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EFFECTS OF LIPOSOMAL SUPEROXIDE DISMUTASE ON HUMAN NEUTROPHIL ACTIVITY

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Study of the effects of liposomal bovine copper superoxide dismutase on human polymorphonuclear neutrophils with respect to production of active oxygen species, chemotaxis and random migration, or bacterial killing show that no significant interference with neutrophil function is observed at levels far exceeding the clinical doses used in the treatment of various pathologies.

Key words: Human neutrophils, liposomal SOD, chemotaxis, oxygen radicals, bacterial killing

INTRODUCTION

Liposomal bovine copper superoxide dismutase Cu-SOD has been used to treat several hundred patients suffering from various inflammatory diseases and post radiotherapeutic sequels[1,2]. It is therefore of interest to present some of the preclinical *in vitro* studies of these liposomes. Pharmacokinetic properties have been reported in rats and rabbits, as well as organ localisation[3,4]. It is to be noted that the circulation life time is not significantly increased relative to free bovine Cu-SOD. The advantages of liposomal encapsulated SOD are multiple. The liposomes do not attach to bacteria or penetrate and therefore would not increase the resistance of pathogens to phagocytic action. If liposomal SOD attaches to PMNs or macrophages, this fixation is followed by fusion with the membrane with interior liberation of the encapsulated protein[5]. Membrane bound enzyme is attached to the inner surface since if the outer surface was involved there would be loss of enzyme to yield the low levels of fixation of the free enzyme (few external sites appear to exist). This does not occur with SOD, glutathione peroxidase or glutathione transferase liposomes and hence it may be concluded that essentially all the membrane bound enzyme is interior and not exterior. Phagocytic activity would thus not be inhibited and protection of ingested bacteria would not occur, the enzyme being on the outside surface of the vacuole. Even within the first hours when the liposome is fixed to the outer membrane surface of the cell

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during endocytosis, protection would not be afforded to ingested bacteria since except for the outer layer, the liposomal encapsulated SOD is not available to O_2^- as shown by *in vitro* tests of liposomal suspensions. In view of possible medical applications these conclusions are of importance since exterior bound enzyme could inhibit the killing action of free radicals during phagocytosis and hence increase the sensitivity of a patient to microbial infection. However, interior bound enzyme in fact protects the polymorphonuclear neutrophils against suicide by over excitation and excessive production of superoxide ions or lipohydroperoxides. This phenomenon thus increases the possible value of liposomal enzymes for treatment of inflammatory conditions.

Other advantages of liposomal SOD lie in improved pharmacokinetic properties leading to a longer life time in the organism, with a slow release of free SOD, concentration in divers organs other than the kidneys, better total distribution, greatly enhanced fixation to the outside of cell membranes compared with free SOD and greatly increased tissue permeability[6]. An example of *in vitro* efficiency is shown by the fact that oxidative damage of mitochondria (due to peroxide formation on UV irradiation of methyl linoleate) is not inhibited by free SOD but is prevented by the liposomal SOD used in this study due to increased membrane fixation[7]. Finally, due to the pharmacokinetic advantages and increased membrane fixation smaller amounts of liposomal SOD and a very reduced schedule of injections are necessary compared with the free enzyme.

The present study concerns the effects of liposomal SOD on human polymorphonuclear neutrophils (PMN).

MATERIALS AND METHODS

Preparation of PMNs

All procedures employed plastic or siliconized glass containers.

Venous blood from human volunteers was drawn on heparin (25 IU/ml blood) and then diluted two fold with phosphate buffered saline medium containing 2.8 mM KCl, 136.7 mM NaCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4. Two volumes of diluted blood were layered on one volume of Ficoll-paque solution (Pharmacia Co., d = 1.077). After centrifugation at 300 g for 25 min at 4°C, the plasma-containing upper layer, mononuclear cell layer and pellet were removed separately. Platelets were eliminated from the plasma by centrifugation at 900 g for 10 min.

The cell pellet, containing PMNs and erythrocytes was washed with three volumes of 0.9% NaCl (centrifugation 10 min at 400 g) and resuspended in 10 ml of plasma adjusted to 1% Dextran (M.W. 177 000) by addition of a 6% solution in 0.9% NaCl. The PMNs were recovered after sedimentation at 37°C for 30–40 min, collected by centrifugation for 10 min at 120 g and the few contaminating erythrocytes were lysed by hypotonic stress on addition of 5 ml of 0.2% NaCl. Thirty seconds later, 5 ml of 1.6% NaCl was added and the suspension centrifuged at 100 g for 10 min.

The PMN cell pellet was washed with 10 ml of phosphate buffered saline medium (centrifugation for 10 min at 100 g) and then suspended in Hank's solution (Nissui Pharmaceutical Co., Tokyo, Japan) pH 7.4 at 10^7 cells per ml.

Light measurements were performed using the apparatus previously described[8], or in an Aloka BLR-102 Luminescence Reader.

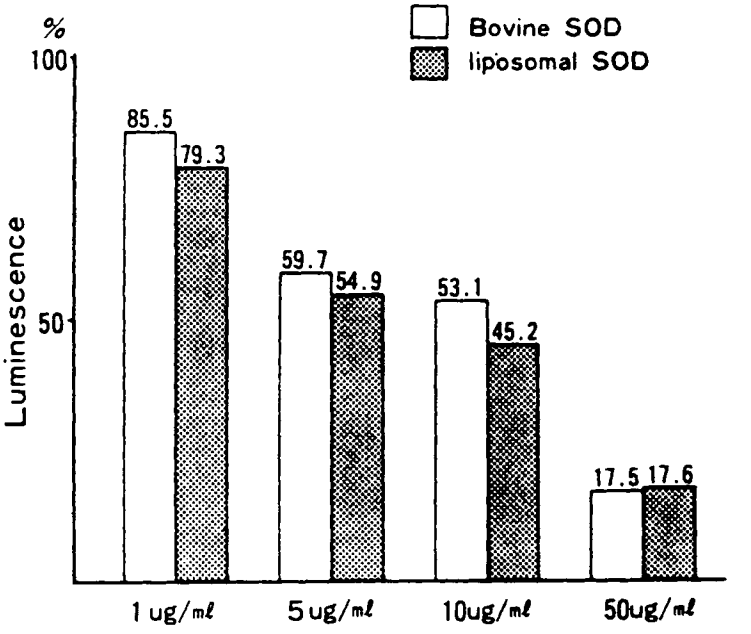
For chemiluminescence studies the incubation mixture contained 10^6 PMNs (100 μl of a suspension at 10^7 cells/ml), 50 μl of opsonized zymosan (at 1.25 mg/ml) and

Hanks solution to a final volume of 0.6 ml. After 2 min incubation at 37°C, 50 μl of a solution of luminol (200 mg/l) were added. Inhibition was estimated as a percentage of quenching of control light emission.

Hydroxyl radicals were estimated by the amount of ethylene produced by 2×10^6 PMNs activated with opsonized zymosan in presence of 1 mM α keto-methiol butyric acid, the total amount of ethylene being measured at 10, 20 and 30 min after initiation.

Chemotaxis and random migration were performed using the Agarose technique described by Nelson *et al.*[9]. Chemotractant (formyl methionyl leucyl phenylalanine, FMLP, at 10^{-7} M or activated serum prepared by incubating homologous serum with zymosan particles at 37°C for 60 min) was placed in an outer well (diameter 3 mm), PMNs in the second well and control medium in a third well in linear radial arrangement. Chemotaxis and random migration were measured by movement of cells from the central well to the two flanking wells.

Bacteriocidal killing was examined by the method of Quie[10]. The phagocytic mixtures were prepared in 12 × 75 mm Falcon polystyrene tubes containing 5×10^6 PMNs (0.5 ml of a suspension at 10^7/ml), 0.4 ml of Opsonin and 0.1 ml of a suspen-



Bovine SOD(cpm)	435×10^4	303×10^4	269×10^4	89.9×10^3
liposomal SOD(cpm)	403×10^4	279×10^4	290×10^4	89.4×10^3

Control 508.5×10^4 cpm/10^6cells

FIGURE 1 a) The effect of bovine Cu-SOD and liposomal SOD on luminol mediated chemiluminescence of human PMNs at different concentrations.

sion of bacteria (*E. coli* or *Staphylococcus aureus* strain 502 A) at 5×10^7 bacteria/ml, i.e. equal numbers of PMNs and bacterial cells. Aliquots to determine live bacteria were taken at 0, 30, 60 and 120 min.

RESULTS AND DISCUSSION

As shown by luminol mediated chemiluminescence, these large multilamellar liposomes can activate neutrophils whether the liposomes contain albumin or superoxide dismutase, that is, they act as opsonized particles. At higher concentrations light emission is inhibited to about equal extents by free SOD or by the equivalent amount of liposomal SOD (Figure 1a). In the case of free SOD this inhibition is almost totally reduced if the cells are washed after two minutes incubation (Figure 1b), that is the effects are almost entirely due to extracellular events. In the case of liposomes the inhibition of light emission after pre-incubation and washing is unchanged due to

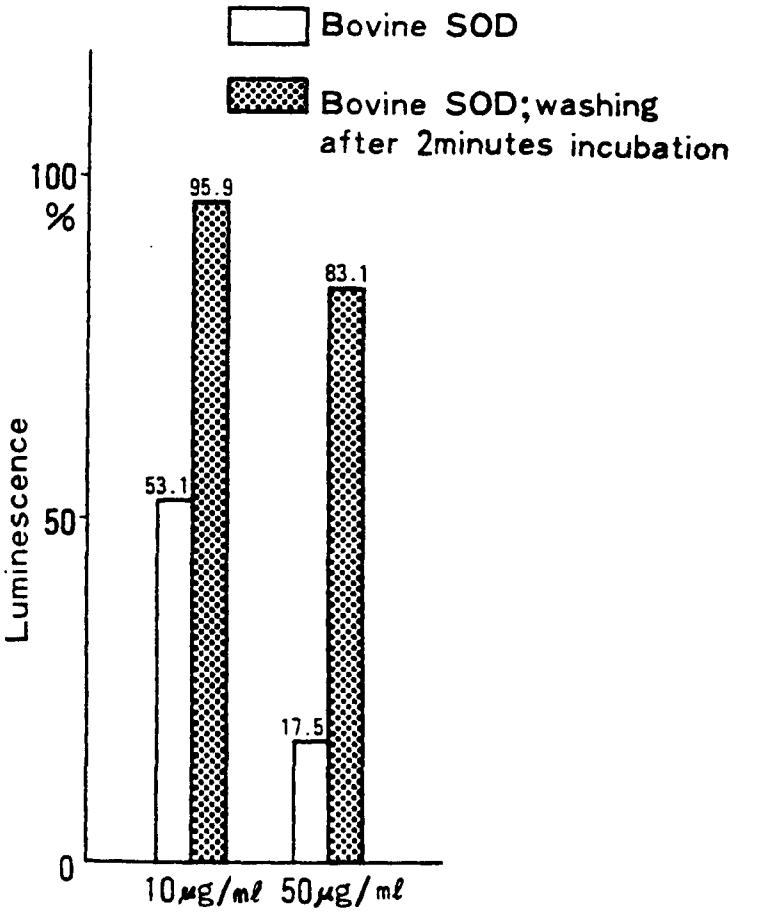


FIGURE 1 b) Effect of washing after 2 min preincubation with SOD.

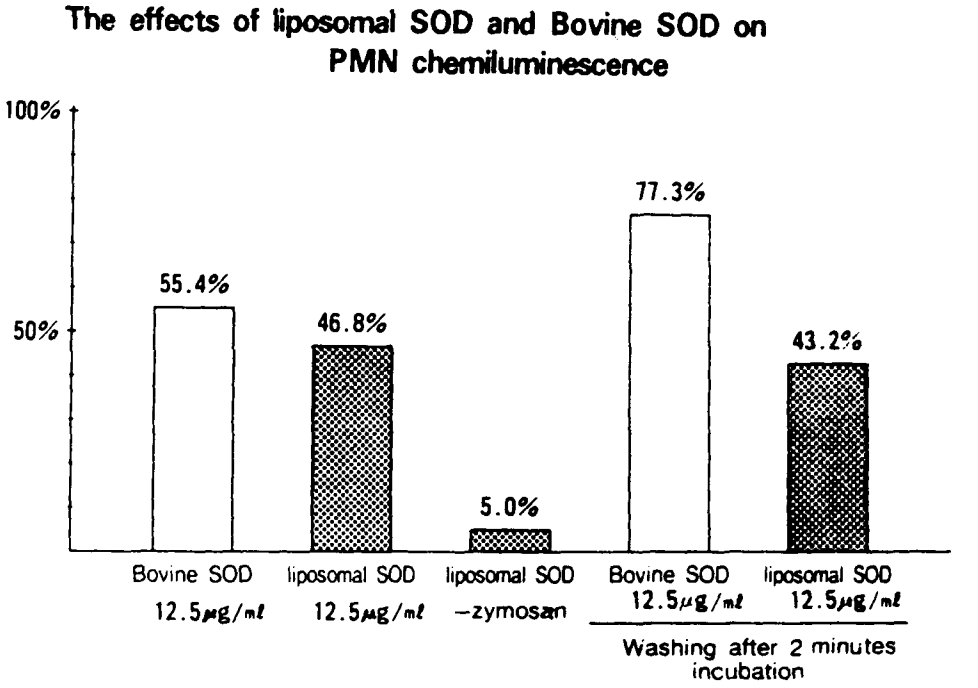


FIGURE 1 c) Comparison of washing between free SOD and liposome SOD.

membrane bound SOD (Figure 1c). As shown in Figure 2 both free SOD and liposomal SOD inhibit hydroxyl radical formation whereas empty liposomes have no effect. Given the quantities necessary to produce such inhibition it is clear that at the clinical doses used (about 50 μg/kilo) liposomal SOD can have no significant effect on neutrophil function.

This is further demonstrated by a study of the effect of bovine Cu-SOD and liposomal SOD on random migration and on chemotaxis induced by formyl methionyl leucyl phenylalanine (FMLP) or Zymosan activated serum (ZAS). As shown in Table I no effects are observed with free SOD or the liposomal form. With the latter at the highest concentration (50 μg/ml) some slight effects are observed in both chemotaxis and random migration, probably due to perturbation of the neutrophil membrane by fusion of liposomes at this massive concentration.

The involvement of oxygen radicals in PMN chemotaxis has been demonstrated with respect to chemio-attractants such as FMLP, leukotriene B4 and complement fragment C5a. Chemotactic stimulation is inhibited[11] dose dependently by SOD (10–100 μg/ml) using the Boyden chamber technique, but with the agarose plate method much larger quantities (1 mg/ml) are necessary. These results are in no way contradictory to those presently described.

Finally, the effect of liposomal SOD on the bactericidal activity of human neutrophils was examined. As shown in Figure 3 no significant reduction in killing was observed with *E. coli* or *S. aureus* when the neutrophils were incubated with 5 μg/ml of liposomal SOD. This quantity represents about 100 times the maximal concentration possible by intravenous injection of 2.5 mg of liposomal encapsulated SOD in an adult human, or 1000 to 10 000 times if i.m. administration is used. Given the rapid

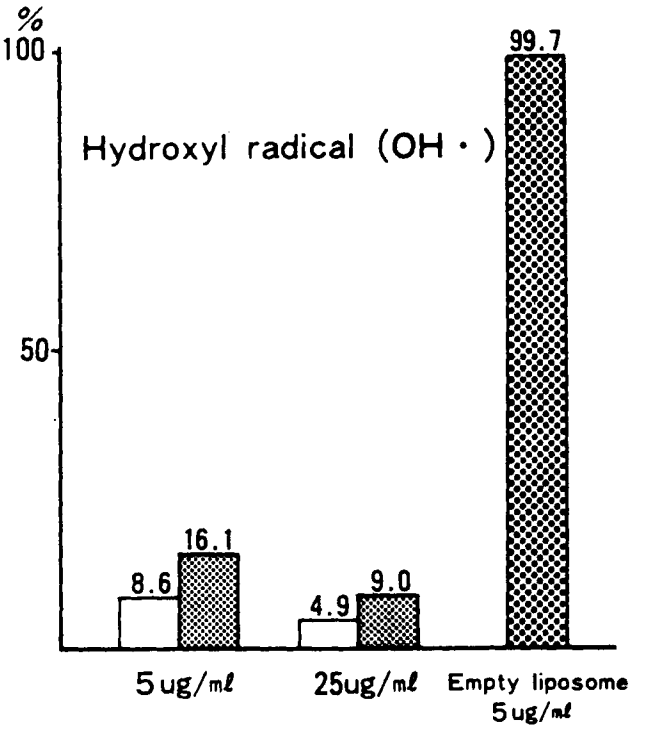


FIGURE 2 Effect of free and liposomal SOD and of empty liposomes on hydroxyl radical production.

TABLE I
The effect of Bovine SOD and liposomal SOD on PMN chemotaxis and random migration

Chemotactic factor		FMLP (10^{-7} M)		ZAS	
		Chemotaxis	Random migration	Chemotaxis	Random migration
	Control	90 ± 5	45 ± 7	94 ± 10	44 ± 8
Bovine SOD	1 μg/ml	87 ± 5	44 ± 2	87 ± 6	42 ± 2
	5 μg/ml	93 ± 2	46 ± 1	86 ± 12	39 ± 1
	10 μg/ml	87 ± 5	41 ± 1	93 ± 7	40 ± 3
	50 μg/ml	94 ± 4	43 ± 0	86 ± 5	38 ± 3
	Control	110 ± 7	45 ± 4	74 ± 11	41 ± 6
Liposomal SOD	1 μg/ml	112 ± 4	38 ± 3	73 ± 8	39 ± 4
	5 μg/ml	104 ± 5	38 ± 3	78 ± 6	41 ± 2
	10 μg/ml	106 ± 5	44 ± 1	63 ± 12**	41 ± 1
	50 μg/ml	98 ± 4*	40 ± 10	48 ± 4*	32 ± 5**

Mean ± SD

*p < 0.01
**p < 0.05

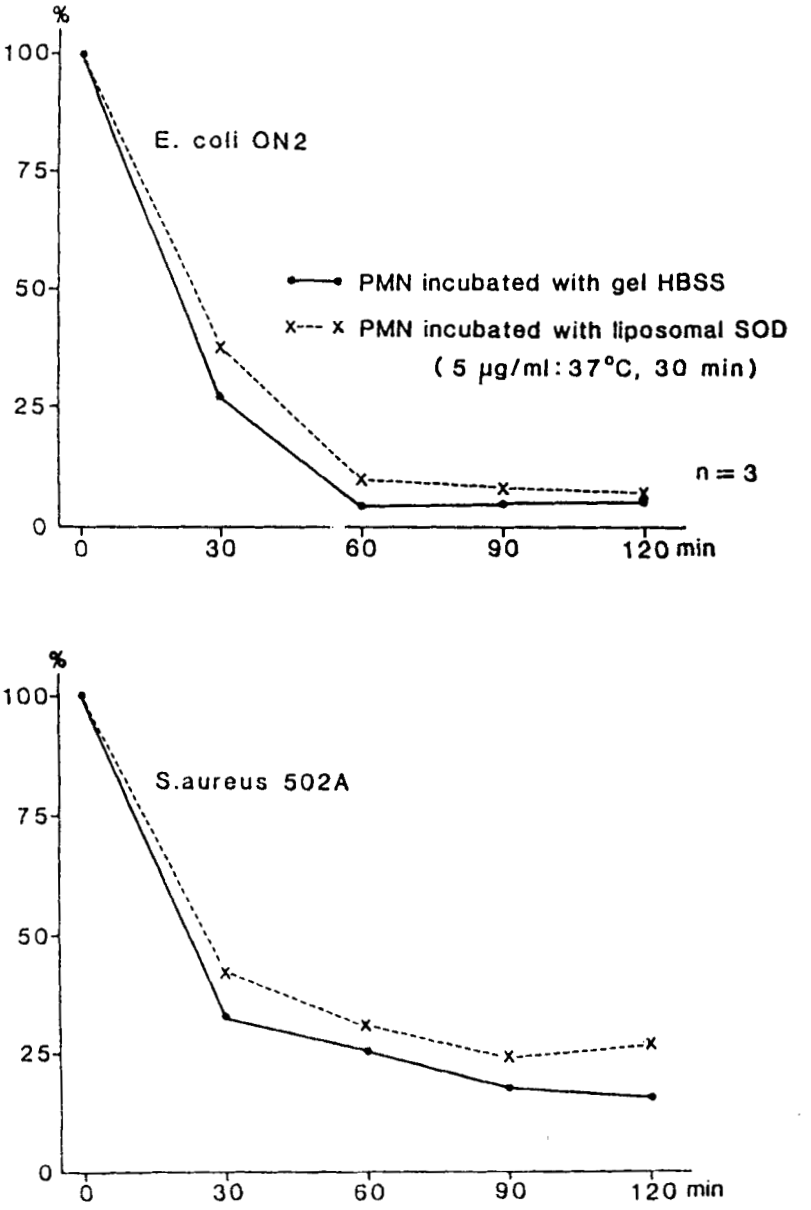


FIGURE 3 Effect of liposomal SOD (at 5 μg/ml) on bacterial activity of human polymorphonuclear neutrophils.

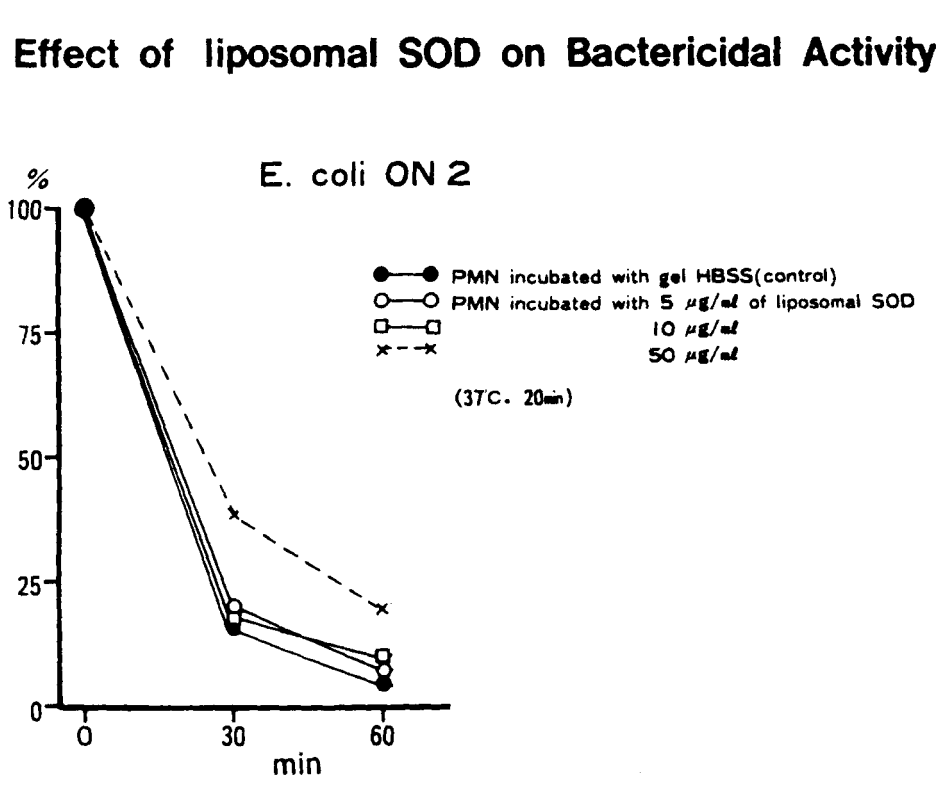


FIGURE 4 Effect of concentration of liposomal SOD on bactericidal activity of PMNs.

loss from the circulation and redistribution in different organs it is clear that intravenous injection (and even less intramuscular or subcutaneous) of clinical doses of liposomal SOD can have no effect whatsoever on the neutrophils. Inhibition begins (drop in killing from 90% to 80%) at 50 μg/ml, which provides a very considerable safety factor (Figure 4).

Studies using both empty liposomes and those containing SOD have shown inhibition of bacterial killing efficiency of PMNs both *in vitro* and *in vivo*[12]. The rather massive amounts of lecithin and cholesterol used in this work were several thousand fold those employed in clinical application of liposomal SOD. At lower levels, (several hundred times clinical concentrations) these effects, independent of SOD and probably due to severe perturbation of the cell membrane, are not observed.

CONCLUSIONS

The innocuity of liposomal SOD has been demonstrated in infants treated for Kawasaki disease (mucocutaneous lymph node syndrome, MCLS) during comparisons made with high dose γ-globulin treatment[13]. No adverse effects were observed in white blood cell counts, α 1-AT, compliments (C3 and C4) or immunoglobulins (IgG, IgA, IgM and IgE). Infants with severe extensive thermal burns, to whom liposomal SOD (at 0.1 mg/kilo) was administered showed good tolerance and again

no reactions or adverse effects were observed[14]. The present results show that *in vitro* experiments also reveal the absence of possible nocive effects of liposomal SOD with respect to neutrophil function and thus refute possible unfounded criticisms, based more on meretricious assumptions than on scientific reality.

Acknowledgements

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