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Effect of Superoxide Dismutase From Bovine Erythrocytes on Different Activity Parameters in Adjuvant-Induced Arthritis

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Background. The purpose of this work was to evaluate the effect of superoxide dismutase (SOD) on primary swelling, lipoperoxidation, body thymus, and spleen weight in the adjuvant-induced arthritis (AIA) model in rats.

Methods. Orally and intraperitoneally administered SOD (100 U/kg) from bovine erythrocytes, as well as naproxen (40 mg/kg) and dexamethasone (25 mg/kg), were evaluated against placebo.

Results. Primary edema was not decreased by SOD; in contrast, naproxen and dexamethasone showed good anti-inflammatory activity. Lipoperoxidation increased 1.8, 2.5, and 2.8 times with intraperitoneal SOD, naproxen, and dexamethasone administration, respectively, while oral SOD decreased lipoperoxidation levels to approximately one-half of that found in the control group. Body weight increased with SOD but decreased with dexamethasone. Naproxen did not change the animal weight. Thymus weight remained unchanged with SOD and naproxen, while it decreased with dexamethasone. Spleen weight remained the same with SOD, but increased with naproxen and decreased with dexamethasone. No side effects were observed in the SOD group, whereas 20% of the rats in the naproxen group died of gastrointestinal hemorrhage, and 50% of the rats in the dexamethasone group, of pulmonary infection.

Conclusions. In conclusion, SOD showed no anti-inflammatory activity but decreased lipoperoxidation when administered orally. No deleterious effects in primary and secondary immunologic organs were observed with this agent.

Key Words: Adjuvant arthritis, Superoxide dismutase, Rat, Naproxen, Dexamethasone.

Introduction

Adjuvant induced arthritis (AIA) develops after injection of complete Freund adjuvant into rats (1). It is a systemic disease with articular and visceral manifestations that resemble rheumatoid arthritis. It is characterized by chronic evolution with recurrent inflammatory bouts resulting in periarticular, articular, and bone lesions (2). It was described originally by Pearson in 1956 (3) using Wistar rats (4,5), although more susceptible strains have since been described. Although the etiology of AIA is not well understood, T-cell mediated autoimmune disease (6) has been postulated. In this model, the mycobacterial 65 KDa heat shock protein (HSP65) present in the adjuvant solution is of crucial significance, because arthritogenic and protective T-cell clones obtained from arthritic rats recognize a 180–185 sequence of HSP65 (7). Three different phases of AIA have been described: first, an acute phase, which is present 7 days after mycobacterium injection and is characterized by local inflammation; second, a subacute phase, usually from days 10–25, with an evident systemic inflammatory response, and a third phase which starts at about day 30, and is characterized by the appearance of trophic lesions (8). Because the anti-inflammatory activity as well as

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some parameters of the immune response can be measured in this type of experimental model, drug effects can be evaluated by measuring decreased lipoperoxidation and primary edema reduction, which are related to an anti-inflammatory activity. In still-growing rats, body and thymus weight increase, as well as spleen weight reduction, are related to a positive influence in some parameters of the immunologic response (9).

The superoxide dismutases are a group of metalloenzymes. Their main function is the scavenging of free radicals (10). These enzymes differ in the nature of their metal prosthetic group and location within the cell component: mitochondrial (Mn-SOD); cytoplasmic (Cu,Zn-SOD); periplasmic space (Fe-SOD), and/or extracellular EC,Cu,Zn-SOD (10). The Cu,Zn-SOD can be obtained from numerous sources; however, bovine erythrocytes are most commonly used (11). This enzyme has been administered with good results in patients with osteoarthritis, rheumatoid arthritis, renal transplant and ischemia-reperfusion, and also in myocardial ischemia (12,13).

Good anti-inflammatory activity of superoxide dismutase from bovine erythrocytes has been reported using the AIA model (8) where primary edema and lipoperoxidation decreased (14). However, the influence of this enzyme on the weight of primary and secondary immunologic organs has not yet been explored.

The purpose of this work was to evaluate the anti-inflammatory effect of superoxide dismutase from bovine erythrocytes as well as its influence on immunologic organ weight in comparison to the well-known antirheumatic drugs, naproxen and dexamethasone.

Materials and Methods

Animals. Male Wistar rats weighing 100–130 g were purchased from Bioterio México (México, D.F.). As soon as the animals were received, they were randomly assigned to different groups, and a period of 4 days for adaptation was allowed. Groups of 10 rats per cage were maintained with food and water *ad libitum*.

Induction of adjuvant arthritis. Complete Freund Adjuvant (CFA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A dose of 0.1 mL at 38°C was administered intraplantarly to each animal in the right hind paw on day 0.

Test groups. Five groups of 10 rats each were formed: group 1 was a control group, to which 1 mL of saline buffer was administered intraperitoneally. In groups 2 and 3, a dose of 100 U/kg of SOD was intraperitoneally and orally administered in a volume of 1 mL. In groups 4 and 5, 40 mg/kg of naproxen (Syntex, Boulder, CO, USA) and 25 mg/kg dexamethasone (Upjohn, Kalamazoo, MI, USA) were administered orally. All drugs were suspended in saline solution and prepared at the beginning of the experiment. Oral administration was always carried out in a 3 mL volume with a number 8 nelaton-type tube, which was introduced about 12 cm deep or until resistance to its passing was noted. Drugs were administered daily from days 0–4, then from days 7–11, and finally, from days 14–18 (terminal date of experiment).

Evaluation of adjuvant induced arthritis. Primary edema was evaluated at the beginning of the experiment, and then every other day until day 18 using the mercury plethysmograph method. Briefly, the fore paw of the rat was ink-marked up to the tibiotarsalis joint and immersed in a mercury-filled chamber, which was connected to a pressure transducer (Grass Cardiovascular Pressure Transducer, P23ID VSA, Quincy, MA, USA). Recording was done in a previously calibrated multichannel polygraph (Grass Polygraph, 79D, Quincy, MA, USA). Results are reported in millimoles of marker displacement. Each animal was used as its own control, taking day 0 measurement as reference.

Serum lipoperoxides were evaluated at day 21 by the thiobarbituric acid technique (15). Briefly, open-chest cardiac punction was performed in rats under anesthesia with ethylic ether using 0.2 mL of anticoagulant (EDTA 10%) in a 10-mL syringe. Blood samples were kept on ice until analysis. Centrifugation (Sol/Bat, Centrífuga Clínica J12, México, D.F.) was done at 4,000 rpm for 5 min to obtain plasma. One milliliter of plasma was mixed with 2 mL of a 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl solution. The test tubes were heated until boiling for 15 min, centrifuged at 3,000 rpm for 3 min, and the supernatant was collected to determine absorbance at 532 nm. The results are reported in nanomoles per liter of malondialdehyde.

Body weight was determined (Oahus Triple Beam Balance 700, Florham Park, NJ, USA) at the beginning of the experiment and then twice a week until day 18. Each rat was its own control. Results are reported in percentage of weight change when comparing day 18 to day 0. Thymus and spleen organs were obtained immediately after cardiac punction and weighed (Sartorious Analytical Balance, Frankfurt, Germany). Results are reported in grams (g).

Statistical analysis. The arithmetic mean value and standard deviation of the results were obtained. Analysis of variance was used for primary edema and lipoperoxidation. Percentage difference was determined using the Z test. Thymus and spleen weight were analyzed using Student's *t* test. Significance level (alpha) was evaluated at 0.05.

Results

Primary edema. The total percentage of edema increase of the injected hind paw was 10.4 ± 3.4% (mean ± standard

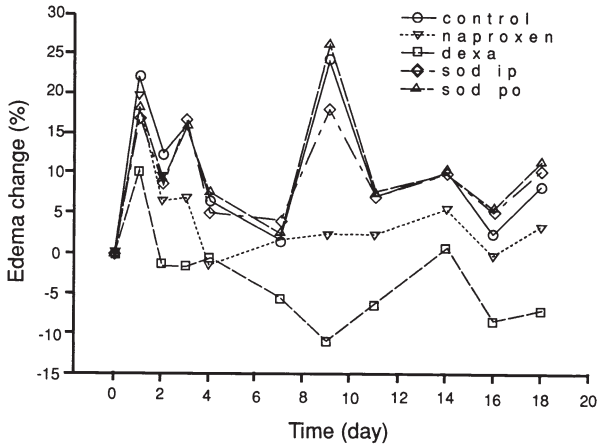

Figure 1. Time-course edema change in the AIA model with different treatments and with respect to control (placebo) group. The dexamethasone group had anti-inflammatory activity throughout the experiment ($p < 0.05$). Naproxen had anti-inflammatory activity which was lost only during hour 7 ($p < 0.05$). SOD groups did not have an effect. Standard deviation (SD) bars are omitted for clarity.

deviation); 10.7 ± 4.1%; 9.6 ± 3.3%; 4.6 ± 2.3%, and −2.14 ± 3.3% for control group, SOD-vo, SOD-ip, naproxen, and dexamethasone, respectively. Only naproxen and dexamethasone showed clear anti-inflammatory activity ($p < 0.05$). Edema increased in all groups at day 1 (Figure 1), decreasing slowly afterward until day 7. An edema increase was evident in the control and the SOD groups at day 9, while naproxen did not change, and dexamethasone had its maximum anti-inflammatory activity.

Lipoperoxidation. All groups tested, except for the group receiving oral SOD, showed an increase in malondialdehyde concentration ($p < 0.05$) when compared to the control group (Figure 2). The control group had a value of 0.1499 ± 0.027 nmol/mL. Lipoperoxidation with naproxen was 0.366 ± 0.06 nmol/mL. The dexamethasone result was 0.416 ± 0.07 nmol/mL. The group with intraperitoneal SOD administration had a lipoperoxidation level of 0.2703 ± 0.044 nmol/L. The oral SOD group had a very low level of lipoperoxidation (0.0674 ± 0.020 nmol/L).

Thymus and spleen weight. There was no difference in thymus weight among the different groups, except in the dexamethasone group, in which there was a weight reduction ($p < 0.05$) when compared to the control group (0.0478 ± 0.008 g vs. 0.3398 ± 0.063 g, respectively) (Figure 3). Thymus weight in the naproxen group was 0.3010 ± 0.1219 g. Intraperitoneally and orally SOD-treated animals had thymus weights of 0.3538 ± 0.1060 g and 0.3292 ± 0.0530 g, respectively. In regard to spleen weight, neither intraperitoneal nor oral SOD groups showed any difference when compared to the control group (0.6036 ± 0.1090 g, 0.5884 ± 0.1390 g, and 0.5 448 ± 0.082 g, respectively). The naproxen group showed a weight increase of 0.8828 ± 0.169 g, while the dexamethasone group had a spleen weight decrease to values of 0.3860 ± 0.022 g.

Body weight. The control group had an average weight increase of 68 ± 10% (Figure 4). Naproxen did not cause any weight difference when compared to the control group (43 ± 27%), while in the dexamethasone group, a body weight loss of −0.4 ± 6% was induced, as compared to the control group ($p < 0.05$). Both intraperitoneal and oral SOD showed a body weight increase of 102 ± 9.3% and 93 ± 16%, respectively, when compared to the control group ($p < 0.05$).

Side effects. We did not observe any side effects in the groups treated with SOD; food and water were well ac-

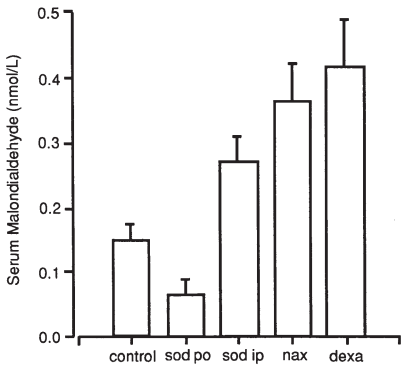

Figure 2. Lipoperoxidation in the AIA serum of Wistar rats with different treatments and with respect to the control (placebo) group. Orally administered SOD decreased malondialdehyde concentration. Intraperitoneal SOD, naproxen and dexamethasone increased lipoperoxidation ($p < 0.05$). (Values are arithmetic mean and standard deviation, $n = 10$.)

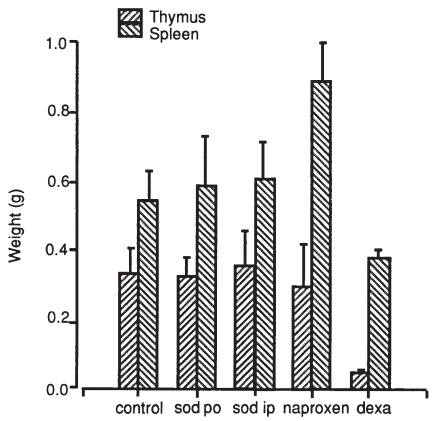

Figure 3. Thymus and spleen weight variation of AIA in Wistar rats with different treatments compared to the control (placebo) group. SOD did not affect weight. Naproxen showed spleen weight increase. Dexamethasone produced spleen and thymus weight decrease ($p < 0.05$). (Values are arithmetic mean and standard deviation, $n = 10$.)

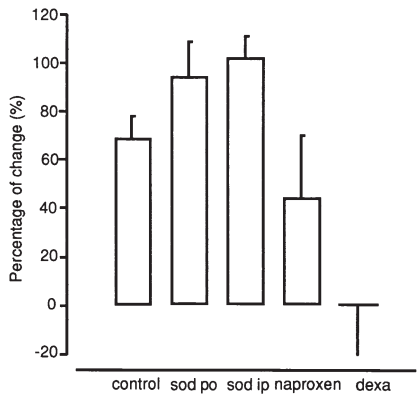

Figure 4. Body weight change of AIA in Wistar rats with different treatments compared to the control (placebo) group. SOD produced weight increase. Naproxen did not induce weight change. Dexamethasone showed a significant weight reduction ($p < 0.05$). (Values are arithmetic mean and standard deviation, $n = 10$.)

cepted. In contrast, there was a mortality incidence of 20% in the naproxen group at day 11; necropsy showed gastrointestinal bleeding. In the dexamethasone group, we had a mortality incidence of 50% at day 11 and another of 50% at day 18; necropsy showed massive pulmonary infection in these cases.

Discussion

Free radicals have been implicated in the inflammatory process (16). The superoxide radical is normally transformed into hydrogen peroxide, a less reactive substance, by the superoxide dismutase enzyme. Under physiologic conditions, hydrogen peroxide is transformed by catalase into water. When an excessive quantity of superoxide radical is produced, it can induce severe tissue damage (17).

Several experimental models have been used in order to study the ability of some drugs to eliminate free radicals (13). Among the most frequently used is superoxide dismutase, which was first identified by McCord and Fridovich (18) in 1969. Since then, a large number of papers have been published regarding the chemical and pharmacologic characteristics of this enzyme, as well as on its effect in different pathologic processes. Superoxide dismutase is obtained from different sources and, according to the source, shows different anti-inflammatory activity (10).

Participation of oxygen-free radicals in several diseases has been demonstrated. In rheumatoid arthritis, an excessive accumulation of polymorphonuclear cells, in addition to the ischemia-reperfusion phenomenon that may occur, favors the production of excess free radicals. Yet the current therapy for inflammation is generally limited to the inhibition of prostaglandin formation. Treatment using SOD seems to be a promising alternative, as it breaks the sequence of free radical-induced events, and its use may reduce inflammation (19).

Our present work was intended to clarify how bovine erythrocyte superoxide dismutase influences different parameters in the AIA model. This enzyme was compared to two antirheumatic drugs. Because the optimal dose of bovine erythrocyte superoxide dismutase as an anti-inflammatory drug is between 20 and 500 U/kg (20), we decided to use an intermediate dose of 100 U/kg.

Primary edema evaluation is a good index of the anti-inflammatory activity of drugs (21). Most anti-inflammatory drugs show an effect on endoperoxide production and decrease primary edema (22). Lipoperoxidation has also been determined in adjuvant-induced arthritis model and was found to be related to disease activity (14).

The immunomodulatory activity of drugs must be evaluated by using different and more sophisticated laboratory methods (23); however, according to a recent report (9), primary and secondary lymphoid tissue weight in growing animals can be related to some influence of the drugs on the immunologic response. In particular, spleen weight reduction and thymus weight increase are related to a positive effect on the immune system in the adjuvant-induced arthritis model (9).

Animal body weight gain in the AIA model is considered as a good index of both anti-inflammatory activity and positive immune effect (8) when testing anti-inflammatory drugs. This effect appears to be related to a decrease in prostaglandin synthesis as well as in cytokine production (23).

Our results showed that with either route of administration, SOD was not effective in decreasing primary edema. Interestingly, the oral route showed only transient benefits during the first 48 h, while intraperitoneal application of SOD was also effective at day 9. Vaille et al. (8), using Lewis rats and a similar dose, reported that intraperitoneal bovine SOD was effective from days 13 to 30 (secondary reaction) in decreasing primary paw edema. During the acute phase, SOD was not effective.

Naproxen showed an edema reduction in comparison to the placebo. Time-course evolution indicated that its efficacy is lost at days 7 and 16. Because the naproxen effect has been related to the inhibition of prostaglandin production, this finding suggests that in the first phase of AIA, prostaglandins may be involved in the development of the inflammatory process induced by mycobacterium (24).

Dexamethasone, however, was effective in decreasing edema intensity throughout the experiment. This may be related to inhibition of phospholipase activation and cytokine production (25).

Lipoperoxidation was modified by all drugs, but to different extents; oral SOD decreased lipoperoxidation, while intraperitoneal SOD, naproxen and dexamethasone produced increases in the concentration of malondialdehyde. Yoshikawa et al. (14) demonstrated that a daily subcutaneous injection of SOD at very high doses (20,000 and 40,000

U/kg) in the AIA in Wistar rats decreased lipoperoxidation in serum from weeks 1 to 2; thereafter, only the higher dose was effective until week 6. This finding demonstrates that at low doses, SOD injected intraperitoneally is not effective in decreasing lipoperoxidation, but rather it increases lipoperoxidation. The fact that all these drugs, at the doses and the administration route utilized, produced an increase instead of a decrease in the lipoperoxide level is not surprising. It is well known that while low concentrations of anti-inflammatory drugs (including SOD) are effective in ameliorating free radical-mediated tissue damage, higher doses may actually exacerbate the injury, possibly by lowering superoxide levels excessively, thus inhibiting the termination step of lipid peroxidation (26).

The fact that orally administered SOD decreases the serum concentrations of malondialdehyde suggests that SOD is absorbed by the gastrointestinal tract. The reason for this absorption may be due to the nelaton tube, which allowed delivery of the drug directly into the small intestine where the pH (7.4) is closer to the isoelectric point of the enzyme (7,8), and the action of proteolytic enzymes (e.g., trypsin) is mild.

Body weight change is a good parameter of disease activity in the autoimmune adjuvant-induced arthritis model (9). Both SOD groups showed significant weight increases when compared to the control group. This finding suggests that low doses of SOD may interfere either with the free radical-mediated production or the action of cytokines (27,28), such as IL-1 or TNF, that are normally increased in this autoimmune disease (29). This action could be accomplished by affecting functions of the phagocytic cells (30). The fact that naproxen was not different from the control group in regard to weight gain shows that this anti-inflammatory drug acts only on cyclo-oxygenase inhibition, and that prostaglandins are not necessarily related to body weight change. The decrease in body weight observed during the adminstration of dexamethasone could be secondary to the antiproliferative and catabolic side effects presented by this drug at high doses.

The fact that spleen and thymus weight was not affected by SOD administration could be interpreted as the absence of the effect of the enzyme in the afferent pathway of the immune response (31). Naproxen showed an increase in spleen weight in a manner similar to that of other nonsteroidal anti-inflammatory drugs (9), suggesting that the dose utilized may be deleterious in the adjuvant-induced arthritis model. Dexamethasone, in contrast, caused spleen weight reduction, which agrees with previous reports; however, because of thymolytic and antiproliferative side effects, an impressive thymus weight reduction was produced (25).

No side effects were observed when superoxide dismutase was administered by either oral or intraperitoneal route. Life-threatening gastrointestinal hemorrhage is commonly observed with naproxen-related inhibition of mucose-protective prostaglandin synthesis (32). The very high rate of mortality due to pulmonary infection found in the dexamethasone group is also a well known, severe side effect present when high doses of corticosteroids are used to treat autoimmune diseases; this is secondary to the immunosuppressive effect on both phagocytic cells and T-cells (25).

In conclusion, bovine erythrocyte SOD was not effective in decreasing the primary paw swelling of the adjuvant-induced arthritis in the Wistar rat model. Lipoperoxidation decreased in the orally administered SOD group, while it increased with intraperitoneal SOD, naproxen and dexamethasone. SOD via any route of administration induced a body weight increase, while dexamethasone had a negative influence on this parameter. Deleterious effects in primary and secondary immunologic organs were found in the groups treated with naproxen and dexamethasone; in contrast, SOD did not show any change in this parameter. No side effects were observed in the SOD groups, while gastrointestinal hemorrhage was present with naproxen, and pulmonary infection was evident in the dexamethasone group.

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