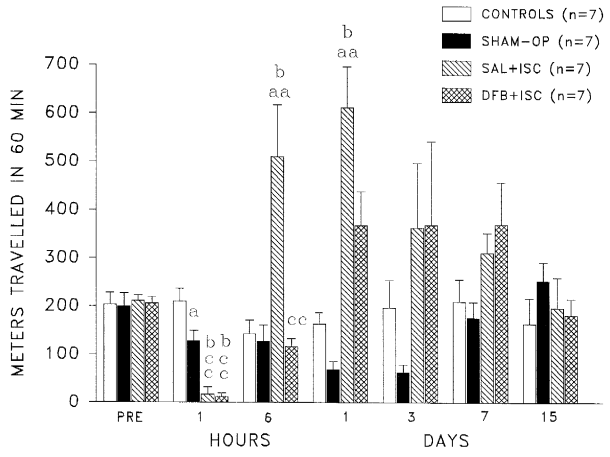

Fig. 3. Effects of defibrotide (DFB) (100 mg/kg, i.v.) and saline (SAL) on spontaneous locomotor activity (mean ± S.E.M.) evaluated in terms of entire distance (meters) travelled in 60 min before and during the post-ischemic period after 10-min bilateral carotid occlusion. [a] $P < 0.05$; [aa] $P < 0.01$ vs. CONTROLS at the same time. [b] $P < 0.01$ vs. SHAM-OP at the same time. [cc] $P < 0.01$ vs. SAL + ISC at the same time (ANOVA test).

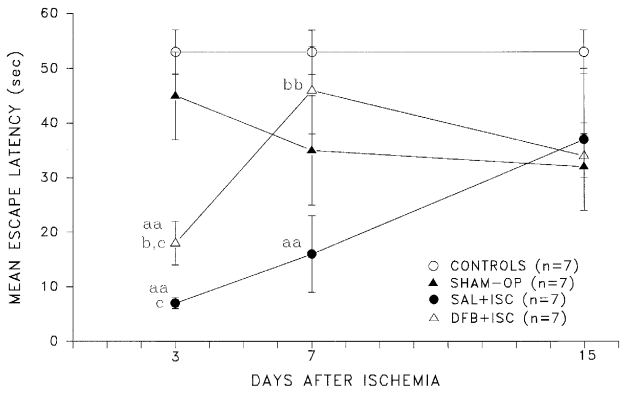

Fig. 4. Mean escape (± S.E.M.) latency evaluated in the passive avoidance task after defibrotide (DFB) (100 mg/kg, i.v.) and saline (SAL) administration to gerbils submitted to 10-min bilateral carotid occlusion. [aa] $P < 0.01$ vs. CONTROLS at the same time. [b] $P < 0.05$; [bb] $P < 0.01$ vs. SAL + ISC at the same time. [c] $P < 0.01$ vs. SHAM-OP at the same time (ANOVA test).

later, the saline-ischemic group was different from the defibrotide group.

There were significant decreases (−70% ± 6, −60% ± 11 and −47% ± 10) in the saline-ischemic group from the pre-ischemic and sham-operated values at 7, 15 and 21 days after recirculation. The decrease observed during the same days in the defibrotide group (30% ± 7, −38% ± 13 and −36% ± 17) was not significantly different from pre-ischemic and sham-operated values.

The number of animals in the defibrotide and saline groups found to be protected (30/90 min/21 days) against the ischemia-induced flattening is shown in Fig. 2B. A significant protection of defibrotide was observed throughout the experiment except for the third day.

3.2. Body temperature

Rectal temperature was significantly decreased only 30 min after occlusion (by about 20%) in all groups except the controls (Tukey test) (data not shown).

3.3. Locomotor activity

One hour after 10 min of bilateral carotid artery occlusion, gerbils showed marked changes between treatments in locomotor activity (Fig. 3). Locomotor activity was significantly less in ischemic animals treated with saline and defibrotide and to a smaller extent also in sham-operated animals, than in controls. This decrease could be due to the depressive effects of anesthesia. Six hours after occlusion, there was significantly greater spontaneus locomotor activity in ischemic animals treated with saline than in the remaining three (Tukey test). Thus, there was a protective effect of defibrotide against hypermotility induced by ischemia. Twenty-four hours later, there was still significant difference between groups. Ischemic animals treated with saline were significantly hyperactive in comparison to controls and sham-operated animals (Tukey test). Treatment with defibrotide decreased the hypermotility to a value not significantly different from that of untreated ischemic gerbils. Between 3 and 15 days, no significant differences were observed between the groups, and a progressive recovery was observed.

3.4. Passive avoidance

The response latency of passive avoidance after 10 min of bilateral carotid occlusion is shown in Fig. 4. ANOVA revealed significant between-groups and days differences when the retention test was carried out 3, 7 and 15 days after ischemia. The Tukey test showed that the latency observed at 3 days in ischemic animals treated with saline and defibrotide differed from that of the control and sham-operated groups. However, the mean escape latency of defibrotide-treated gerbils was significantly higher than in saline-treated animals. Seven days after occlusion, the latency in ischemic gerbils treated with saline was slightly

Table 1
Neuronal cell count, 7 days after reperfusion, in the CA1 region of the hippocampus of sham-operated or ischemic gerbils treated with saline (SAL) or defibrotide (DFB) (100 mg/kg, i.v.) immediately after a 10 min bilateral carotid occlusion

Treatment	Cell count (mean ± S.E.M.)
SAL + isc ($n = 6$)	284 ± 9
DFB + isc ($n = 6$)	681 ± 22 [a,b]
Sham-op ($n = 6$)	748 ± 14 [a]

For each animal, mean of five hippocampal sections from the same coronal plane.
[a] $P < 0.01$ vs. SAL + isc.
[b] Not significantly different from Sham-op.

increased but still significantly different from that of the control group; the defibrotide group had had complete recovery. Fifteen days after occlusion there were no significant differences between the groups.

3.5. Histology

Histological examination of the hippocampus, made 7 days after ischemia, revealed some significant modifications. As shown in Table 1, there was a significant loss of neurons in the CA1 region of ischemic gerbils treated with saline (Tukey test). Counts of these cells revealed a mean loss of 60% in comparison with the sham-operated group. A significant protective effect was observed in the ischemic group treated with defibrotide, as compared with the ischemic saline group.

4. Discussion

Our results confirm that quantitative analysis of cortical or hippocampal EEGs is one of the most sensitive ways to evaluate functional disturbance of the brain during and after ischemia in the gerbil (Suzuki et al., 1983; Araki et al., 1986). In fact, it is well known that severe interference with the cerebral blood flow has a pronounced and prompt effect on neuronal function.

Numerous electrophysiological studies have indicated that in conditions of severe ischemia there is a rapid suppression of the electroencephalogram to flat within 15–30 s. Using this technique, several anti-ischemic drugs ([+]-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate), vindeburnol and phencyclidine, employed in doses from 2 to 10 mg/kg) have been found to significantly decrease the ischemia-induced flattening of the EEG in gerbils after carotid occlusion (Barzaghi et al., 1986), in rats after anoxia and carotid occlusion (Barzaghi et al., 1985; Peruche et al., 1995) and in cats after middle cerebral artery (MCA) occlusion (Stevens and Yaksh, 1990; Deszi et al., 1992, 1994). However, in many parts of these studies the potential therapeutic efficacy of these drugs was not completely demonstrated, since they were given before (or both before and during) the ischemic insult. For these reasons, we decided to study the potential anti-ischemic effect of defibrotide administered after reperfusion on the post-ischemic recovery of brain functions.

Defibrotide played an important role as protective agent in cerebral ischemia, since the EEG total spectral power in the treated ischemic group was significantly higher than that in the untreated ischemic gerbils.

In the saline group, ischemia induced complete flattening of the electroencephalogram within 20 s, which persisted until 80 min and progressively disappeared after 6 h. From 1 day to 3 days, the spectral power progressively returned to the baseline value. This flattening is in good agreement with previous reports (Araki et al., 1986; Suzuki et al., 1983; Barzaghi et al., 1985). In addition, Peruche et al. (1995) reported that in the rat 1 h after ischemia the spectral power was characterized by a dominant peak in the delta band (0.5–3.5 Hz) followed by a slight increase in the portion of frequencies higher than 5 Hz. Only 1 day later, the spectral power of the ischemic group was not distinguishable from the pre-ischemic pattern. In the ischemic group treated with defibrotide, there was an early EEG recovery, observed 30/90 min after treatment. This recovery was characterized by the appearance of slow frequency powers (1–3 Hz, δ band). Within 1 h, the powers shifted into higher frequencies (4–8 Hz). Six hours later, the EEG pattern of defibrotide-treated gerbils had completely returned to the baseline value.

There was a second decrease in the control ischemic-group 7 and 15 days after ischemia, suggesting, as reported by Peruche et al. (1995), that the late EEG power decrease may be related to pronounced damage of neurons, which are replaced by astrocytes in the hippocampal subfield. In this context, defibrotide protection observed within 1 h was able after 7 days to protect against the ischemia-induced EEG spectral power decrease, suggesting that EEG power is a good measure of cell death. However, on the 21st day, there was a slight but not significant recovery in the saline ischemic group, suggesting a possible compensatory mechanism of surviving neurons.

These findings are in line with previous reports that a range of doses from 16 to 200 mg/kg, infused i.v. or given p.o., reduced infarct size after myocardial or kidney ischemia, in rabbits (Thiemermann et al., 1989; Bianchi et al., 1990), in cats (Niada et al., 1985, 1986a,b) and in rats (Marni et al., 1990). A small amount of information comes from Zirh et al. (1992) reporting that defibrotide (200 mg/kg, i.v.) significantly preserved neuronal structures of the rat when administered 30 min before the occlusion of middle cerebral artery.

It can be excluded that the protective anti-ischemic effect of defibrotide is related to hypothermia, as shown for other anti-ischemic drugs such as [+]-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate (Hoffman and Boast, 1995). In fact all groups, except for the controls, had a slight and similar decrease of body temperature only 30 min after surgery, which can be attributed to the hypothermic effect of the anesthetic.

The protective effects of defibrotide are further sustained by the data for memory. In fact, defibrotide administered immediately after blood recirculation prevented ischemia-induced impairment of memory retention, as shown by the longer mean escape latency in comparison to that of untreated ischemic gerbils. It is well known that a brief period of cerebral ischemia induces neurological disfunction in laboratory animals which is reflected in the deterioration of memory function (LaPoncin-Lafitte et al., 1981; Kiyota et al., 1985). This memory disturbance has been clinically demonstrated. In fact, some survivors of cardiac arrest have revealed an amnesic syndrome charac-

terized by impaired learning and memory of events after their injury and a gradient of memory loss for premorbid events (Volpe and Hirst, 1983). The hippocampus is an area of predilection for ischemic damage and, within the hippocampus, the CA1 pyramidal cells are more vulnerable to reduced cerebral blood flow than cells in the CA2, CA3 and dentate gyrus fields (Schmid-Kaster and Freund, 1991). This selective vulnerability has been shown in several mammalian species including man, so that animal models may have analogous neuronal mechanisms.

Defibrotide had slight but significant antiamnesic effects 3 days after occlusion and recovery was complete only after 7 days. The compound was able to completely antagonize the ischemia-induced hypermotility only within 6 h after occlusion but not 1 day after ischemia. This lack of effect could have affected normal learning during training in the passive avoidance test. However, 7 days after ischemia, the motor activity had returned to normal.

It can be argued that the only partial recovery of performance in the passive avoidance task observed 3 days after occlusion may be related not only to an early phase of CA1 neuronal death, as previously suggested (Nunn and Hodges, 1994), but also to hyperlocomotion. Wang and Corbett (1990) have reported that this abnormal activity can reflect a functional deficit such as impairment of spatial mapping ability.

Memory deficit was completely recovered 7 and 15 days after occlusion in the defibrotide-treated group, since most of the pyramidal cells of the CA1 subfield had survived. In the saline-ischemic group the cell loss observed at 7 days was strictly related to the memory impairment, while 15 days later there was recovery. It remains to be clarified why there was this recovery at 15 days since it is well known that a mild ischemia such as occlusion for 5–10 min causes a loss of CA1 neurons but the extent of the destruction increases day by day (up to 14 days), suggesting further, delayed neuronal death (Araki et al., 1986). It can be argued, based also on our electrophysiological data observed 21 days after occlusion, that a compensatory mechanism of survival neurons can counterbalance the deficit of dead neurons. Alternatively, memory deficit could be unrelated to the delayed neuronal death in the CA1 subfield as suggested by Katoh et al. (1992). In this line, Nunn and Hodges (1994) reported that there is a highly significant relationship between the extent of cell loss in the CA1 region and duration of ischemia, while the correlation between CA1 cell loss and mnemonic measures is essentially zero. Memory disturbances are reported after 2-vessel cerebral occlusion in rats, even though no delayed neuronal death is observed (Jasper et al., 1990).

Many explanations have been proposed for the mechanism of neuronal death after ischemia. Among these the 'excitotoxic' hypothesis is based on the massive release of excitatory amino acid neurotransmitters, such as glutamate and aspartate (Simon et al., 1984). These trigger further membrane depolarization and additional accumulation of free cytosolic Ca^{2+} by cellular influx. The accumulation of Ca^{2+} seems to play a key role in propagation of the process toward irreversible neuronal damage (Rudolphi et al., 1992a). Hagberg et al. (1986) reported that the excessive concomitant glutamatergic excitation seems to contribute to the increase of extracellular adenosine following ischemia. In fact, adenosine release during ischemia was significantly decreased in rats pretreated with a competitive glutamate receptor antagonist. This effect of adenosine is mediated by presynaptic adenosine A_1 subtype receptor linked via G proteins to both Ca^{2+} and K^+ ion channels (Fredholm and Dunwiddie, 1988). In the postsynaptic neuron, adenosine helps to maintain intracellular Ca^{2+} homeostasis, which is of particular importance for neuroprotection.

Data from radioligand binding assay in rat brain synaptosomal preparations have indicated that stranded DNA chains, such as defibrotide, bind selectively and specifically to the adenosine A_1 and A_2 subtype receptors (Palmer and Goa, 1993; Bianchi et al., 1991) with an IC_{50} for adenosine A_1 and A_2 subtype receptors of 10 and 28 μM, respectively (Barone et al., 1992). These concentrations are not different (one order of magnitude) from the plasma level concentration found in humans of 25–30 mg/l, suggesting a possible interaction with adenosine A_1 and A_2 subtype receptors. Thus, beside its important neuromodulatory effects via adenosine A_1 subtype receptors, defibrotide acting on adenosine A_2 subtype receptors in the cerebrovasculature (increase in E_2 and I_2 prostaglandin levels with consequent regional vasodilatation, inhibition of platelet aggregation, reduction of leukotriene B4 levels) may increase oxygen and substrate supply using a range of doses between 30 and 200 mg/kg in different species (Palmer and Goa, 1993). What is relevant, defibrotide has been recently shown to directly inhibit lipid peroxidation activated during ischemic events in the rat (unpublished data). In addition, defibrotide has been shown to inhibit the activation of neutrophils through adenosine A_2 subtype receptors, so indirectly reducing the release of free oxygen radicals and other cytotoxic substances (Di Perri et al., 1991).

Until now there is no evidence that defibrotide gains access to the brain or cerebral resistance vessels; however, it is likely that an active carried-mediated transport enables defibrotide to traverse the blood-brain barrier as already reported for nucleosides (Pardridge, 1983).

Finally, it can be hypothesized that defibrotide, as a polydeoxyribonucleic acid, could interact with polyamines whose synthesis can be greatly stimulated in response to ischemia (Paschen et al., 1988).

In conclusion, these findings suggest that defibrotide administered i.v. after ischemia improves the post-ischemic EEG recovery, prevents memory impairment by protecting against neuronal loss in the hippocampus. Whether or not defibrotide, which has been shown to possess a high tolerability in a large number of clinical trials after chronic

i.v. administration (Palmer and Goa, 1993), may be considered a potential therapeutic agent, remains to be established. Preliminary results indicate a complete protection even when defibrotide is administered 1 h after ischemia. Further investigations into the dose-response curve relationship, the replicability of antischemic properties using a more invasive test (such as medial cerebral artery occlusion) and the possible influence of adenosine receptor antagonists in defibrotide-induced anti-ischemic effects are scheduled for the future.

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