

Influence of Zymosan A on the Content of Ascorbic Acid in Mice

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Abstract

The aim of the study was to determine the effects of single intraperitoneal injections of zymosan A on changes in the content of ascorbic acid (ASC) in the brain, liver, spleen and kidneys of mature male mice, line Swiss. The experiments were carried out on 54 mice divided into 3 control groups and 6 experimental groups. Samples for analysis were collected after 3 h (experimental group I), 6 h (experimental group II) and 24 h (experimental group III) after the injection of zymosan A at the dose of 1 mg/kg body weight (b.w.). For groups IV, V and VI, the organs were removed at the same time as the previous groups, but the animals were administered zymosan A at the dose of 100 mg/kg b.w. The content of ASC was then determined. The results showed that zymosan A significantly reduced the content of ASC in the brain of the mice in all the experimental groups, in the spleen in all the experimental groups except for group I (after 3 h since injection of zymosan A at 1 mg/kg b.w.), in the liver only in experimental groups IV, V and VI (after the injection of zymosan A at 100 mg/kg b.w.), while in the kidneys the effects were observed for groups III, V and VI. The data suggest that the observed decrease in the content of ASC is caused by the oxidative activity of zymosan A.

Key Words: inflammation, mouse, reactive oxygen species, ascorbic acid, zymosan A

Introduction

Zymosan A, a constituent of the cell wall of the yeast (*Saccharomyces cerevisiae*) causes acute inflammation through the induction of various cytokines, pro-inflammatory lipid intermediates and also free radicals (9, 17, 31, 42). The inflammatory reaction is accompanied by an increase in the level of nitrite, nitrate, in the activity of myeloperoxidase and an increase in the levels of nitrotyrosine (5, 6, 13) and malondialdehyde and 4-hydroxyalkenals in the liver, kidneys, spleen and small intestine of rats (10, 33).

The main role in the inflammatory process is attributed to reactive oxygen species (ROS); superoxide radical anions, hydrogen peroxide, hydroxide radical and singlet oxygen, and also to nitric oxide (NO) and peroxynitrite (39, 40), because a positive correlation

was shown between the intensity of the inflammatory process and the amount of production of ROS (26). Reactive nitrogen species and ROS also play positive and important roles in the process of cellular communications. The maintenance of certain levels of radicals in cells is necessary for information flow between cells and the inside of cells. The suppression of the presence of free radicals or the increase in their levels may to some extent disturb the cellular communications. Even slight fluctuations in the basic level of ROS significantly affect changes in cellular metabolism, expression of genes and posttranslational modification of proteins (1).

Increased gene expression of important enzymes such as mitochondrial superoxide dismutase (MnSOD) and inducible nitric oxide synthase (iNOS) and protein kinase MAPK activation affects many cellular func-

tions including growth, proliferation and adaptation. ROS serves as communication molecules, activates adaptive responses through effects on signaling pathways in the cell to maintain redox homeostasis (15, 20).

The control of the amount of endogenous ROS involves enzymes of the antioxidant system as well as uric acid, glutathione, vitaminum E and ascorbic acid (ASC) (36, 41). In the inflammatory process, ASC plays a special role because of its wide spectrum of different ROS quenching and also because it participates in the regeneration of other low-molecular-weight antioxidants (16, 29). The significant role of ASC in the inflammatory process caused by the administration of zymosan A can be shown through changes in its content and this was the aim of the present study.

Materials and Methods

All experiments were carried out on 54 (4-month-old) male Swiss mice weighing 27 grams. The animals were housed at room temperature ($20 \pm 2^\circ\text{C}$) under a standard 12-h light/dark cycle (lights on at 8.00 am) with free access to standard laboratory food (LSM, Motycz near Lublin, Poland) and tap water. In the experiments, the mice were divided into three control groups ($n = 18$) and six experimental groups ($n = 36$). Mice of all control groups received intraperitoneally 0.3 ml of 0.9% NaCl solution (Polfa, Cracow, Poland) at 8.00 am (the beginning of the light phase L of the cycle LD 12:12). Experimental animals of groups I, II and III received intraperitoneally zymosan A (Sigma, St. Louis, MO, USA) at the dose of 1 mg/kg body weight (b.w.) dissolved in 0.9% NaCl solution at the same time of the day, *i.e.* at 8.00 am. Mice of groups IV, V and VI received intraperitoneally zymosan A at the dose of 100 mg/kg b.w., at 8.00 am. In the experiment at the doses of applied zymosan A and after 24 h, there was an 8% mortality rate in the animals. However, when very high doses of zymosan A (500 mg/kg b.w.) were administered, weight loss and an increase in the mortality rate of the animals have been reported (8, 12). Both control and treated animals were anaesthetized by an intramuscular injection of vetbutal (Biowet, Poland) at the dose of 35 mg/kg b.w., dissolved in 0.9% NaCl (Polfa), 3, 6 and 24 h after the injection of zymosan A. The organs under investigation, that is, the liver, spleen and kidneys, were quickly removed and ~100 mg of each tissue was homogenized in 2 ml 10% trichloroacetic acid (TCA; Sigma). The whole brain was extracted and weighed, and was homogenized in 2 ml 10% TCA. The brain homogenate was diluted to 100 mg of tissue per 2 ml of 10% TCA. Homogenates of all investigated organs were centrifuged in a MPW-365 centrifuge at $2.000 \times g$ for 10 min.

In the supernatants of the brain, liver, spleen and kidneys obtained after the centrifugation the

amount of ASC was determined according to the method of Omaye *et al.* (28). The following solutions were added to 1 ml of the supernatant: 0.4 ml phosphoric acid (V) (Sigma) made by dissolving of 50 ml of the acid in 50 ml of redistilled water, 0.4 ml of 4% solution in ethanol of 2,2'-dipyridyl (Sigma) and 0.2 ml of 3% solution of FeCl_3 (III) (Sigma). After the mixing of the content, the test tubes were placed in a water bath at 37°C for 1 h. In this method, oxidoreducing properties of ASC were utilized. In the acidic environment, ASC reduces the iron ion (III) Fe^{3+} to the iron ion (II) Fe^{2+} which is, next, bound to 2,2'-dipyridyl and forms a chelate complex with it which shows a characteristic absorption at $\lambda = 525$ nm. The measurements of the absorption were done with a spectrophotometer Marcel S330 (Marcel Sp. z o.o., Warsaw, Poland). The content of ASC was read from the calibration curve obtained on the basis of solutions of different concentrations of a standard solution of ASC (Sigma). The concentration of ASC in tissue extracts was expressed in $\mu\text{g}/100$ mg tissues. The mean and standard deviation of the ASC concentrations of the studied organs were calculated for each control and experimental group of mice. Percentage changes in the ASC content were also calculated by comparison of an experimental group with the relevant control group.

In order to confirm statistical significance in changes in the concentration of ASC in the studied organs after the injection of zymosan A at the dose of 1 mg/kg b.w., and separately at the dose of 100 mg/kg b.w., within the analyzed periods of time (*i.e.* 3, 6 and 24 h after injections) for the given experimental groups, analysis of variance (ANOVA) test was applied. Results were analyzed by two-way ANOVA and Tukey's *post-hoc* test. All statistical calculations of the data obtained during experiments were done with the computer program STATISTICA 9 (StatSoft, Cracow, Poland).

Results

The results concerning the content of ASC in the brain, liver, spleen and kidneys of mice which were administered intraperitoneally zymosan A at the dose of 1 or 100 mg/kg b.w. are presented in Figs. 1-4. In comparison with the control values, the administration of zymosan A at the dose of 1 mg/kg b.w. caused a statistically significant decrease in the concentration of ASC in the brain in all analyzed periods of time, *i.e.* after 3, 6 and 24 h after the injection. The decrease in the brain was 12.43% after 3 h, 21.43% after 6 h and 27.56% after 24 h (Fig. 1). In the spleen, a statistically significant decrease in the content of ASC was found only after 6 h (20.93%) and 24 h (10.43%) after the injection of zymosan A (Fig.

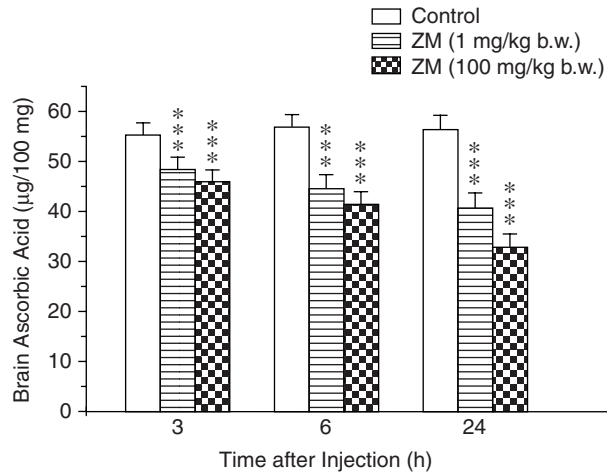


Fig. 1. The content of ascorbic acid (in µg/100 mg tissue) in the brain of 4-month-old control male mice, line Swiss and experimental ones 3, 6 and 24 h after the intraperitoneal injection of zymosan A (ZM) at the dose of 1 and 100 mg/kg b.w. ***indicates statistically significant at $P < 0.001$.

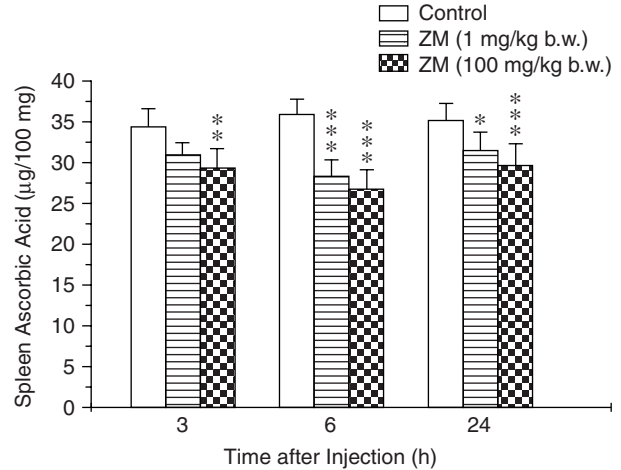


Fig. 3. The content of ascorbic acid (in µg/100 mg tissue) in the spleen as in the experiment in Fig. 1. *** $P < 0.001$; ** $P < 0.01$ and * $P < 0.05$.

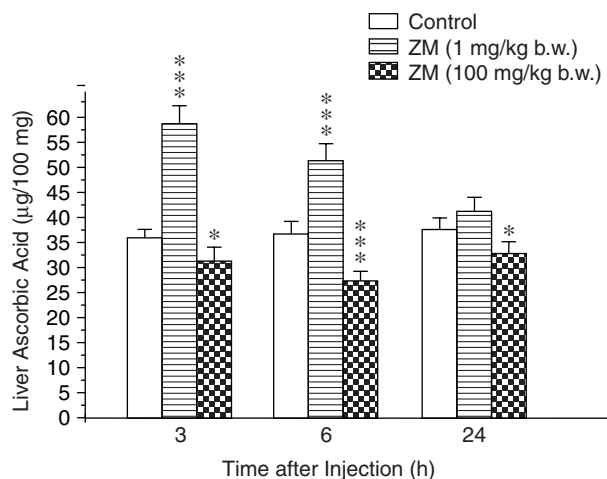


Fig. 2. The concentration of ascorbic acid (in µg/100 mg tissue) in the liver. The experiment was as in Fig. 1. ***indicates statistically significant at $P < 0.001$ and *indicates $P < 0.05$.

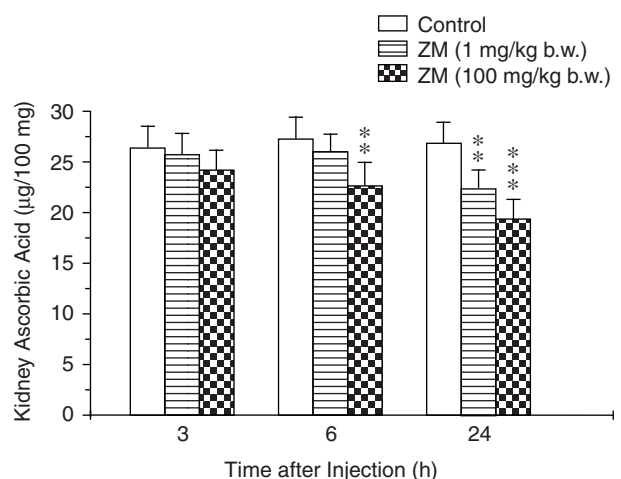


Fig. 4. The concentration of ascorbic acid (in µg/100 mg tissue) in the kidneys as in Fig. 1. *** $P < 0.001$ and ** $P < 0.01$.

3). For the kidneys, a statistically significant decrease (16.37%) in the ASC content was found only 24 h after the zymosan A injection (Fig. 4). In the liver, a significant increase in the concentration of ASC was found 3 and 6 h after the injection of zymosan A at the dose of 1 mg/kg b.w. The increase was 62.49% after 3 h and 39.28% after 6 h. After 24 h, the increase in the content of ASC (9.54%) was not statistically significant (Fig. 2).

The administration of zymosan A at the dose of 100 mg/kg b.w. resulted in a statistically significant decrease in the concentration of ASC in the brain, liver

and spleen 3, 6 and 24 h after the beginning of the experiment (Figs. 1-3), while in the kidneys it was after 6 and 24 h (Fig. 4). The decrease of 41.38% was the highest in the brain after 24 h (Fig. 1); in the liver, the decrease was 25.09% after 6 h (Fig. 2), in the spleen, the decrease was 25.35% after 6 h (Fig. 3), and in the kidneys, the decrease was 27.6% after 24 h (Fig. 4).

Two-way ANOVA in all the groups showed that these changes in the ASC concentrations are statistically significant in the brain ($F_{2,45} = 44.241$, $P = 0.00000$), liver ($F_{2,45} = 22.878$, $P = 0.00000$), spleen ($F_{2,45} = 4.376$, $P = 0.01834$) and kidneys ($F_{2,45} =$

12.202, $P = 0.00006$).

Discussion

In the reported experiments, it was found that the administration of zymosan A resulted, generally, in statistically significant decreases in the content of ASC in the brain, kidneys, spleen and liver of mice (with the exception of the dose of zymosan A of 1 mg/kg b.w.). Zymosan A-induced shock has been associated with an increased production pro-inflammatory cytokines and mediators, tumor necrosis factor alpha (TNF- α), interleukin 2 (IL-2) and interferon gamma (INF- γ) causing a generalized dysfunction of liver, lung and kidneys (8) due to the disrupted endothelia layer integrity of these organs (7, 30). Inflammatory cytokines also cause alterations in the permeability of the brain blood barrier and hence allowing inflammatory cell migration and the occurrence of oxidative stress in the brain (34).

In the examined organs in this study, the action of zymosan A might have depended on various factors, among others on individual susceptibility, but mostly on the dosage. Many drugs administered at appropriate doses could become medicinal. The same compounds administered at higher doses may cause dangerous poisoning as pointed out by the German alchemist Paracelsus (4).

Higher levels of ASC after the administration of low dose of zymosan A was observed. This suggests that low doses of zymosan A actually stimulate the synthesis of ASC in the examined organs of mice. The highest increase in this synthesis was observed in the liver. The liver is the site of ASC synthesis in mammals. Increased concentrations of ASC in the studied organs of mice after administration of small doses of zymosan A can be explained as an adaptive response to overproduction of proinflammatory cytokines and ROS. ASC is an essential nutrient for some vertebrates, such as humans, other primates and guinea pigs, which lack L-gulonolactone oxidase, the final enzyme in the pathway of ASC synthesis from glucose (21, 23, 27). ASC is present in many organs of humans and other animals. The highest concentration of ASC has been found in adrenal and pituitary glands, and in a little lower concentration in the brain, liver, spleen, pancreas, kidneys and thymus. However, ASC concentration depends generally on the type of diet (18) and oxidative stress to which an organism is subjected to (22).

ASC supplementation decreases training efficiency because it reduces the exercise-induced expression of key transcription factors involved in mitochondrial biogenesis: peroxisome proliferator-activated receptor co-activator 1, nuclear respiratory factor 1 and mitochondrial transcription factor A.

ASC also prevents expression of cytochrome c and the antioxidant enzymes SOD and glutathione peroxidase. On this basis, it is suggested that ASC supplementation prevents some cellular adaptation to exercise (14).

ASC is a key component of antioxidant defense and influences the antioxidant response in neutrophils, erythrocytes and lymphocytes. Many studies in human and animals have shown that nutrients that contribute to antioxidant defense within the body (ASC, GSH and its precursors) in general have an anti-inflammatory and immunostimulatory influence (19). Diet supplementation with ASC decreases neutrophil GSH/GSSG ratio and reduces iNOS levels and NO production in neutrophils, erythrocytes and lymphocytes (37, 38).

Inflammation may lead not only to the development of cancer but also to the occurrence of atherosclerosis, arthritis and Crohn's disease. For therapy of these diseases, it is important to localize the inflammatory reaction and eliminate it by supplementing relevant antioxidants (3, 32). Diet rich in ASC is recommended to prevent these diseases; administration of drugs preventing infections and inflammatory processes which can lead to oxidative injuries are also recommended (11).

In the process of inflammation, ASC fulfills all the criteria of an effective antioxidant. Due to its low reduction potential, ASC is an efficient electron donor for different radicals and oxidants (2) which works in the aqueous environment of the cell and in the intercellular fluid. Protective action of ASC involves mainly direct reduction of ROS. In the physiological conditions, this action stops reactions caused by radicals and protects the cell against intercellular injuries. However, if inflammation injuries, mainly to mitochondria, are inflicted as a result of, for example, disorders in redox reactions (24, 42), this may lead to a decrease in the synthesis of ASC in mice. It is possible that the above is one of the causes of the observed decrease in the amount of this vitamin. As a result of the reduction of ROS, ASC is oxidized to dehydroascorbic acid and this process can also be detected as a decrease in its amount (25). ASC also acts indirectly on ROS to change the oxidized form of vitamin E into its active form (35).

The results of this work indicated that in the studied organs of mice, there was a close relationship between the ASC content and inflammation caused by the administration of zymosan A. The observed changes in the ASC contents could have been caused by the disturbances in the synthesis or the increased consumption of the vitamin tested. Based on the above literature data and the results obtained in this study, it can be suggested that ASC has a significant impact on the process of inflammation in animals.

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