

EVALUATION OF ANTIINFLAMMATORY ACTIVITY OF LIPOSOME ENCAPSULATED SUPEROXIDE DISMUTASE IN RATS PERITONITIS

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Abstract

This work describes the effect of superoxid dismutase (SOD) in the prevention and treatment of chemical induced peritonitis in rats. SOD was administered i.p., both as a solution and loaded in liposomes, in a dose of 500 U/kg body weight. SOD efficiency was compared with diclofenac, vitamin E and a nonselective nitric oxide synthase inhibitor, N-nitro L-arginine methyl ester, noted as NAME. The antiinflammatory effect was evaluated through bone marrow acute phase response and some global and specific oxidative stress parameters. SOD liposomes were more efficient than the solution of SOD in the prevention of peritonitis and their effect was similar in the treatment of peritonitis.

Rezumat

Această lucrare prezintă efectul lipozomilor cu superoxidismutază (SOD) în prevenirea și tratamentul peritonitei induse chimic la șobolani. SOD a fost administrată i.p., atât ca soluție, cât și încorporată în lipozomi, în doze de 500 U/kg corp. Eficiența SOD a fost comparată cu cea a diclofenacului, vitaminei E și a unui inhibitor neselectiv de nitric oxid sintetază, esterul metilic al N-nitro L-argininei, notat NAME. Efectul antiinflamator a fost evaluat prin răspunsul medular de fază acută și prin parametrii generali și specifici de stres oxidativ. Lipozomii cu SOD au fost mai eficienți decât soluția cu SOD în prevenirea peritonitei, iar efectele lor au fost similare în tratamentul peritonitei.

Key words: superoxide dismutase (SOD); liposomes; peritonitis; inflammation.

Introduction

Reactive oxygen species (ROS) are known to be involved in the inflammatory process developed in many diseases, the most abundant being the superoxide radical. Under normal conditions, a balance is maintained between the formation of ROS and their removal by the endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase [1]. The inflammatory process is associated with the

excess production of superoxide radicals and the alteration of the cellular oxidation/reduction balance which leads to potential oxidative damage [2,3].

SOD is an antioxidant enzyme that catalyzes the dismutation of superoxide radical into hydrogen peroxide and oxygen. SOD is widespread in eukaryotic and prokaryotic organisms and is considered as one of the most potent antioxidants known in nature [4, 5]. Using SOD for treating the diseases in which the oxidative stress has been involved seems to be a promising alternative to conventional therapies. For this purpose, an appropriate delivery system for this enzyme is needed, that would be able to protect the enzyme against inactivation, prolong its circulation lifetime, attain a more effective site-directed delivery and, ideally, provide intracellular delivery [6].

Liposomes were chosen as delivery system for SOD in this study because the free enzyme is cleared very rapidly from blood and local injection sites (half-life in blood is about 6 min in rats and 30 min in humans), limiting its therapeutic effects [7]. The antiinflammatory activity of liposome entrapped SOD was evaluated using a model of experimental induced peritonitis in rats. The treatment was administered either before (in the preventive study) or after the induction of peritonitis (the therapeutic evaluation study) by a carrageenan injection. The antiinflammatory activity of the encapsulated enzyme was compared with the free enzyme (SOD solution), a nonsteroidal antiinflammatory drug (diclofenac), an antioxidant (vitamin E) and a nonselective NOS (nitric oxide synthase) inhibitor (N-nitro-L-arginine methyl ester, NAME). The major aim of this study was to compare the performance of liposome entrapped SOD with that of a SOD solution.

Materials and methods

Materials

Soybean phosphatidylcholine (PC), cholesterol were purchased from Merck. Bovine erythrocytes Cu/Zn-superoxide dismutase (SOD; Cu,Zn-SOD), Cytochrome C from horse heart, Xanthine Oxidase and Xanthine were purchased from Sigma. All other chemicals used were reagent grade.

Liposomes preparation

The liposomes were prepared using the "film method". Briefly, PC (50 μ M/mL) and cholesterol (25 μ M/mL) were dissolved in 5 mL ethanol in a 300 mL round-bottomed flask. After complete dissolution, the solvent was evaporated under reduced pressure at 50°C in a rotary evaporator, leading to the formation of a thin film of lipids on the surface of the flask. In order to completely remove the residual solvent, the film was maintained under a

nitrogen gas flow for 1h. Then the film of lipids was hydrated with 5 mL SOD aqueous solution (0.5 mg/mL) in phosphate buffered saline (PBS, pH=7.8) for 15 minutes at 37°C. The resulted liposomes dispersion was maintained 1h at 4°C and then the excess unloaded SOD was removed by centrifugation (30 minutes, 8000×g).

SOD encapsulation in liposomes

The liposome-encapsulated SOD activity was determined after the liposomes were lysed with Triton-X-100, in order to obtain a clear solution.

SOD activity was measured by the xanthine/xanthine-oxidase/cytochrome C method according to McCord and Fridovich [7]. This method is based on superoxide radical production by the xanthine/xanthine-oxidase system. The superoxide radical reduces the cytochrome C and its reduced form can be assessed spectrophotometrically at 550nm. SOD competes with cytochrome C for the dismutation of superoxide radical and SOD activity in the sample is measured as the inhibition of the rate of reduction of cytochrome C by the superoxide radical. One SOD unit is equivalent to the SOD activity that inhibits the rate of reduction of cytochrome C by 50%. All activity measurements were performed using a blank containing the same Triton-X 100 concentration as the liposome sample.

Animal experiments

Induction of inflammation

All animals used in this study were maintained in facilities fully accredited and the experiments described here were performed in compliance with European Communities Council Directive 1986 (86/609/EEC) and Ordinance No. 37 of the Romanian Government from 2nd February 2002.

The experiments were performed on male Wistar rats weighing 150-200 g. Animals were fed with standard laboratory food and water *ad libitum*. Groups of 10 animals each were used to evaluate the preventive and therapeutic effect of Cu, Zn-SOD. For this purpose, two separate sets of experiments were performed. The treatments were administered in a 0.5 mL single dose 30 minutes before peritonitis induction in the preventive study and 30 minutes after peritonitis induction in the therapeutic evaluation.

The peritonitis was induced by the injection of a carrageenan solution (25 mg / kg body weight) [9]. The animals were randomly divided into groups of 10 animals each and were put in separate cages. For both experiments, the treatment consisted in an intra peritoneal injection consisting in one of the following preparations: (1) liposomes encapsulated SOD (500 U/kg body weight) [10]; (2) SOD solution (500 U/ kg body weight); (3) vitamin E solution (20 mg/kg body weight) [11]; (4) diclofenac

solution (20 mg/ kg body weight) [12]; and (5) N-nitro-L-arginine methyl ester (NAME) (10 mg / kg body weight) [13].

Six hours after the induction of peritonitis, blood samples were withdrawn via the retro bulbar vein puncture on EDTA solution in order to evaluate the phagocytosis properties of the blood and to count the white blood cells, and without EDTA for the biochemical parameters determination.

Evaluation of the anti-inflammatory activity

The anti-inflammatory activity was assessed through multiple parameters: the evaluation of bone marrow acute phase response, using total leukocyte count [14], differential leukocyte count [8] and *in vitro* phagocytosis test [15]; oxidative stress, using global tests as total oxidant status (TOS) [16], total antioxidant response (TAR) [17] and oxidative stress index (OSI) [18], and by specific tests as nitrite and nitrate [19], malonaldehyde (MDA) [20] and plasma reduced glutathione (GSH) [21].

Statistical analysis

The results for each group were expressed as mean $\pm$ standard deviation. The groups were compared using Student t test. The differences between groups were considered significant for $p < 0.05$.

Results and discussion

The “film method” was found to be suitable for the preparation of large sized liposomes encapsulating SOD. The activity of the enzyme was well preserved during the preparation, about 20% of the enzyme used being recovered in the final liposome dispersion.

Bone marrow acute phase response and the test of *in vitro* phagocytosis

The results for the leukocyte count and the phagocytosis test are presented in Table I and Figure 1 for the preventive study and in Table II and Figure 2 for the therapeutic evaluation study. Concerning the total leukocytes, their count significantly increased when peritonitis was induced, compared with control group ($p < 0.001$).

In the preventive study, compared with the control group, all treatments did not change significantly the total leukocytes count compared with the control group ($p > 0.05$). Moreover, the reduction of the leukocyte count compared with the peritonitis group was significant for all treated groups ($p < 0.001$). The effect of SOD, both as free form or in liposomes, on the total leukocytes count, was more important than that of diclofenac. The total leukocyte count was significantly lower for the SOD liposomes pretreated group than for the SOD solution pretreated group ($p < 0.05$).

In the therapeutic evaluation study, diclofenac did not reduce significantly the total leukocyte number ($p > 0.05$), while all the other treatments administered were able to importantly reduce the total leukocyte number ($p < 0.001$ vs. peritonitis group). The effect of SOD liposomes and SOD solution on the leukocyte number was more intense in this case than in the preventive study.

The effect of SOD on bone marrow acute phase response can be explained by the reduction of ROS that will consequently reduce the cell inflammatory response. SOD liposomes proved to be more effective than the SOD solution and SOD products than diclofenac.

Table I
Leukocytes count and differential leukocyte count in the preventive study

	Leucocytes/ mm ³	Neutrophils (%)	Monocytes (%)	Lymphocytes (%)
Control	3332.1 ± 493.9	63.0 ± 4.1	8.0 ± 2.1	29.0 ± 4.1
Peritonitis	6002.3 ± 632.4 ^{###}	89.8 ± 3.9	4.4 ± 1.9	6.5 ± 3.8
Diclofenac	3806.2 ± 829.5 ^{***}	81.5 ± 3.7 ^{***}	5.1 ± 1.9	13.5 ± 4.1
SOD liposomes	3013.9 ± 532.0 ^{***}	89.8 ± 4.1	4.4 ± 2.4	5.8 ± 2.9
SOD solution	3640.9 ± 649.4 ^{***}	80.0 ± 3.8 ^{***}	4.8 ± 1.7	15.2 ± 3.7
Vitamin E	3113.9 ± 1388.7 ^{***}	80.0 ± 2.7 ^{***}	4.0 ± 0	16.0 ± 2.7
NAME	2392.5 ± 927.7 ^{***}	85.2 ± 2.7 [*]	4.0 ± 0	10.8 ± 2.7

(* $p < 0.05$ vs. peritonitis; *** $p < 0.001$ vs. peritonitis; ^{###} $p < 0.001$ vs. control)

Table II
Leukocytes count and differential leukocyte count in the therapeutic evaluation study

	Leucocytes/ mm ³	Neutrophils (%)	Monocytes (%)	Lymphocytes (%)
Control	3332.1 ± 493.9	63.0 ± 4.1	8.0 ± 2.1	29.0 ± 4.1
Peritonitis	6002.3 ± 632 ^{###}	89.8 ± 3.9 [*]	4.4 ± 1.9	6.5 ± 3.8
Diclofenac	5900.0 ± 600 ^{***}	86.0 ± 3.4	4.4 ± 1.3	9.6 ± 3.9
SOD liposomes	2697.5 ± 308 ^{***}	86.8 ± 4.2 ^{***}	4.8 ± 1.7	9.6 ± 2.1
SOD solution	2136.1 ± 507 ^{***}	80.8 ± 4.5 ^{***}	5.2 ± 1.9	14.0 ± 4.3
Vitamin E	4297.5 ± 100 ^{***}	83.6 ± 3.5 ^{***}	5.2 ± 1.9	11.2 ± 2.5
NAME	4135.0 ± 168 ^{***}	85.2 ± 5.3 [*]	5.6 ± 2.1	9.6 ± 4.3

(* $p < 0.05$ vs. peritonitis; *** $p < 0.001$ vs. peritonitis; ^{###} $p < 0.001$ vs. control)

Concerning the differential leukocyte count, the peritonitis group was significantly characterized by an increase of the neutrophils percentage compared with the control group ($p < 0.001$). In both our experiments, the administration of SOD solution and vitamin E had the major ability to

reduce the neutrophils percentage ($p < 0.001$ vs. peritonitis group), while SOD liposomes did not have a significant effect on the neutrophils percentage ($p > 0.05$). Also, diclofenac caused an important reduction of neutrophils percentage in both the preventive ($p < 0.001$) and the therapeutic evaluation study ($p < 0.05$), the reduction being more important in the preventive study. The NAME treatment either before or after the induction of peritonitis was followed by a significant reduction of neutrophils, the rate of neutrophils percentage reduction being similar for both studies ($p < 0.05$).

In all studied groups leukocytes decrease was due to the reduction of neutrophils. This mechanism was expected because in acute inflammation the bone marrow is stimulated to release neutrophils.

As shown in Figure 1 and Figure 2, the induction of peritonitis was characterized by an important increase of the percentage of active phagocytes expressed as phagocytes index (PI) and of the phagocytes activity (PA). All the treatments administered through the preventive and the therapeutic evaluation study were able to significantly reduce the two parameters vs. peritonitis group ($p < 0.001$), but for vitamin E treated group the reduction was not statistically significant ($p < 0.05$). The most important reduction of the PI was obtained in the groups pretreated or treated with SOD liposomes. The PI values were lower for the groups that received SOD liposomes before or after the induction of peritonitis than for the peritonitis group. The effect of SOD liposomes was significantly higher than that of the free enzyme in the preventive study ($p < 0.001$). In the therapeutic evaluation study the SOD solution had a higher effect compared with the SOD liposomes on the PI ($p < 0.001$), and a similar effect on the PA ($p > 0.05$). Concerning the influence of SOD liposomes on the PA, the reduction of this parameter compared with the peritonitis group was also very important ($p < 0.001$), the PA for the liposomes group being close to that of the control group.

Diclofenac also caused a significant reduction of PI and PA vs. the peritonitis group ($p < 0.001$), but the effect was not as important as in the case of SOD liposomes and solution, and was higher in the preventive study. NAME and vitamin E had a better effect than diclofenac, and both were more effective in the preventive than in the therapeutic evaluation study.

By reducing ROS production in the phagocytes, SOD formulations decreased the rate of phagocytes activity and their activation. That is why SOD reduced the PI and PA. The results are positive also due to the fact that SOD effects are better than that of diclofenac.

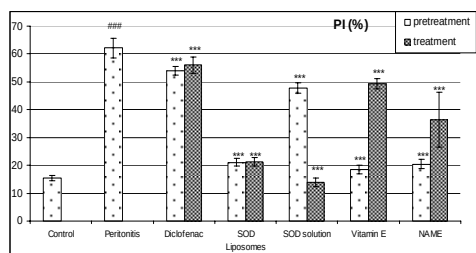


Figure 1

Phagocytes index (PI) for the preventive and therapeutic evaluation study. Data are expressed as mean $\pm$ SD (n=10). (***) $p < 0.001$ vs. peritonitis; ### $p < 0.001$ vs. control)

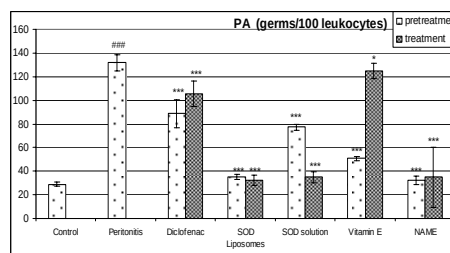


Figure 2

Phagocytes activity (PA) for the preventive and therapeutic evaluation study. Data are expressed as mean $\pm$ SD (n=10). (***) $p < 0.001$ vs. peritonitis; ### $p < 0.001$ vs. control)

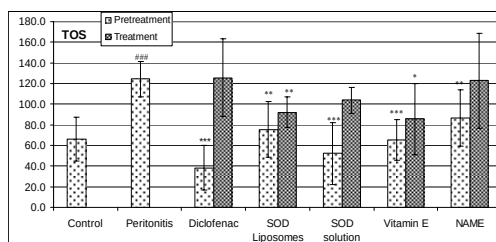
Total oxidant status, total antioxidant response and oxidative stress index

Figure 6 presents the total oxidant status (TOS) while Figure 7 shows the values for total antioxidant response (TAR). In the peritonitis group, the level of serum oxidant species expressed as TOS, significantly increased compared with the control group ($p < 0.001$). This increase of TOS was also accompanied by the reduction of serum TAR ($p < 0.001$).

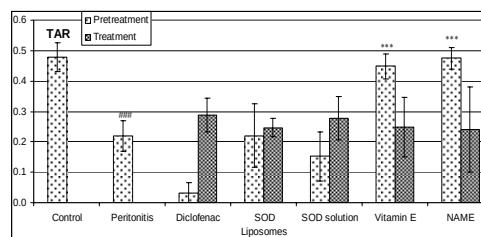
When our treatment was administered prior peritonitis to induction, all groups had significantly lower total peroxide levels compared with the peritonitis group ($p < 0.01$). For the preventive study, the most effective in reducing the TOS was diclofenac. SOD liposomes were less effective in reducing the TOS than SOD solution. In this study, the level of TOS reduction by liposomes was close to that obtained for the vitamin E group.

In the therapeutic evaluation study, only SOD liposomes ($p < 0.01$) and vitamin E ($p < 0.05$) reduced significantly the TOS. SOD solution was also effective but the difference vs. the peritonitis group was not significant. However, all treated groups had a higher TOS than in the preventive study.

Concerning the antioxidant reactivity, we expected an increase of the antioxidant potential associated with the reduction of the oxidative status, but the results were not as we expected. In the preventive study, only the groups that have received vitamin E and NAME had TAR significantly higher than the peritonitis group ($p < 0.001$). In the therapeutic evaluation study, the antioxidant reactivity was better for all treated groups, but the level of serum antioxidants was not significantly higher than that of the peritonitis group.

**Figure 3**

Total oxidant status expressed as total peroxide level ($\mu\text{mol H}_2\text{O}_2$ equiv. /L). Data are expressed as mean $\pm$ SD (n=10). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. peritonitis; #### $p<0.001$ vs. control)

**Figure 4**

Total antioxidant response against potent free radicals, expressed as Trolox equivalents (mmol Trolox equiv. /L). Data are expressed as mean $\pm$ SD (n=10). (*** $p<0.001$ vs. peritonitis; #### $p<0.001$ vs. control)

Table III

Oxidative stress index (OSI) for the preventive and the therapeutic evaluation study.

Data are expressed as mean $\pm$ SD (n=10)

	Control	Peritonitis	Diclofenac	SOD liposomes	SOD solution	Vitamin E	NAME
OSI pretreatment	13.9 $\pm$ 4	54.5 $\pm$ 15####	68.6 $\pm$ 15	34.9 $\pm$ 16*	82.9 $\pm$ 28	14.6 $\pm$ 4*	18.2 $\pm$ 5
OSI treatment			45.0 $\pm$ 15	38.3 $\pm$ 9*	40.0 $\pm$ 15	36.8 $\pm$ 16	69 $\pm$ 29

(* $p<0.05$ vs. peritonitis; #### $p<0.001$ vs. control)

OSI, expressed as the ratio of total peroxide to total antioxidant potential, appreciates the degree of oxidative stress. As shown in Table III, the induction of peritonitis was characterized by an important increase of the OSI levels ($p<0.001$) compared with control group. This suggested that an important oxidative damage was involved in the evolution of peritonitis. Not only the peritonitis group but all the groups treated presented an oxidative stress index significantly higher than the control group ($p<0.01$). However, in the preventive study, SOD liposomes ($p<0.05$) and vitamin E ($p<0.01$) were able to importantly reduce the OSI vs. the peritonitis group. The administration of control anti-inflammatory drug, diclofenac, did not significantly influence the OSI ($p>0.05$) vs. the peritonitis group. The groups treated with SOD liposomes either before or after the induction of peritonitis had an OSI significantly lower than the peritonitis group ($p<0.05$). Compared with the free form of SOD, the liposomes were much more effective in reducing the OSI. While in the therapeutic evaluation study the difference between the liposomes and SOD solution was not important, in the preventive study the OSI of the group treated with SOD solution was higher for the solution than for the liposomes ($p<0.01$), which reflects the higher ability of SOD liposomes to reduce serum oxidative status.

Plasma nitrite and nitrate

The nitrites/nitrates levels in serum were significantly higher in the peritonitis group compared with the control group (Figure 5), due to the induction of nitric oxide synthase iNOS which produces the nitric oxide (NO), during the inflammatory process. All the administered treatments reduced the nitrite/nitrate levels compared with the peritonitis group, but the reduction was not significant excepting the group treated with SOD encapsulated in liposomes. When SOD liposomes were administered either before or after the induction of peritonitis the nitrites/nitrates concentrations were significantly reduced compared with the peritonitis group ($p < 0.05$). We can also remark that nitrite/nitrate levels after the administration of SOD loaded liposomes were not significantly different compared with the levels of the control group ($p > 0.05$).

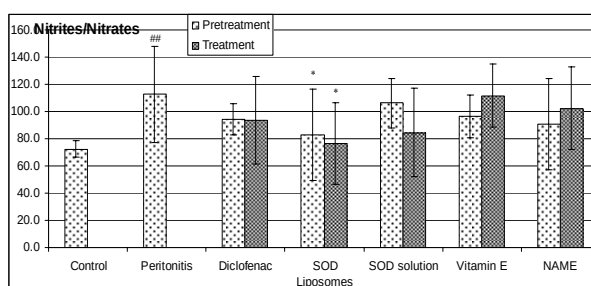


Figure 5
Nitrites/nitrates levels (μmol/ml) for the preventive and the therapeutic evaluation study. Data are expressed as mean $\pm$ SD (n=10). (* $p < 0.05$ vs. peritonitis; ## $p < 0.01$ vs. control)

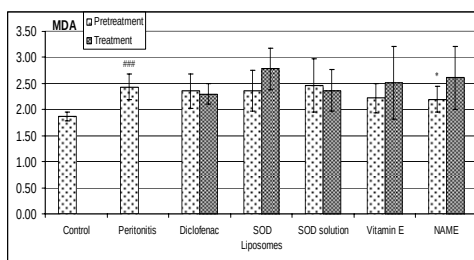
Knowing that SOD and NO act by competition on ROS, the increase of SOD will induce a ROS reduction and, by that, will diminish iNOS expression and the NO secretion. This explains the decrease of nitrite/nitrate serum levels after SOD treatment.

Malonaldehyde (MDA)

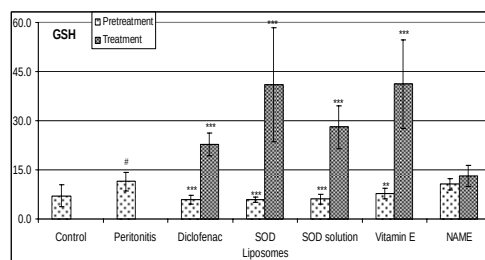
Lipid peroxidation, evaluated by the determination of malonaldehyde in serum, significantly increased when peritonitis was induced (Figure 6). The malonaldehyde serum levels were not significantly influenced by the treatments administered neither in the preventive nor in the therapeutic evaluation study, excepting the case of pretreatment with the inhibitor of nitric oxide synthesis, NAME ($p < 0.05$).

Plasma reduced glutathione

The values for plasma reduced glutathione (μmol/ml) for the preventive and the therapeutic evaluation study are presented in Figure 7.

**Figure 6**

Malonaldehyde serum concentrations (nmol/ml) in the preventive and therapeutic evaluation study. Data are expressed as mean $\pm$ SD (n=10). (* p <0.05 vs. peritonitis; ### p <0.001 vs. control)

**Figure 7**

Plasma reduced glutathione concentrations (μ mol/ml) for the preventive and the therapeutic evaluation study. Data are expressed as mean $\pm$ SD (n=10). (** p <0.01, *** p <0.001 vs. peritonitis; # p <0.05 vs. control)

In both studies, all the treatments administered, excepting NAME, significantly influenced plasma glutathione levels (p <0.05). In the peritonitis group, glutathione level was importantly higher compared with the control group (p <0.05). The increase of reduced glutathione level in the peritonitis group can be explained by the induction of the enzymes involved in glutathione synthesis after the exposure to oxidative stress associated with the induction of peritonitis. This induction have been previously shown both *in vivo* and *in vitro* studies on lung epithelial cells exposed to oxidative damage [23, 24], and probably the same phenomenon occurs during the induction of peritonitis. In the preventive study, the glutathione levels of the groups treated with diclofenac, SOD (either encapsulated in liposomes or free) and vitamin E groups were close to that of the control group, and significantly lower than that of the peritonitis group for diclofenac (p <0.001), SOD (p <0.001) and vitamin E (p <0.01). So we can conclude that the administration of enzymatic antioxidants, that reduce the level of reactive oxygen species, and the administration of diclofenac, that reduces nitric oxide synthesis, prior to peritonitis induction, can prevent glutathione synthesis. If the same treatments were administered after the peritonitis induction, glutathione levels increased significantly vs. the peritonitis group. These results may be explained by fact that the ROS were already released when our treatment was administered, and the over expression of glutathione synthesis has been initiated.

Conclusions

Our results provide evidence that superoxide dismutase can reduce the bone marrow acute phase response and the oxidative stress associated with the development of the inflammatory process in peritonitis.

In the preventive study, SOD liposomes had a significantly better influence than the SOD solution on total leukocyte count and neutrophils percentage, phagocytes index, total antioxidant reactivity, oxidative stress index and nitrite/nitrate serum levels.

In the therapeutic evaluation study, SOD liposomes were more effective than the solution in reducing the total oxidative status, the oxidative stress index and the serum nitrite/nitrate level. Also, total antioxidant reactivity was higher for the group treated with liposomes, but the differences were not significant. On the contrary, the SOD solution better reduced the total leukocyte number, the neutrophils and the phagocytes index.

The results showed that SOD liposomes are more effective than the solution in the prevention of the diseases characterized by high ROS levels, while the treatment did not show important differences between the two formulations.

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