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TREATMENT OF BRAIN TRAUMA WITH LIPOSOMAL SUPEROXIDE DISMUTASE

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Brain trauma was induced in rats by impact of a steel bar on the head with a force such that damage (as measured by neurological scoring) was reversible in fourteen days. Systemic treatment (intraperitoneal injections) with free bovine copper superoxide dismutase or a liposomal form of the enzyme considerably shortened recovery time to less than half. Tests included cranial nerves – cornean and aural reflexes, and sensorial motricity functions – gripping reflexes, displacement reactions, recovery and flexion reflexes, equilibrium tests and spontaneous mobility. Normalisation of EEG recordings was also greatly accelerated in the case of treated animals. No changes of brain glutathione peroxidase, glutathione transferase or Mn superoxide dismutase in traumatized animals were observed. However a slight decrease in Cu-SOD occurs. Cerebral lipoperoxidation is increased in the traumatized animals compared with controls. This increase is reduced on treatment of the rats with liposomal SOD (or the free enzyme). Very small amounts of the exogenous SOD pass the brain barrier, the permeability of which is increased in traumatized animals. The enzyme is particularly concentrated in the cortex. Despite apparent total neurological recovery at 15 days for untreated traumatized animals, significant differences in EEG recordings, in percentage cerebral water content and in histological examination of brain tissue of these controls compared with treated animals were observed with a net improvement in the latter case. The results obtained with this model suggest that clinical treatment of coma states and brain traumas with liposomal superoxide dismutase may have certain advantages over orthodox treatments.

KEY WORDS: Liposomes, superoxide dismutase, lipoperoxides, brain trauma, neurological behaviour, brain barrier.

INTRODUCTION

In France (population 53×10^6) an average of 30 deaths per day occur as a result of road accidents. Apart from such irreversible phenomena a large number of accidents result in brain injury and cerebral traumas in general, often leading to coma, with more than 100 000 cranial trauma cases per year. A majority recover as a result of progress in reparative and corrective surgery, improved techniques of re-education and medical treatment. Various approaches to decrease irreversible destruction of brain cells include hypothermia[1] but the most generally used is treatment with barbiturates[2] though the precise mechanism of action is not entirely clear as yet. Although the standard re-animation techniques are often successful, no direct treatment with respect to oxidative damage occurring in cerebral edema is presently available.

Two types of edema occur as a result of diffuse encephalic lesions 1) rupture of the blood brain barrier and vasomotor paralysis with infiltration of liquid in the ex-

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tracellular space (vasogenic) and 2) membrane lesions and astrocyte swelling (cytotoxic). The cerebral edema is a source of intracranial hypertension which increases the effects by compression of the veins resulting in local ischemic conditions. Lesions of the blood brain barrier occur very early in cranial traumatism and are rapidly aggravated by cellular changes (sodium and calcium pumps) and hemodynamic modifications, with collapse of the microcirculation and increase in platelet aggregation. Free radicals increase membrane lipid peroxidation and are probably responsible at least in part for disorganisation of the ionic homeostasis.[3]

Trauma provokes a decrease of cerebral vasoconstriction of blood volume which with hematomas blocking veinous return favorises exudation of plasma fluid[4] also increased under the induced ischemia. Superoxide anions are also involved in the breakdown of endothelium-derived vascular relaxing factor[5] which mediates vasodilator responses induced by substances such as acetylcholine and bradykinin. *In vitro* breakdown is protected by SOD but not by catalase. Of interest also is the inhibition of normal neutrophil activity by trauma serum.[6] Trauma serum significantly decreased O_2^- production (cytochrome c assay) and chemiluminescence compared with incubation in normal serum. Neutrophil-membrane depolarization response to latex particles was also inhibited as measured by flow cytometry.

The role of free radicals in brain trauma has received considerable attention in recent years, particularly with respect to hypoxic or ischemic conditions. It is known[7] that liberation of hemoglobin and iron occurs with extravasation of erythrocytes in head trauma with contusion or cortical laceration as well as in hemorrhagic or ischemic cortical infarction and intracerebral hematomas. This can give rise to free radical production with subsequent peroxidation of neural lipids resulting in a localised edema.[8] Peroxidative changes in brain cortical fatty acids and phospholipids stimulated by ferrous ions have been observed *in vivo*[9] and *in vitro*.[10] In contrast, ischemic conditions (in absence of decompartmentalization of ferrous derivatives) do not appear to result in significant peroxidation of membrane lipids of the brain *in vivo*[11].

Studies of fluid-percussion brain injury in cats equipped with cranial windows have demonstrated the role of superoxide radicals in a variety of the lesions induced.[12] Apart from direct detection of O_2^- production, post treatment with topical SOD and catalase eliminated the sustained arteriolar dilation and reduced responsiveness to the vasoconstrictor effects of arterial hypocapnia. This form of experimental brain injury causes appearance of O_2^- in the extracellular fluid space, and production continues for at least one hour. That the vascular damage is not irreversible is shown by the effects of locally applied SOD. Acute hypertension induces generation of O_2^- in association with accelerated arachidonate metabolism. The radical enters the cerebral extracellular space via the anion channel and causes vasodilatation by relaxation of cerebral vascular smooth muscle. Implication of oxygen radicals in the cerebral vascular abnormalities seen in experimental acute hypertension has been reviewed by Kontos.[13] Brain edema in dogs after trepanation has been treated directly[14] with aqueous solutions of bovine Cu-SOD, apparently with some success, but no details have been described.

We have previously reported the successful application of SOD and liposomal SOD in various inflammation models in the rat, such as carrageenan[15] and adriamycin[16] induced edemas and adjuvant induced polyarthritis[17] and more recently in a simple form of ischemia, the rat tourniquet poditis model.[18] In this study we use a model (direct impact on the rat cranium) of concussive brain injury more directly related to

clinical situations than the fluid-percussion system and we present results of systemic treatment which confirm the reversibility of much of the damage, as well as the role of superoxide radicals.

MATERIALS AND METHODS

Animals and Trauma Technique

Male Wistar AF-gnotoxenic rats maintained under sterile microclimate controlled conditions[17] weighing 300 ± 20 g were used. A cylindrical stainless steel bar 82.5 cm long with a diameter of 1 cm (weight 719 g) is dropped through a guide tube (80 cm long, 1.8 cm diameter) from a distance of exactly 12 cm (determined in preliminary trials) on the centre of the head of the non-anaesthetized animal maintained in a stereotactic support on a rigid surface. All animals so treated were unconscious for 30–60 seconds after impact. For each series: undamaged controls, untreated trauma controls, treatment with free bovine Cu-SOD or the liposomal form, 14 rats were used. Controls I. Non-traumatised rats receiving no treatment. Controls II. Trauma-induced animals which receive an i.p. injection of 1 ml 0.9% NaCl each day for 15 days. Treatment with free bovine Cu-SOD: i.p. injections of 100 μg/kg of Cu-SOD in 1 ml saline were performed each day. Liposome encapsulated SOD was administered at the same dose rate. All solutions were pre-warmed to 37°C before injection and administered at 9.00 a.m. As in earlier studies, i.p. injection is preferred to i.v. since anaesthesia is not necessary, thus avoiding shock or stress. A radiographic control of the skull was performed the same day as impact.

For EEG studies, five cortical electrodes (for electroencephalograms) were implanted in each rat to measure cortical activity by four bipolar derivations. As control this was done three weeks before induction of the cranian trauma, using 14 animals in each series. Electroencephalograms were recorded at days 0, 1, 5, 10 and 15 and at day 15 blood was removed for various clinical estimations. Finally the animals were sacrificed to remove the brain for study by optical microscopic histological techniques and for water content.

Neurological Scoring

Neurological examination for estimation of reduced capacities and efficiency of treatment was based on the following criteria.[19]

1 Corneal reflex. The cornea is gently touched with a hair fixed to a Pasteur pipette to provoke closing of the eyelid. Score: normal = 1, absence = 0 for each eye.

2 Aural response (Pinna reflex). The operator blows through a flexible rubber tube into the ear of the animal which responds with a rapid rotation of the head. Score: normal = 1, absence = 0 for each ear.

3 Gripping reflex. The rat is held by the skin of the back and a steel rod 15 cm long and 2 mm in diameter is placed in contact with the bottom of the front paws. This provokes gripping of the bar and resistance to removal. Score: normal = 1, absence = 0 for each front paw.

4 Position reactions. (i) Visual. The animal, held by the tail is slowly placed near a table on which it places the two front paws. Score: two paws = 2, 1 paw = 1, absence = 0. (ii) Loss of contact. The rat is held at the edge of a table and each paw (front and hind) is successively placed over the edge. Response is immediate retreat to the surface of the table. Score: normal = 1, absence = 0 for each of the four paws.

5 Readjustment reflexes. (i) Placed on the back of a flat surface the animal immediately returns to its four feet. Score: normal = 1, absence = 0. (ii) The rat is maintained horizontal at the level of the lumbo-sacral region. Rotation of the body about the longitudinal axis provokes a rotation of the head to maintain the initial position. Score: reaction of both sides = 2, one side = 1, absence = 0. (iii) Free fall. The animal is released from a height of 40 cm onto a soft surface. Score: falls on four feet = 1, falls on the side = 0.

6 Equilibrium tests. (i) Inclined surface. The animal is placed on a small-meshed grill fixed to a wood plank (50 × 15 cm) inclined at 45° to the horizontal in open space, with the head pointing downwards. Reaction involves turning 180°C to face upwards. Score: turn-around in < 15 sec = 2, between 15 and 30 sec = 1, absence = 0. (ii) Horizontal bar. The two front paws are placed on a rough wooden bar, 30 cm long, diameter 3 cm supported at a height of 50 cm. The animal clings to the bar with both rear paws. Score: for each rear paw placed on the bar in less than 5 sec = 1.

7 Bending reflexes. The toes of each of the rear paws are successively nipped in a Bulldog clamp. This provokes an immediate flexion of the paw. Score: normal = 1, absence = 0, for each of the rear paws.

8 Spontaneous mobility. The rat is removed from its cage and placed freely on a table. Score: normal movement = 2, unsteady displacement = 1, immobility = 0.

A total of 12 neurological tests were thus applied with a maximal score of 24. For the 14 control (non-traumatized) rats the average score was 23.43 ± 0.20. Neurological scores were estimated at 1, 24, 36 hrs, 4, 7 and 14 days after induction of the trauma.

Statistical tests. Scoring of responses in the interior of each group of animals was analysed by Kruskal–Wallis non-parametric tests and by the techniques of Mann and Whitney.[20]

Preparation of Brain Homogenates

The tissue was disintegrated at 4°C in a Potter for 1 min in freshly degassed (passage of argon) buffer containing 0.05 M sodium phosphate, 0.015 M NaCl and 0.145 M KCl pH 7.0 using 10 ml of buffer per g of brain.

Estimation of lipoperoxides (malondialdehyde) was based on the method of Yagi.[21] The honmogenate (0.5 ml) was added to 6 ml of 0.083 N H_2SO_4 plus 1.5 ml of a solution of 10% aqueous phosphotungstic acid, mixed on a Vortex and left 30 min in ice. The mixture was centrifuged at 1000 g for 10 min and the supernatants removed by aspiration. To the precipitate was added a mixture of 3 ml H_2O and 1 ml of a 0.67% solution of thiobarbituric acid in 50% acetic acid. After mixing on a Vortex the suspension was heated for 60 min at 95°C, then centrifuged for 10 min at 14 600 g. Optical absorption of the supernatants (against a water blank) was measured at

532 nm and MDA estimated using a calibration curve using 1–10 nmoles of MDA in three ml H_2O heated for 60 min at 95 C with 1 ml of 0.67% thiobarbituric acid. For estimation of soluble lipoperoxide the homogenate was centrifuged for 20 min at 14 600 g and MDA measured in the supernatants (1 ml) by the above technique.

Protein was measured by the method of Lowry.[22] Cu-SOD and Mn-SOD wre estimated by enzymic activity using 2×10^{-3} M KCN to inhibit the copper enzyme in the total value using the riboflavin-NBT method.[23] Radioactive labelled enzyme was prepared as previously described by incorporation of ^{57}Co in the apoenzyme from bovine Cu-SOD.[24] Liposomes containing either the cold enzyme or radioactive material were also prepared as described earlier.[25] A correction for radioactivity in the blood present in the brain was applied using the value of 0.024 ml of blood per g wet weight[26] and the cpm per ml of blood.

RESULTS

Preliminary experiments of treatment of force induced brain trauma in rats with *E. coli* Mn-SOD, bovine Cu-SOD or liposomal bovine Cu-SOD at 33 μg/kg showed no detectable improvement. The dose rate was therefore increased to 100 μg/kg i.p. in the present studies and at this level highly significant results were obtained. Immediately after impact on the head the animals are unconscious for 30–60 sec, then slowly recover. In the control batch of 14 rats two animals (15%) died on the seventh day. No deaths were observed in the treated batches (out of 28). Radiographic control on day 1 of the traumatized animals showed no severe fractures, but relatively distinct fissures in the frontal and parietal bones. Bone alterations were very homogeneous in the three groups (untreated, SOD and liposome treated).

Neurological Scoring

The similarity of the scores at 1 hour after impact in the three groups (Table I) justifies comparison of each series. The neurological tests were based on various types of scoring and included the following examinations:

Cranial nerves:
- cornean reflex
- aural reflex.

Sensorial motricity functions:
- gripping reflexes, left and right rear paws
- reaction of visual displacement
- reaction of displacement due to loss of support
- recovery reflexes – back, rotation, fall
- equilibrium tests – sloping plane, horizontal bar
- flexion reflexes
- spontaneous mobility.

The results are presented in Table I. After 14 days no differences can be seen among controls, trauma controls or treated animals, and except for two deaths in the trauma controls at day 7, all animals appear to reach total recovery with neurological scores corresponding to those obtained before cranial concussion. The model is thus essenti-

TABLE I
Neurological scoring

Time after impact	Traumitzed controls n = 14		Bovine Cu-SOD 100 μg/kg i.p. daily n = 14		Liposomal bovine Cu-SOD 100 μg/kg i.p. daily n = 14	
	Score	% recovery	Score	% recovery	Score	% recovery
1 hr	13.86 ± 1.09	59.2	14.14 ± 0.82	60.3	14.57 ± 1.07	62.2
24 hr	16.29 ± 0.73	69.5	18.43 ± 0.80	78.7	17.36 ± 0.96	74.1
36 hr	17.57 ± 0.88	75.0	21.43 ± 0.40	91.5	19.71 ± 1.14	84.1
4 days	17.86 ± 0.61	76.2	22.14 ± 0.39	94.5	21.57 ± 0.87	92.1
7 days	20.28 ± 0.25 (n = 12)	86.6 (2 deaths)	23.28 ± 0.20	99.4	22.86 ± 0.45	97.6
14 days	23.00 ± 0.27 (n = 12)	98.2	23.57 ± 0.21	100.6	23.43 ± 0.30	100.0

Non-traumatized controls 23.43 ± 0.20 (n = 14)
Maximum score = 24.0

	Days to reach neurological recovery of 85%	90%	95%	97.5%
Controls	6.6	9.1	12.0	13.5
Bovine Cu-SOD	1.2	1.4	4.3	6.0
Liposomal Cu-SOD	1.7	3.3	5.5	6.7

For comparisons of trauma controls with non-traumatized animals from 1 hr to day 7, $p < 0.001$.
For comparisons of treated animals compared with trauma controls from 24 hr to day 7, $p < 0.001$.

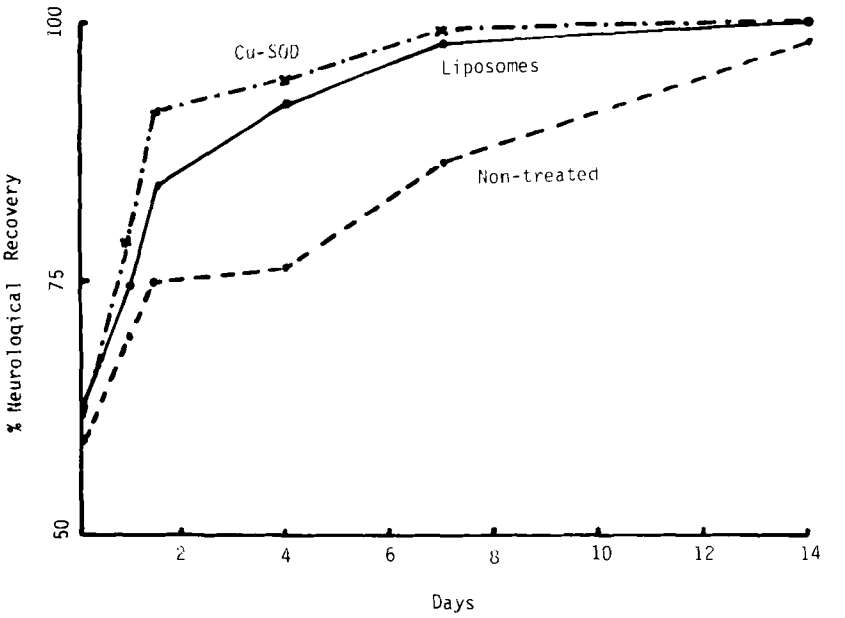

FIGURE 1 Neurological recovery as a function of time for untreated traumatized rats (– –) and for free bovine Cu-SOD (—·—) and liposomal SOD (———) treated animals.

ally an induced reversible brain trauma. It can be readily calculated that impact of a mass of 719 g dropped from a height of 12 cm corresponds to an impact of 1.1 kg metres/sec on a surface of 0.785 cm^2 i.e. 1.4 kg metres/sec per cm^2 ($d = 1/2\,gt,^2$ $t = 0.1565$ sec; $v = gt$, $v = 1.534$ m/sec; $mv = 0.719$ kg $\times$ 1.543 m/sec). With respect to the surface of contact with the skull (0.785 cm^2) this corresponds to impact of 100 g at 40 km/hr for the rats used in this work. On a human scale this is very roughly equivalent to impact of 4 kg at 100 km/hr. Since the elasticity of rat skulls is not available the force involved cannot be calculated.

With respect to rapidity of recovery, extremely important differences are seen. Untreated animals require the full 14 days to approach 100% recovery, whereas in treated animals this is greatly accelerated (Figure 1). Thus 85% neurological improvement is observed in control animals at 6–7 days whereas treated rats reach this same level between the first and second day (Table I). Even for 97.5% recovery, the time for treated animals is half that of non-treated controls. Treatment with bovine Cu-SOD or the liposomal SOD thus has a very significant effect on the recuperation of normal neurological behaviour in traumatized rats.

EEG Recording

The most readily visible changes in EEG recordings after impact brain trauma in the rat with appearance of an edema are profound perturbations of the cortical electrogenesis characterized by a degradation of the θ and β rhythms, increase in slow α waves and in particular, appearance of δ waves, sign of deterioration of cerebral function. EEG recordings were made before impact and at days 1, 5, 10 and 15, over a period of 15 min between 9.00 and 11.00 a.m. For comparisons, each wave was scored 0 to 3 and the ratio $\theta + \beta/\alpha + \delta$ calculated. For normal non-traumatized rats this ratio (6/1) was considered as 100%. In each series $n = 14$.

In non-treated traumatized animals this ratio was decreased by $65 \pm 7\%$ at day 1, essentially due to increase in δ waves and decrease in $\theta + \beta$ rhythms. At days 5, 10 and 15 this decrease was reduced to $51 \pm 4\%$, $34 \pm 6\%$ and $19 \pm 4\%$ respectively. For the treated rats, the number of pathological δ waves was significantly decreased without modification of the physiological θ rhythms, indicating an effect limited to the post-traumatic cerebral edema, source of the perturbations. For rats treated with free SOD the percentage decreases in ratio were reduced to $60 \pm 2\%$, $30 \pm 3\%$, $11 \pm 6\%$ and $4 \pm 2\%$ at days 1, 5, 10 and 15 respectively. Corresponding results for traumatized animals treated with liposomal SOD were $64 \pm 4\%$, $35 \pm 2\%$, $8 \pm 2\%$ and $3 \pm 1\%$. Both treatments thus lead to a much more rapid normalisation of EEG recordings compared with controls and at 15 days a significant difference ($p < 0.001$) still exists (Figure 2). Differences between trauma controls and treated animals were non-significant at day 1, the first day of treatment, but were highly significant at days 5, 10 and 15 ($p < 0.001$ in all cases).

Biochemical Estimations

No significant changes in the brain glutathione peroxidase or glutathione transferase were observed in untreated animals sacrificed four days after trauma compared with non-traumatized controls. A slight but significant decrease in total brain SOD was observed, almost entirely with respect to Cu-SOD, the Mn-SOD being unchanged, as shown in Table II. This decrease was not reversed in treated traumatized animals

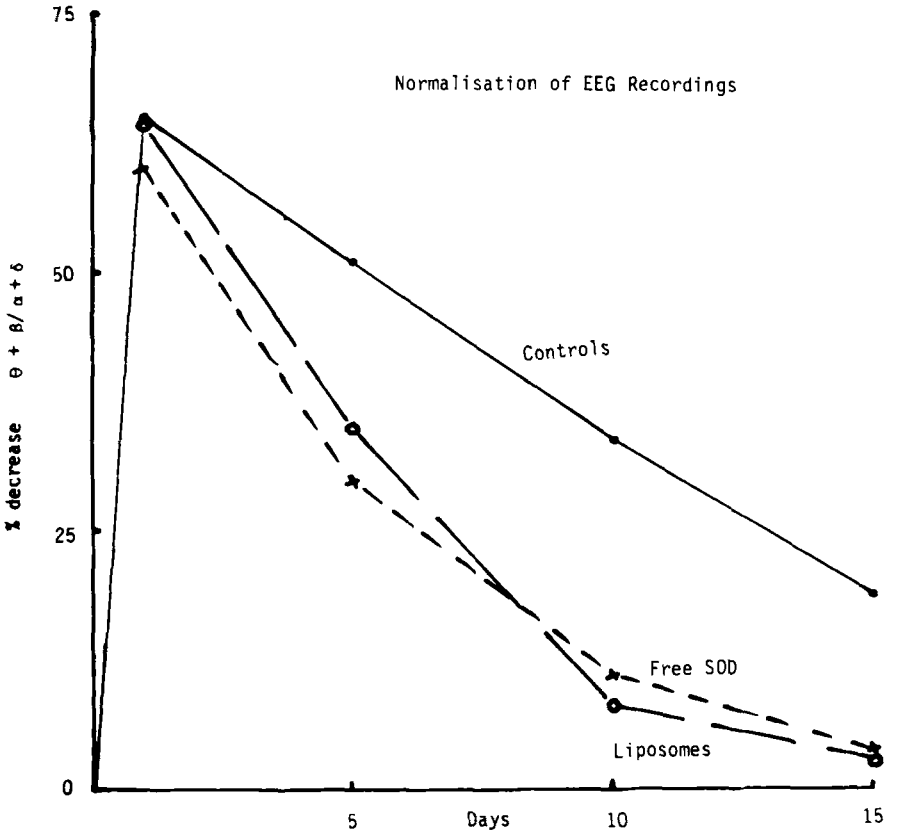

FIGURE 2 Normalisation of EEG recordings as a function of time using the ratio of $\theta + \beta/\alpha + \delta$ as indication.

sacrificed at the fourth day. Examination of serum proteins in the rats at 15 days showed an increase in the traumatized rats (compared with controls) of globulins, particularly $\alpha 1$, β and γ. This increase was significantly decreased in the rats treated with liposomal SOD (Table III). A curious side effect was observed in the treated

TABLE II
Changes in brain SOD four days after trauma

Units/mg protein (±SD)	Total SOD	Mn-SOD	Cu-SOD
Control (n = 5)	7.22 ± 0.42	2.18 ± 0.25	5.04 ± 0.41
Trauma (n = 8)	6.45 ± 0.58	2.10 ± 0.14	4.35 ± 0.54
	(−10.7%)	(−3.7%)	(−13.7%)
p<	0.05	NS	0.05
Units/g tissue			
Control (n = 5)	268.75 ± 17.7	81.07 ± 8.8	187.68 ± 18.3
Trauma (n = 8)	237.36 ± 20.9	77.23 ± 6.7	160.13 ± 18.7
	(−11.7%)	(−4.7%)	(−14.7%)
p<	0.01	NS	0.05

TABLE III
Blood analyses

Globulin g l	Controls	Trauma controls	Free SOD	Liposomes
αl	1.16 ± 0.03	1.65 ± 0.27 (+42.2%)	1.05 ± 0.25 (−9.5%)	1.28 ± 0.13 (+10.3%)
β	17.12 ± 0.41	23.70 ± 2.25 (+38.4%)	22.92 ± 0.89 (+33.9%)	14.50 ± 1.76 (−15.3%)
γ	3.90 ± 0.42	7.57 ± 0.71 (+94.1%)	7.08 ± 0.50 (+81.5%)	5.98 ± 0.69 (+53.3%)
Cholesterol (mmoles/l)	1.64 ± 0.08	1.67 ± 0.04	1.38 ± 0.08 (−17.4%)	1.53 ± 0.07 (−8.4%)
Triglycerides (mmoles/l)	1.91 ± 0.08	1.44 ± 0.11	1.13 ± 0.12 (−21.5%)	1.26 ± 0.15 (−12.5%)

$p < 0.001$ in all cases.

animals in that blood cholesterol and triglycerides were both reduced compared with the trauma controls.

Liperoxidation

As shown in Table IVa and IVb, lipoperoxidation as measured by malondialdehyde estimation is significantly increased in the brain of traumatized animals, compared with controls. This increase (global 13.6%) is observed both in the "membrane" or insoluble fraction (10.5%) as well as the "soluble" supernatants (15.5%) three days after the impact trauma. In the treated animals peroxidation is considerably decreased, liposomal SOD being more efficient than the free enzyme. Indeed at high levels (1 mg/kg) the MDA material is lower than in the non-traumatized controls. Thus from a biochemical viewpoint, treatment of the traumatized rats shows a marked effect in reduction of the formation of lipoperoxides in the brain, and this effect is a function of the dose of enzyme administered.

Passage of SOD in the brain

Animals were traumatized at 9.00 a.m. the first day and one hour later treated i.p. with radioactive SOD or liposomal SOD. The same treatment was applied at the second and third days (at 9.00 a.m.) and the animals sacrificed three hours after the last injection by decapitation. The brain was removed and the cortex separated. All fractions were weighed and radioactivity measured in a Kontron γ counter. All injections contained 210 μg ^{57}Co SOD (or the liposomal form) in PBS (725 μg enzyme/kg) and each injection contained 1.26×10^6 cpm, the specific radioactivity of the protein being 6000 cpm/μg.

The results are shown in Table V. It can be seen that increased passage of the brain barrier by a factor of 2 to 3.5 occurs in the case of traumatized animals. This increase is less in the case of liposomes after three days treatment suggesting that repair of the brain barrier and decrease in permeability occurs more efficiently with liposomal SOD than with the free enzyme. In terms of concentration highest amounts are observed in the cortex compared with the rest of the brain and this is particularly so in the case

TABLE IVa
MDA-like material in rat brain

	Soluble fraction				Membrane	Total
	mg protein/ml	mg soluble protein/g tissue	nmoles MDA per mg soluble protein	nmoles MDA per g tissue	nmoles MDA per g tissue	nmoles MDA per g tissue
Controls n = 5	3.39 ± 0.16	37.25 ± 1.77	2.75 ± 0.36	102.3 ± 12.54	55.5 ± 3.69	157.8 ± 11.93
Trauma controls n = 8	3.35 ± 0.10	36.84 ± 1.10	3.21 ± 0.22	118.0 ± 7.63	61.3 ± 3.45	179.3 ± 9.05
p<	(trauma against controls)		0.01	0.01	0.01	0.01
SOD 100 μg/kg i.p. n = 5	3.33 ± 0.15	36.67 ± 1.66	3.05 ± 0.27	111.6 ± 8.99	56.1 ± 6.84	167.7 ± 10.42
p<	(treated against trauma)		NS	NS	0.05	0.05
Liposomes 100 μg/kg i.p. n = 5	3.51 ± 0.21	38.63 ± 2.29	2.82 ± 0.27	108.8 ± 10.23	49.5 ± 7.20	158.4 ± 12.38
p<	(treated against trauma)		0.01	0.05	0.001	0.01
SOD 1000 μg/kg i.p. n = 3	3.65 ± 0.13	40.19 ± 1.38	2.57 ± 0.15	103.3 ± 5.88	57.9 ± 4.99	161.3 ± 0.93
p<	(treated against trauma)		0.001	0.01	NS	0.001
Liposomes 1000 μg/kg i.p. n = 3	3.33 ± 0.17	36.67 ± 1.87	2.39 ± 0.29	87.4 ± 6.23	50.8 ± 5.36	138.2 ± 7.47
p<	(treated against trauma)		0.001	0.001	0.01	0.001

TABLE IVb
% Change in MDA-like material compared with controls

	Soluble per mg protein	Soluble per g tissue	"Membrane" per g tissue	"Membrane" + soluble per g tissue
Trauma	+16.7	+15.3	+10.5	+13.6
100 μg SOD	+10.9	+9.1	+1.1	+6.3
100 μg liposomes	+2.5	+6.4	−10.8	+0.4
1000 μg SOD	−6.5	+1.0	+4.3	+2.2
1000 μg liposomes	−13.1	−14.6	−8.5	−12.4

of traumatized rats. The total amount which penetrates the brain is extremely small, about 0.002 to 0.008% of the total injected material, but is nevertheless significant if a mechanism involving fixation of the enzyme to cell membranes is considered, rather than circulating extracellular levels.[27] Increased permeability of the brain barrier as a result of trauma is established, and confirmed by the present results. Even in normal controls, slight penetration occurs and this may well be of importance for the treatment of diseases such as multiple sclerosis. It may be noted that earlier studies with various inflammation models (carrageenan, adriamycin and adjuvant-induced

TABLE V
Passage of SOD in brain tissue

	Cortex		Brain minus cortex		Total brain		Concentration ratios, cortex relative to brain minus cortex
	ng/cortex	ng/g	ng/tissue	ng/g	ng/brain	ng/g	
Controls Free SOD	5.47 ± 1.91	24.03 ± 8.16	10.62 ± 5.79	6.25 ± 3.15	16.09 ± 7.48	8.38 ± 3.64	3.84
Trauma Free SOD	18.92 ± 3.28	90.69 ± 20.08	33.17 ± 16.56	19.67 ± 9.29	52.09 ± 19.70	27.57 ± 9.77	
Factor increase	3.46	3.77	3.12	3.15	3.24	3.29	4.61
$p <$	0.01	0.01	0.05	0.05	0.01	0.01	
Controls Liposomes	5.12 ± 1.65	27.43 ± 10.99	12.00 ± 4.02	7.29 ± 2.79	17.12 ± 5.31	9.31 ± 3.26	3.76
Trauma Liposomes	14.17 ± 6.86	64.99 ± 27.49	24.32 ± 7.70	15.38 ± 4.72	38.49 ± 12.06	19.65 ± 6.54	
Factor increase	2.77	2.37	2.03	2.11	2.25	2.11	4.23
$p <$	0.05	0.05	0.05	0.05	0.01	0.05	

n = 4 for each experiment. Three days' treatment i.p. Sacrifice 3 hr after last injection.

TABLE VI
Distribution of exogenous SOD in soluble and "membrane" fractions of the cortex

	ng/cortex			ng/g cortex		
	membrane	soluble	total	membrane	soluble	total
Controls free SOD (n = 3)	2.65 ± 0.64 (62.5%)	1.59 ± 0.60 (37.5%)	4.24 ± 0.45 –	11.72 ± 4.08 (62.2%)	7.13 ± 4.08 (37.8%)	18.85 ± 6.24 –
Trauma free SOD (n = 4)	5.21 ± 0.37 (55.9%)	4.11 ± 1.12 (44.1%)	9.32 ± 1.39 –	22.27 ± 4.52 (55.5%)	17.86 ± 7.09 (44.5%)	40.13 ± 11.34 –
Factor increase	1.97	2.58	2.20	1.90	2.50	2.13
p <	0.001	0.01	0.001	0.05	0.05	0.05
Controls liposomes (n = 3)	1.81 ± 0.35 (42.7%)	2.43 ± 0.43 (57.3%)	4.24 ± 0.45 –	7.85 ± 1.70 (42.7%)	10.55 ± 2.02 (57.3%)	18.40 ± 2.46 –
Trauma liposomes (n = 4)	2.92 ± 1.62 (41.0%)	4.20 ± 2.22 (59.0%)	7.12 ± 3.80 –	11.27 ± 5.85 (40.7%)	16.44 ± 8.59 (59.3%)	27.71 ± 14.30 –
Factor increase	1.61	1.73	1.68	1.44	1.56	1.51
p =	NS	NS	NS	NS	NS	NS

Injection i.p. 420 μg ^{57}Co SOD (5900 cpm μg) per rat (1450 μg·kg) 30 min after trauma. Decapitation 3 hours later.

arthritis) in the rat[15-17] have demonstrated that heterologous SOD is necessary for biological activity and that endogenous homologous enzyme is inefficient. In view of the neurological comportment tests with animals treated daily for 14 days it may be concluded that the small amounts of free SOD or the liposomal form which pass the brain barrier are totally non-toxic.

Distribution of Exogenous SOD in the Cortex

In order to determine whether the SOD was membrane bound (insoluble fraction) or soluble (extracellular and cytoplasmic) the homogenates of cortex excised from brains of animals injected with 420 μg ^{57}Co Cu-SOD (5900 cpm/μg) i.e. 1450 μg/kg 30 min after trauma (or the equivalent of liposomes) followed by decapitation three hours later were centrifuged to give a "soluble" supernatant and a residue "insoluble membrane fraction". Radioactivity in each fraction was measured. The results are shown in Table VI expressed as ng SOD per cortex and per g of cortex. Again the increase in passage of the brain barrier is observed in the case of traumatized animals. In the case of free SOD, somewhat more enzyme is membrane (or organelle) bound than is soluble whereas this tendency is reversed in the case of liposomes, perhaps because of greater cellular penetration to liberate free SOD in the cytoplasm. In both cases the presence of membrane bound SOD is highly significant in terms of mechanism of protection and recuperation.

Fixation in Organs

A remarkable increase of fixation of SOD to various organs (liver, kidneys, spleen, heart, lungs and plasma) was observed in traumatized animals compared with con-

TABLE VIIa
Organ distribution: μg SOD/g tissue

	Liver	Kidneys	Spleen	Heart	Lungs	Plasma
Controls free SOD	1.14	7.79	0.44	0.19	0.12	0.38
	±0.07	±1.25	±0.01	±0.02	±0.01	±0.05
Trauma free SOD	1.95	11.09	1.01	0.28	0.23	0.68
	±0.13	±0.84	±0.08	±0.03	±0.02	±0.13
% increase	71.05	42.36	130.11	47.36	89.58	78.89
Control liposomes	1.29	6.78	0.70	0.20	0.14	0.28
	±0.16	±0.63	±0.04	±0.02	±0.01	±0.02
Trauma liposomes	1.78	7.38	1.06	0.24	0.16	0.35
	±0.23	±0.38	±0.25	±0.01	±0.01	±0.04
% increase	37.98	8.89	51.78	20.00	14.29	25.00

n = 4 for each value. 210 μg per injection (725 μg/kg). Three days' treatment i.p. Sacrifice 3 hr after last injection.

TABLE VIIb
Organ distribution: μg SOD/g tissue

	Liver	Kidneys	Spleen	Heart	Lungs	Erythrocytes	Plasma
Control free SOD n = 3	1.31 ± 0.16	13.02 ± 2.57	0.63 ± 0.08	0.22 ± 0.02	0.22 ± 0.02	0.14 ± 0.02	0.65 ± 0.08
Trauma free SOD n = 4	1.46 ± 0.24	14.69 ± 0.69	0.69 ± 0.08	0.27 ± 0.02	0.21 ± 0.01	0.10 ± 0.04	0.63 ± 0.06
Controls liposomes n = 3	0.57 ± 0.10	9.72 ± 0.61	0.90 ± 0.32	0.21 ± 0.03	0.20 ± 0.05	0.09 ± 0.005	0.47 ± 0.03
Trauma liposomes n = 4	0.62 ± 0.15	9.91 ± 1.29	1.02 ± 0.20	0.19 ± 0.03	0.17 ± 0.03	0.07 ± 0.02	0.49 ± 0.02

Single injection (420 μg SOD per rat, 1450 μg kg) 30 min after trauma, sacrifice 3 hr later.
No significant differences between controls and traumatized animals treated with free SOD or with liposomes.

trols injected with the same amount of enzyme (210 μg per rat daily for three days, ^{57}Co SOD) as shown in Table VIIa. This was also seen to a lesser extent with animals treated with liposomal SOD. The increase in organ fixation was not observed in a short term experiment in which a single injection (i.p.) of free SOD or liposomes (420 μg/rat, 1450 μg/kg) was made 30 min after trauma and the animals sacrificed 3 hours later (Table VIIb).

DISCUSSION

The above results give a strongly positive indication that brain trauma including coma states in humans could be successfully treated with liposomal bovine Cu-SOD or with the free enzyme. Given the slow release mechanism of SOD from liposomes this is the preferred approach since it implies fewer injections with longer intervals. In various models we have shown that longer lasting effects are obtained with liposomes compared with the free enzyme (Table VIII). It may also be noted that enzymic activity

TABLE VIII
Comparison of free and liposomal bovine Cu-SOD in the rat paw adriamycin edema model

	1 Hour			5 Days		
	Plethysmometric measure ± SEM	Percentage change	p<	Plethysmometric measure ± SEM	Percentage change	p<
Adriamycin controls	+62.7 ± 5.3	–	–	+112.7 ± 9.8	–	–
Liposomes						
33.3 μg/kg	+37.3 ± 6.1	−40.5	0.001	+80.5 ± 6.3	−28.6	0.001
100 μg/kg	+26.3 ± 4.5	−58.0	0.001	+64.4 ± 7.0	−42.9	0.001
Adriamycin controls	+58.4 ± 4.3	–	–	+100.7 ± 7.3	–	–
Free bovine Cu-SOD						
33.3 μg/kg	+50.3 ± 6.4	−13.9	0.01	+100.3 ± 9.4	−0.3	NS
100 μg/kg	+48.3 ± 7.3	−17.3	0.005	+90.4 ± 8.28	−10.1	0.02

Injection i.p. at 24 hours before and 3 days after adriamycin only. Measurements at 1 hour and five days after injection of the adriamycin. Ten rats in each group.

The results indicate a much more significant long term efficiency of the liposomal form compared with free bovine Cu-SOD.

is necessary for the expression of biological activity since if the metal in the active centre is replaced e.g. Cu-SOD or Mn-SOD → Fe-SOD, with loss of enzymic properties but total retention of the protein structure, no biological effects are seen (Table IX). A strong dose rate response is observed in such models, ranging from 3 to 100 μg/kg in general. We have previously discussed mechanisms of the biological activity[27] and it is clear that intracellular penetration or extracellular circulation is not of importance. The essential aspect is fixation of SOD (10–100 molecules per cell) to

TABLE IX
Rat paw adriamycin edema model. Dose reponse relationships

	1 Hour			5 Days		
	Plethysmometric measure ± SEM	Percentage change	p<	Plethysmometric measure ± SEM	Percentage change	p<
Adriamycin injected controls	+62.7 ± 5.3	–		+112.7 ± 9.8		–
E. coli Mn-SOD						
3.3 μg/kg	+63.3 ± 6.4	+1.0	NS	+105.4 ± 10.4	−6.5	NS
9.9 μg/kg	+60.4 ± 9.2	−3.6	NS	+98.3 ± 7.1	−12.8	0.005
16.7 μg/kg	+43.3 ± 6.1	−30.9	0.001	+78.25 ± 8.3	−30.6	0.001
33.3 μg/kg	–	−81.3	0.001	–	−73.3	0.001
E. coli (Mn-SOD → Fe-SOD)						
33.3 μg/kg	+60.3 ± 8.9	−3.8	NS	+115.4 ± 10.2	+2.4	NS

Male Wistar AF-gnotoxenic rats, 280 ± 25 g.

Percentage volume change compared with non-treated controls. *E. coli* Mn-SOD i.p. daily for 5 days. Ten animals in each group.

E. coli Mn-SOD was converted to the apoenzyme followed by addition of ferrous ions to give an iron containing protein. Unlike the natural Fe-SOD of E. coli this protein showed less than 2% of the original enzymic activity. Addition of manganese ions did not restore activity showing that the active site was occupied by Fe. This is perhaps the best control material since no amino acids are changed. Similar procedures gave non-active Fe-SOD from bovine Cu-SOD. The results with such controls show that enzymic activity is necessary for biological (anti-inflammatory) activity even if certain SODs have zero anti-inflammatory properties despite full enzymic activity.

the cellular membrane to inhibit free radical cascade reactions resulting from an initial lipoperoxidation on and in the membrane matrix. Thus very little of the injected SOD may in fact be operational, but the traces which reach the site of the edema are sufficient to overcome at least part of the local biochemical pathology involved. Alternatively action of the heterologous SOD on various circulating plasmatic factors may be important, thus causing amelioration of the cerebral damage indirectly. At the moment this possibility cannot be excluded.

The greatly increased rates of normalisation of EEG recordings in the treated animals, in recovery of neurological behaviour and of changes in various biochemical parameters indicate that apart from immediate effects on recuperation, use of liposomal SOD (or the free enzyme) could influence subsequent processes. That treatment may effect long term sequels of brain trauma was shown by histological studies and determination of water content of the brain for the different groups of animals. This was done at 15 days when untreated controls and treated animals all showed essentially total neurological behaviour recovery (Figure 1), although EEG recordings in untreated trauma controls were still abnormal, in contrast with the treated rats.

Histological examination of brain tissue showed very significant differences between trauma controls and treated animals. Intracerebral hematomas, diffuse axonal lesions and cerebral swelling were easily observed in the controls whereas in animals treated with SOD or liposomal SOD, hematomas were absent and axonal lesions and diffuse swelling very considerably reduced.

Similarly, water content of the brain (measured by the usual drying techniques) and of structures such as the cortex, cerebellum and sub-cortex was signficantly reduced ($p < 0.001$) in the case of treated animals compared with trauma controls at 15 days. These controls showed an increased water content compared with non-traumatized animals. In all cases of treated groups the percentage water was reduced to that of the non-trauma controls. As an example, percentage loss in weight representing water content of total brain was $76.9 \pm 0.1\%$ in normal rats ($n = 5$), $78.6 \pm 0.2\%$ in trauma controls ($n = 4$), $76.8 \pm 0.1\%$ in rats treated with free SOD ($n = 4$) and $76.7 \pm 0.2\%$ in those treated with liposomal SOD ($n = 4$).

The pharmacological application of liposomal SOD to various human inflammatory pathologies[28,29] as well as radio-induced fibrosis[30] has been reported. A controlled clinical trial is presently underway at the Centre Hospitalier et Universitaire de la Timone at Marseille (Service de Neurochirurgie Infantile) for the treatment of brain trauma with liposomal SOD. Some 1000-1200 infants are hospitalised per year of which 60–80 are in coma for more than two days and are treated in the pediatric reanimation unit. Preliminary results suggest that intracranial edema can be reduced by treatment with liposomal SOD, with improvement of short term prognostics and reduction of the severity of post-traumatic sequels. A second trial with adult trauma cases (Service de Neurochirurgie, Adultes) at the same hospital has also been initiated.

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