Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmaceutics and Biopharmaceutics 60 (2005) 47–51
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

The effect of liposomes with superoxide dismutase on A2182 cells

Ružica Galović Rengel[a,*], Jelena Filipović-Grčić[b], Ivana Čepelak[a], Tihana Žanić-Grubišić[a], Karmela Barišić[a]

[a]Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia
[b]Department of Pharmaceutics, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

Received 8 June 2004; accepted in revised form 7 December 2004
Available online 18 January 2005

Abstract

Differently charged liposomes were examined for the efficiency of delivery of Cu/Zn superoxide dismutase (CuZnSOD) to human lung epithelial cells, A2182, and their prospects of cell protection from oxidative agents. A2182 cells were treated with cationic, neutral and anionic liposomes with encapsulated CuZnSOD. Untreated cells and cells pre-treated with liposome-encapsulated CuZnSOD were exposed to oxidative stress caused by xanthine/xanthine oxidase. Cellular antioxidant response was monitored for 4 or 24 h after the beginning of oxidative stress induced by the activity of superoxide dismutase (SOD) and total glutathione concentration. CuZnSOD-loaded liposomes increased the SOD activity of A2182 cells 24 h after treatment. The highest increase of cellular SOD, by 108%, was achieved using anionic liposomes. Neutral and cationic liposomes increased cellular SOD by 83 and 85%, respectively. Cationic liposomes were the most cytotoxic. Exposure of untreated cells to oxidative stress increased the cellular glutathione level after 24 h. Cells pre-treated with liposome-encapsulated CuZnSOD were protected from oxidative stress, as shown by the unchanged concentration of cellular glutathione.

Keywords: A2182 cells; Glutathione; Liposomes; Superoxide dismutase; Oxidative stress

1. Introduction

Respiratory epithelium, often exposed to environmental and pathophysiological oxidants, is an important target for antioxidant therapy. Superoxide dismutase (SOD), the enzyme catalyzing the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide, is a potential antioxidant therapeutic agent [1]. SOD is found abundantly in many organisms, from microorganisms to plants and animals. Three SOD types have been found in mammalians: cytoplasmic CuZnSOD, extracellular EcSOD (both containing copper and zinc in their active sites) and mitochondrial MnSOD (manganese in the active site) [2].

However, the results of animal and clinical studies of the therapeutic usage of SOD showed only a modest protective effect against oxidative stress attributable to inadequate delivery of the enzyme to the sites of therapeutic action [1]. Therefore, an appropriate drug delivery system, which could protect the enzyme against inactivation, prolong its circulation lifetime and attain a more effective site-directed delivery, would be desirable. Liposome-encapsulated antioxidant enzymes enter cells in the active form, either by fusion with plasma membranes or by endocytosis, and render the treated cells more resistant to oxidants [3]. Liposome-encapsulated SOD, injected intravenously and subcutaneously into a rat model of rheumatoid arthritis [4,5] or instilled into the reactive oxygen-injured airway of adult rabbit [6], appears to be an effective way of protecting the tissue from oxidative damage. In in vitro experiments, liposome-encapsulated SOD showed also protective effects on the cells exposed to oxidants [3,7]. Nevertheless, certain types of liposomes could be toxic to the cells [7–9].

We have previously shown that SOD could be efficiently encapsulated into differently charged (cationic, neutral and anionic) liposomes [10]. Hereby we wanted to examine these liposomes for in vitro toxicity, efficiency of SOD

* Corresponding author. Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, P.O. Box 156, 10000 Zagreb, Croatia. Tel.: +385 1 4612606; fax: +385 1 4612716.
E-mail address: ruzica.galovic@ka.htnet.hr (R.G. Rengel).

0939-6411/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2004.12.002

delivery to the lung epithelial cells and its prospects for cell protection from oxidative agents.

2. Materials and methods

2.1. Chemicals

Soybean natural phosphatidyl choline (SPC) and soybean natural phosphatidyl glycerol (SPG) were obtained from Lipoid GmbH, Germany. Bovine erythrocytes CuZnSOD, stearylamine (SA), Triton X-100, xanthine (X) and xanthine oxidase (XO) were purchased from Sigma Chemical Company, USA. All other chemicals were of analytical grade and were purchased from Kemika, Croatia.

2.2. Liposome preparation

Positively charged, cationic (CatL), neutral (NeuL) and negatively charged, anionic (AnL) liposomes were prepared by the proliposome method as previously described [10]. Briefly, a mixture of lipids was dissolved in ethanol, an aqueous solution of CuZnSOD was added, and then the proliposome mixture was stirred while the phosphate buffered solution (PBS) was added dropwise to form the liposome suspension. Concentrations of phospholipids (16 mM) and CuZnSOD (0.5 mg/ml) were constant in all preparations. The lipid composition of the obtained liposomes and the molar ratio of lipids used for the preparation of liposomes were: SPC only, for NeuL; SPC:SA = 9:1 for CatL; and SPC:SPG = 7:3 for AnL. The prepared NeuL, CatL and AnL entrapped SOD with high efficiencies: 39, 60 and 65% of the initial enzymatic activity, respectively [10]. Empty liposomes were prepared without CuZnSOD in the aqueous solution. The produced vesicles were large and multilamellar (mean diameter 0.640 ± 0.532 μm), with wide size distributions. In order to obtain smaller liposomes with a homogenous size distribution, liposomal preparations were extruded once through 400 nm polycarbonate membrane filters (LiposoFast, Canada). Extruded liposomes retained more than 90% of the originally encapsulated CuZnSOD and they were uniformly dispersed, with a mean diameter 0.283 ± 0.157 μm [10]. Liposome-entrapped CuZnSOD was separated from the unentrapped enzyme by centrifugation at $113{,}000 \times g$ for 45 min at 4 °C in a Beckman Optima LE-80-K ultracentrifuge, washed twice in a 0.9% NaCl solution and resuspended in a cell culture medium.

2.3. Cell culture

A2182, the human lung carcinoma cell line, mycoplasma free, was maintained in continuous culture at 37 °C with 5% CO_2 in Dulbeecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), with addition of penicillin, streptomycin and amphotericin B. The cell culture medium was purchased from Gibco Invitrogen Corporation, UK.

2.4. Cell exposure to liposome-treatment and/or oxidative stress

Monolayers of confluent A2182 cells were prepared by seeding 0.5×10^6 cells/well in a six-well plate and their reculturing in DMEM with 10% FBS for 24 h. Confluent monolayers were rinsed two times with PBS, test suspensions (2 ml) of native (liposome-free) CuZnSOD, CuZnSOD-loaded liposomes or empty liposomes in DMEM without FBS were added, and the plates were reincubated for 24 h. The amounts of liposomes used for the treatment were determined according to the activity of entrapped CuZnSOD (CuZnSOD loaded liposomes) or as a quantity of liposomal suspension (empty liposomes). Control cells were also rinsed two times with PBS, DMEM medium (without FBS) was added, and the plates were reincubated for 24 h.

For oxidative stress, cells were washed three times with PBS and exposed to various concentrations of xanthine/xanthine oxidase (X/XO): 50 μM/2.5 mU/ml, 50 μM/5.0 mU/ml, 50 μM/10.0 mU/ml in DMEM (without FBS). Cellular SOD activity and total glutathione were studied 4 or 24 h after the beginning of induced oxidative stress. At the end of the treatment, the cell culture medium was removed and used for measurement of the lactate dehydrogenase activity (LDH); the cells were then washed with PBS, detached with trypsin-EDTA and centrifuged at $500 \times g$ for 5 min at 4 °C. Finally, the cell pellet was resuspended in PBS.

An aliquot of cells was diluted with trypan blue, counted in a hemocytometer, and, based on the number of viable and dead cells, the cell viability was calculated. Cell membrane integrity was estimated by measuring the catalytic activity of lactate dehydrogenase in cell lysate as well as in culture medium [11].

2.5. Determination of SOD activity

The liposome-encapsulated SOD activity and cellular SOD activity were determined in cell lysate. Cells were lysed with Triton X-100 in a final concentration of 0.5%. Cellular SOD activity was calculated by dividing the total activity by the number of cells.

The SOD activity assay was based on the ability of the enzyme to convert superoxide radicals, produced by X/XO, to hydrogen peroxide, inhibiting indicator oxidation and colour formation (RANSOD test kit, Randox Laboratories Ltd, UK). The possible influence of detergent on the SOD activity determination was compensated by using the SOD standard dissolved in 0.5% Triton X-100.

2.6. Determination of total glutathione concentration

Total glutathione (GSH) concentration was assayed in the supernatant obtained after deproteinisation of the total

cell homogenate with 10% metaphosphoric acid (cell lysate:metaphosphoric acid = 1:1, v/v). The sulphydryl group of GSH reacts with 5,5′-dithio-bis(2-nitrobenzioc acid) in the glutathione reductase recycling method [12]. GSH concentration per cell was calculated by dividing the total concentration by the number of cells.

2.7. Statistical analysis

The results are expressed as the mean ± SD. Significance of the differences between experimental groups was tested using the analysis of variance, followed by the Dunnet or Tukey multiple comparison procedure. Statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Toxicity of liposomes

In this study, we used differently charged liposomes that encapsulated CuZnSOD with high efficiency [10]. A2182 cells were exposed to various amounts of AnL, NeuL and CatL with entrapped CuZnSOD. The amounts of liposomes used for the treatment were 10, 20, 50 and 100 U CuZnSOD/ml of liposomal suspension. Cells were treated with the same concentrations of native CuZnSOD or with the same amounts of empty liposomes. Control cells were grown without any treatment. AnL were not toxic to A2182 cells. They caused no change in the viable cell number (Fig. 1A) or LDH release (Fig. 1B). Higher amounts of NeuL aggregated and settled on the cells, so NeuL with 50 and 100 U/ml CuZnSOD caused small, but not significant decreases in the number of cells (Fig. 1A), and no change in LDH release (Fig. 1B). CatL showed limited cytotoxicity at a concentration of 20 U/ml CuZnSOD, with no significant change in the number of cells (Fig. 1A), but with a significant increase in LDH release (Fig. 1B). Higher concentrations of CatL caused significant cytotoxicity (Fig. 1A and B).

A2182 cells were also treated with the same quantities of empty AnL, NeuL, and CatL with the same outcomes: AnL were non toxic, depletion of cell growth appeared in the cultures treated with NeuL due to liposomal aggregates that settled on the cells, and CatL were significantly cytotoxic (data not shown). Therefore, it was not CuZnSOD but liposome compositions that were responsible for the NeuL and CatL side effects.

Toxicity of cationic liposomes has been reported in the literature [8]. It was shown that among differently charged liposomes only cationic liposomes induced inflammation in mouse lung. In another study [9], in vitro experiments showed that cationic stearylamine liposomes induced apoptosis in WEHI 231 cells, while anionic and neutral liposomes did not cause this effect.

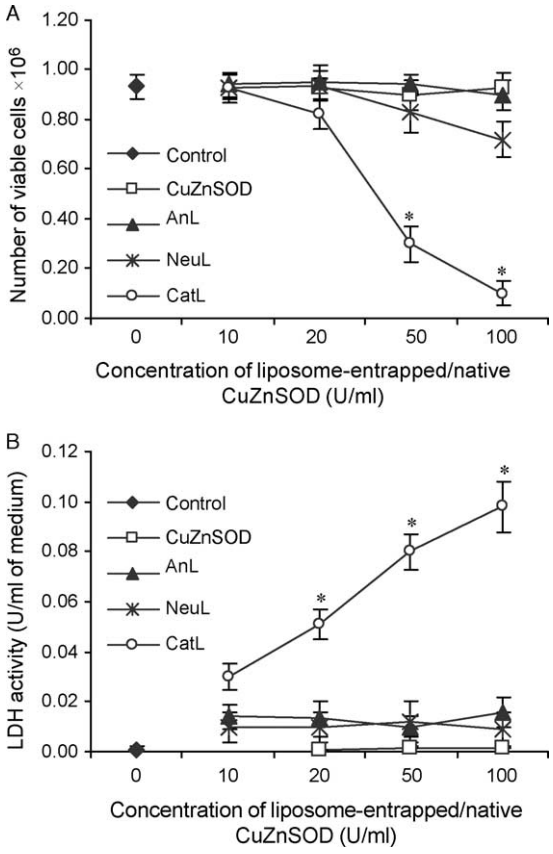

Fig. 1. Cytotoxic effects of differently charged liposomes with CuZnSOD on A2182 cells. (A) The number of viable cells and (B) LDH activity of the cells treated with different concentrations of native CuZnSOD and AnL, NeuL, CatL-entrapped CuZnSOD for 24 h. Each symbol is the mean ± SD of six experiments. *Significant difference compared to control value ($P < 0.05$).

3.2. Activity of SOD

The ability of different types of liposomes to deliver SOD to the cells was tested. For this purpose, cell cultures were exposed to AnL, NeuL and CatL containing 20 U CuZnSOD/ml of liposomal suspension or to native CuZnSOD for 24 h. The results are summarized in Fig. 2.

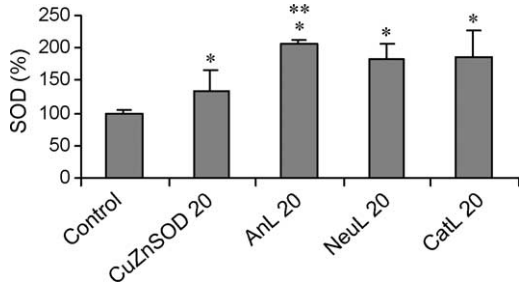

Fig. 2. Effect of 20 U/ml native CuZnSOD and AnL, NeuL, CatL-entrapped CuZnSOD on relative cellular SOD activity of A2182 cells after 24 h of treatment. The activity of control cells was set at 100%. Each bar is the mean ± SD of four experiments. *Significant difference compared to control value ($P < 0.05$). **Significant difference compared to native CuZnSOD ($P < 0.05$).

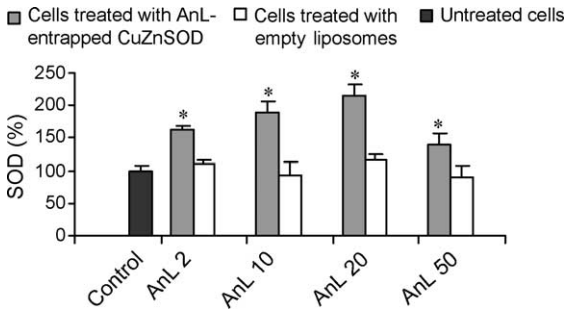

Fig. 3. Effect of 2, 10, 20 and 50 U/ml of AnL-entrapped CuZnSOD and adequate quantities of empty liposomes on relative cellular SOD activity of A2182 cells after 24 h of treatment. The activity of control cells was set at 100%. Each bar is the mean ± SD of six experiments. *Significant difference compared to the control value ($P < 0.05$).

The activity of SOD of untreated cells was 2.4 ± 0.3 U/10^6 cells ($n = 12$). The highest increase, by 108%, was detected in the cells treated with AnL. SOD activity in the cells treated with NeuL and CatL with entrapped CuZnSOD increased by 83 and 85%, respectively (Fig. 2). We found that native CuZnSOD was able to enter the cells and increase SOD activity by 35% compared to SOD activity in control cells.

In subsequent experiments, the cells were treated with AnL only, because of their highest ability to deliver entrapped CuZnSOD to the cells. On the other hand, AnL were not toxic to the cells with regard to cell viability and LDH leakage. We examined the increase in SOD within the cells due to their 24 h-treatment with different amounts of AnL, empty or containing 2, 10, 20, and 50 U CuZnSOD/ml liposomal suspension. We found that AnL with entrapped 20 U CuZnSOD/ml liposomal suspension were the most potent in increasing SOD activity within the cells, as shown in Fig. 3. This increase could not be attributed to SOD induction by liposome particles without the enzyme (Fig. 3). Our results are in agreement with the previously reported results of some authors, who successfully delivered catalase to lung cells [13], and catalase and SOD to the lungs of newborn rats [7]. In both mentioned cases, cationic stearylamine liposomes were used. However, the use of anionic liposomes for successful delivery of SOD and catalase to lung cells has also been reported [3].

3.3. Exposure of A2182 cells to oxidative stress

Capacity of mammalian cells to maintain homeostasis of cellular functions during oxidative stress depends on rapid induction of protective antioxidant enzymes [14]. We addressed the question whether SOD delivered to A2182 cells by AnL could protect the cells against oxidative stress.

As previously reported, in vivo experiments of administration of SOD-loaded liposomes demonstrated a good protective effect on the rat model of rheumatoid inflammation [4,5]. However, administration of liposomal SOD and catalase to the lungs showed miscellaneous results.

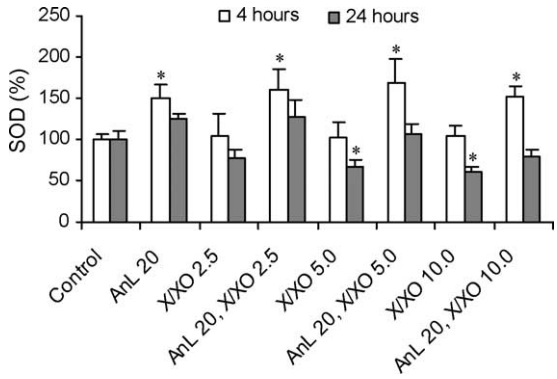

Fig. 4. Relative SOD activity of A2182 cells, untreated and treated with AnL with encapsulated CuZnSOD (20 U/ml medium) for 24 h, exposed to oxidative stress caused by xanthine/xanthine oxidase (X/XO). Adequate cells were exposed to X/XO (50 μM/2.5 mU/ml, 50 μM/5.0 mU/ml, 50 μM/10.0 mU/ml). Four or 24 h after the beginning of induced oxidative stress, the cells were harvested and SOD activity was measured. The activity of control cells was set at 100%. Each bar is the mean ± SD of three experiments. *Significant difference compared to the control value ($P < 0.05$).

Certain authors reported the results where only catalase seemed to be effective in protection from oxidative damage [7,13], while others suggested that both antioxidant enzymes, SOD and catalase, were equally effective in the protection from active oxygen [3,6].

In the cited literature, antioxidative effects of delivered antioxidant enzymes were evaluated on the basis of the regression of oedema in the inflammation model [4,5] or according to survival after exposure to oxidative stress in the lung model [7]. In in vitro experiments [3,13], antioxidant protection of SOD and catalase was evaluated according to the decrease of cytotoxicity by decreased LDH release. In our experiments, the protective effect of

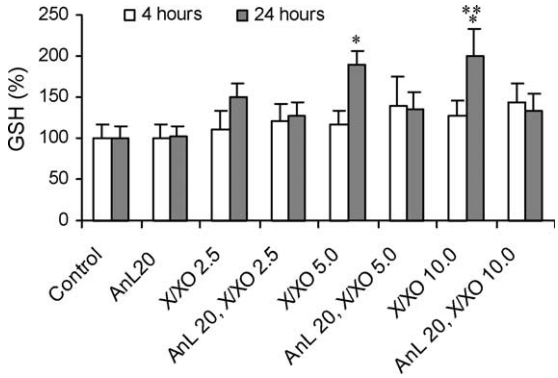

Fig. 5. Relative glutathione amount of A2182 cells, untreated and treated with AnL-encapsulated CuZnSOD (20 U/ml medium) for 24 h, exposed to oxidative stress caused by xanthine/xanthine oxidase (X/XO). Adequate cells were exposed to X/XO (50 μM/2.5 mU/ml, 50 μM/5.0 mU/ml, 50 μM/10.0 mU/ml). Four or 24 h after the beginning of induced oxidative stress, the cells were harvested and GSH concentration was measured. Each bar is the mean ± SD of three experiments. *Significant difference compared to the control value ($P < 0.05$). **Significant difference compared to cells treated with AnL 20 and then exposed to the same oxidative stress (X/XO 10 vs. AnL 20, X/XO 10) ($P < 0.05$).

the delivered SOD was examined by measuring the concentration of GSH, a ubiquitous cellular nonprotein antioxidant. It has been reported that exposure to oxidants produced an elevation in GSH levels [14,15].

Untreated and treated A2182 cells (with AnL-entrapped 20 U CuZnSOD/ml) were exposed to oxidative stress caused by xanthine/xanthine oxidase (X/XO) (50 μM/2.5 mU/ml, 50 μM/5.0 mU/ml, 50 μM/10.0 mU/ml), an O_2^- generating system. We studied the SOD activity and GSH amount of untreated and treated cells 4 and 24 h after the beginning of induced oxidative stress. Four hours after exposure to all concentrations of X/XO, there was no change in the cellular SOD activity of untreated cells compared to control cells (Fig. 4). Cells pre-treated with AnL-entrapped CuZnSOD kept the obtained increased cellular activity of SOD (Fig. 4). Four hours after exposure to X/XO, no change in cellular GSH concentration was observed (Fig. 5).

After 24 h, the untreated cells exposed to X/XO (50 μM/5.0 mU/ml, 50 μM/10.0 mU/ml) decreased the SOD activity compared to the control cells (Fig. 4). Decrease in the SOD activity could be explained by the inactivation of CuZnSOD through interaction with excessive hydrogen peroxide produced by superoxide dismutation [16,17]. This effect could probably not be seen 4 h after the beginning of induced oxidative stress because of shorter exposure time to X/XO. Proteolytic degradation of the excessive SOD is also a possible explanation for the decrease of SOD activity after 24 h. However, 24 h of exposure to X/XO affected GSH in such a manner that GSH levels increased by 50–100% (Fig. 5). In the cells first treated with AnL-entrapped CuZnSOD, exposed to X/XO and monitored after 24 h, SOD activity was the same as that detected in the control cells (Fig. 4). We also found that GSH levels were not changed in the cells pre-treated with AnL-entrapped CuZnSOD and then exposed to X/XO (Fig. 5). The difference in GSH concentration between the cells pre-treated with AnL-entrapped CuZnSOD and the cells exposed only to X/XO reflects the protective action of CuZnSOD delivered to the cells by AnL.

Elevation of GSH levels in the cells exposed to X/XO (without pre-treatment with AnL-entrapped CuZnSOD) is probably caused by the induction of enzymes for GSH synthesis after longer exposure to oxidative stress and insufficient removal of reactive oxygen species by dismutation [14]. In contrast, no elevation of GSH levels was observed in the cells pre-treated with liposomal CuZnSOD, because CuZnSOD efficiently dismutated $O_2^{-\cdot}$.

In conclusion, the presented results demonstrate that negatively charged liposomes prepared of SPC and SPG can be used for efficient delivery of CuZnSOD to A2182 cells, thus increasing their antioxidative strength. Using the induction of GSH as a sensor for oxidatively stressed cells, we have confirmed that liposome-delivered SOD to the lung cells could protect the cells from oxidative damage, as reported previously on different experimental models [3,6].

Acknowledgements

This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia, grants 0006631 and 006251.

References

[1] V.R. Muzykantov, Delivery of antioxidant enzyme proteins to the lung, Antioxid. Redox Signal. 3 (2001) 39–62.
[2] I. Fridovich, Superoxide dismutases, Annu. Rev. Biochem. 44 (1975) 147–159.
[3] F.J. Walther, R. David-Cu, M.C. Supnet, M.L. Longo, B.R. Fan, R. Bruni, Uptake of antioxidants in surfactant liposomes by cultured alveolar type II cells is enhanced by SP-A, Am. J. Physiol. 265 (1993) L330–L339.
[4] M.L. Corvo, M.B.F. Martinis, A.P. Francisco, J.G. Morais, M.E.M. Cruz, Liposomal formulations of Cu, Zn-superoxide dismutase: physico-chemical characterization and activity assessment in an inflammation model, J. Control. Release 43 (1997) 1–8.
[5] M.L. Corvo, O.C. Boerman, W.J.G. Oyen, J.C.S. Jorge, M.E.M. Cruz, D.J.A. Crommelin, G. Storm, Subcutaneous administration of superoxide dismutase entrapped in long circulating liposomes: in vivo fate and therapeutic activity in an inflammation model, Pharm. Res. 17 (2000) 600–606.
[6] M.L. Barnard, R.R. Baker, S. Matalon, Mitigation of oxidant injury to lung microvasculature by intratracheal instillation of antioxidant enzymes, Am. J. Physiol. 265 (1993) L340–L345.
[7] A.K. Tanswell, B.A. Freeman, Liposome-entrapped antioxidant enzymes prevent lethal O_2 toxicity in the newborn rat, J. Appl. Physiol. 63 (1987) 347–352.
[8] S. Dokka, D. Toledo, X. Shi, V. Castranova, Y. Rojanasaku, Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes, Pharm. Res. 17 (2000) 521–525.
[9] Y. Aramaki, S. Takano, H. Arima, S. Tsuchiya, Induction of apoptosis in WEHI 231 cells by cationic liposomes, Pharm. Res. 17 (2000) 515–520.
[10] R. Galović Rengel, K. Barišić, Ž. Pavelić, T. Žanić Grubišić, I. Čepelak, J. Filipović-Grčić, High efficiency entrapment of superoxide dismutase into mucoadhesive chitosan-coated liposomes, Eur. J. Pharm. Sci. 15 (2002) 441–448.
[11] J.A. Cook, J.B. Mitchell, Viability measurements in mammalian cell systems, Anal. Biochem. 179 (1989) 1–7.
[12] F. Tietze, Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissue, Anal. Biochem. 27 (1969) 502–522.
[13] B.J. Buckley, A. Tanswell, B. Freeman, Liposome-mediated augmentation of catalase in alveolar type II cells protects against H_2O_2 injury, J. Appl. Physiol. 63 (1987) 359–367.
[14] I. Rahman, A. Bel, B. Mulier, K. Donaldson, W. MacNee, Differential regulation of glutathione by oxidants and dexametasone in alveolar epithelial cells, Am. J. Physiol. 275 (1998) L80–L86.
[15] K. Miura, T. Ishii, Y. Sugita, S. Bannai, Cystine uptake and glutathione level in endothelial cells exposed to oxidative stress, Am. J. Physiol. 262 (1992) C50–C58.
[16] E.K. Hodgson, I. Fridovich, The interaction of bovine superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation, Biochemistry 14 (1975) 5299–5303.
[17] J.C. Koningsberger, B.S. van Asbeck, E. van Fassen, L.J.J.M. Wiegman, J. van Hattum, G.P. van Berge Henegouwen, J.J.M. Marx, Copper, zinc-superoxide dismutase and hydrogen peroxide: a hydroxyl radical generating system, Clin. Chim. Acta 230 (1994) 51–61.