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# Anti-inflammatory activity of superoxide dismutase obtained from *Debaryomyces hansenii* on type II collagen induced arthritis in rats

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**ABSTRACT**

**Introduction.** Rheumatoid arthritis is an autoimmune inflammatory disease of unknown etiology, free radicals have been implicated in the genesis and perpetuation of damage in this pathology. **Objective.** To evaluate the anti-inflammatory effect of Cu,Zn-superoxide dismutase (SOD) obtained from two different sources (bovine erythrocytes, Be-SOD, and Debaryomyces hansenii, Dh-SOD) with Type II Collagen-induced Arthritis model in rats. **Material and methods.** Arthritis was induced by repeated injection of a porcine type II collagen-incomplete Freund adjuvant suspension on the back of Dark Augui (DA) rats. Arthritis was clinically evaluated throughout the study. Body weight was determined at three different times. Two different doses for each treatment (Be-SOD, Dh-SOD) were tested: 100 and 1,000 U/kg. At the end of the trial (day 28), histological analyses of the most inflamed ankle joint, as well as serum anti-collagen antibodies, were determined. **Results.** Both sources of SOD decreased, although to a different extent, the incidence and severity of the disease. Arthritis score was lower in all treatments, except for the low dose of Be-SOD. Groups receiving either source of SOD showed a significant weight increase compared to the placebo group. Histological damage was similar in all groups. Only the group that received the highest dose of Dh-SOD showed a significant lower antibody titer; nevertheless, no correlation appears to derive from arthritis score and antibody titer. **Conclusions.** Our findings suggest that, although unable to counteract the arthritis syndrome, SOD may still be beneficial due to its anti-inflammatory activity. In the case of Dh-SOD, the best effect was observed at the highest dose tested.

**Key words.** Collagen Induced Arthritis. Rat; Anti-inflammatory activity. Debaryomyces hansenii. Superoxide Dismutase.

***Efecto anti-inflamatorio de la superóxido dismutasa obtenida de Debaryomyces hansenii en la artritis inducida por colágena tipo II en ratas***

***RESUMEN***

***Introducción.*** *La artritis reumatoide es una enfermedad inflamatoria de etiología autoinmune en la cual se ha postulado la participación de radicales libres en su génesis y perpetuación.* ***Objetivo:*** *Evaluar el efecto anti-inflamatorio de la Cu,Zn superóxido dismutasa obtenida de dos diferentes fuentes, eritrocitos de bovino y la levadura marina Debaryomyces hansenii en el modelo de artritis inducida por colágena tipo II en ratas.* ***Material y métodos.*** *La artritis se indujo mediante la inyección (día 0) de colágena porcina tipo II en el dorso de ratas Dark Augui. Se evaluaron frecuentemente la incidencia y la intensidad artritis (clínicamente), el peso, la histología y la presencia de anticuerpos anti-colágena (al final del experimento el día 28). Se estudiaron dos dosis de cada una de las enzimas: 100 y 1,000 U/kg, administradas diariamente por vía intraperitoneal.* ***Resultados.*** *La enzima de ambas fuentes disminuyó la incidencia y la intensidad de la artritis, exceptuando la dosis menor de eritrocitos de bovino. Se presentó un adecuado incremento en el peso. No hubo diferencia en la histología. Sólo el grupo que recibió la dosis mayor de la enzima de D. hasenii, disminuyó la producción de anticuerpos.* ***Conclusiones.*** *La enzima superóxido dismutasa tiene actividad anti-inflamatoria en este modelo animal. La dosis mayor de la enzima de D. hansenii tuvo mejor efecto.*

***Palabras clave.*** *Artritis reumatoide. Modelos animales. Superóxido dismutasa. Actividad anti-inflamatoria. Debaryomyces hansenii.*

## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease of unknown etiology with several distinctive features such as chronic, symmetric, and erosive synovitis of peripheral joints.[1] RA affects 2% of the adult population, it has a high incidence in some geographic areas, and produces severe physical and psychological disability.[2] Antibodies against the highly specific articular type II collagen have been detected in sera and synovial fluids of patients with rheumatoid arthritis,[3] suggesting that hypersensitivity to collagen may, at least in part, contribute to inflammation and joint destruction. Treatment for this disease has evolved rapidly during the last decade. However most therapies have a moderate percentage of efficacy and a high incidence of side effects.[1]

Collagen-induced arthritis (CIA) is an animal model for the study of RA. It was described in 1977 by Trentham[4] who observed that at least 40% of a group of rats injected with heterologous and homologous type II collagen developed an arthritic-like syndrome; that is, a chronic, symmetric, proliferative, and destructive inflammation. In this model, denatured type II collagen, and the genetically distinct collagen forms types I, III, and IV, are unable to elicit a similar response. Both, passive immunization with antibodies against type II collagen, and administration of sensitized lymph node and spleen, induce clinically obvious inflammation and synovitis in rats.[5] Increased urinary excretion of nitrate and of the immune/inflammatory nitric oxide synthase (iNOS) mRNA during joint inflammation in CIA were also recently reported,[6] indicating the involvement of free radicals in the process. Arthritis can be also induced when type II collagen is intradermally injected in primates and susceptible strains of mice and rats. Griffiths, *et al*.[7] showed that Dark Augui (DA) rats are among the most reactive strains and can develop arthritis in the CIA model with an incidence of more than 95%. The same authors observed that the synovitis appears very suddenly, even overnight, two weeks after a collagen injection and reaches a maximum intensity at day 25, with decreased joint inflammation and osseous fusion thereafter.[7] Thus, CIA has been considered a very good model to evaluate the anti-inflammatory and anti-rheumatic effects of drugs.[8]

Oxygen free-radicals are molecules, or molecular fragments, with unpaired electrons in the outer orbital. As such, these entities are very reactive and tend to initiate chain reactions that result in the irreversible chemical alteration of lipids, proteins, nucleic acids and carbohydrates.[9] They are ubiquitous in biological systems and are produced within the cell under controlled situations. Under particular conditions, excessive production or limited elimination of free radicals induce several of the symptoms observed during the progress of certain diseases.[10] For example, free radicals participate actively in all kinds of inflammatory conditions and have been implicated in the genesis, damage, and perpetuation of rheumatoid arthritis.[11]

The eukariotic cells possess a well developed protection mechanism against the reactive oxygen species (ROS). They can be enzymatic or non-enzymatic. Among the former is the Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (12). This enzyme catalyses the dismutation of the very reactive ion superoxide, producing the less toxic, but still reactive non-free radical hydrogen peroxide. Superoxide dismutase can be obtained from different sources and they all have different anti-inflammatory activities, which still remain to be explained. At our laboratory, a novel Cu,Zn-SOD has been obtained from the marine yeast *Debaryomyces hansenii* (Dh-SOD) which appears to have physicochemical and molecular characteristics different to those of other sources.[13,14] The SOD has been already tested as an anti-inflammatory drug in type II collagen- induced arthritis in mice showing promising results.[15] Therefore, we considered further testing of this property in a rat CIA model a point of interest. Bechaump *et al*.[16] have already studied the effect of bovine erythrocyte Cu,Zn-SOD (Be-SOD) in this model and found that a modified polyethylene glycol Cu,Zn-SOD, parenterally administrated after arthritic signs appeared, showed only a moderate benefit. In addition, no difference in the arthritic index from placebo was found. Hence a therapeutic effect of Be-SOD in the CIA model with rats has been questioned. The purpose of the present work was to evaluate, and compare, the preventive anti-inflammatory activity of these two different sources of SOD (Be-SOD and Dh-SOD). Dh-SOD showed to be more efficient than Be-SOD in reducing the arthritis index. We conclude that, although Dh-SOD cannot completely prevent, nor reverse, the chronic arthritis induced by type II collagen in rats, an important symptom relief may derive from the anti-inflammatory effect of this enzyme. In addition, compared to Be-SOD, Dh-SOD was not only more effective, but also very efficient in lowe-

ring the arthritis index, thus indicating that at least the severity of disease could be attenuated by this enzyme.

## MATERIAL AND METHODS

### Animals

The protocol was accepted by the Animal Ethics Committee of The Scripps Research Institute (TSRI). Forty two female, seven week old Dark Augui (DA) rats were purchased from Harlan Sprague. A one week period of adaptation was allowed before experiments began. Animals were kept in the animal facility of the TSRI with antiseptic conditions, constant temperature of 25 grades, 50% of humidity and light-dark cycle of 12 hours. Three animals per cage were allowed, with filtered air device, and food and water *ad libitum*.

### Experimental groups

Six groups were analyzed; each one consisted of seven rats. Group 1, was the negative control and was integrated with naive animals; Group 2, was the positive control and thus, collagen was applied for arthritis induction and phosphate buffer was administrated as a placebo; Groups 3 and 4, were treated with 100 U/kg and 1000 U/kg Dh-SOD, respectively. Groups 5 and 6 were treated with 100 U/kg and 1,000 U/kg Be-SOD, respectively.

### Drug administration

All drug treatments were administered intraperitoneally by injecting a 1 ml volume of the enzyme daily from day 0 until the end of the experiment (day 28). Be-SOD (3300 U/mg) was purchase from Sigma (St. Louis Mo., US). Dh-SOD (9,900 U/mg) was obtained as previously reported (13); briefly, considerable amounts of biomass of the halotolerant yeast *Debaryomyces hansenii* were produced with a simple fermentation process using a culture medium formulated with seawater; the culture was carried out in 60-liter carboys in the presence of chlorine dioxide to further repress the growth of any contaminant microorganism; the SOD was extracted by mechanical cell disruption (using glass beads) and further chemical extraction (with chloroform-ethanol); approximately, 0.6% of protein was obtained (on a dry matter basis) with a SOD activity of 9,900 U/mg was obtained in a single step. The enzyme stock solutions were prepared in phosphate buffer (pH 7.4) one day before the experiment started and were kept in refrigeration until use.

### Collagen

Native porcine type II collagen immunization grade was purchased from Dr. Griffiths (University of Utah, School of Medicine, UT, US). All procedures were performed at 9°C (cold room). One day before injection, the collagen was dissolved in cold 0.1N acetic acid (Sigma, St Louis MO, US) at 2 mg/mL, and allowed to mix on a rotator in the cold room overnight. The day of the injection, the collagen was manually emulsified with cold Incomplete Freund Adjuvant (IFA) (Sigma, St Louis MO, US) in 1:1 proportion.

### Arthritis induction

Rats were shaved, and under a mild inhalation anesthesia, arthritis was induced (day 0) by repeated injection of 0.1 mL of collagen-IFA suspension on the back of the animal at multiple sites up to a total dose of 2 mg per kg rat weight. A boost with 0.1 mL of the collagen-IFA suspension was given at the seventh day in the base of the tail of each animal.

### Arthritic score

Clinical evaluation of arthritis was achieved by determining the degree of inflammation according to Trentham.[1] The maximum score was 6 for each of the forepaws, assigning a value of 1 for each of the four major fingers inflamed, and 0-2 value for inflammation severity in the metacarpal region (0 for no inflammation, 1 for mild, and 2 for severe inflammation). For the hindpaws, the maximum assigned score was 11: 1 for each of the four major digits inflamed, 0-3 for the severity of inflammation of the metatarsal region, and 0-4 for the severity of inflammation of the ankle joint. Hence, the total maximum score of arthritis inflammation was 34. The observation and assignment of evaluation was openly performed by one of us (AG), well trained in experimental inflammatory animal models, and by chosen randomly any cage; then determining the degree of inflammation and afterwards registration of the cage control number. The evaluation was carried out on days 0, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26, and 28.

### Arthritis intensity

Arthritis was classified as mild (0-8), moderate (9-22), and/or severe (23-34), summing up the assigned arthritic score values.

### Body weight

Animal body weight was determined at days 0, 7, 16, and 26 using a triple beam balance (Ohaus 700, Florham Park NJ).

### Histology analysis

At the end of the study (day 28), the animals were exanguinated by cardiac punction under inhalation anesthesia and sacrificed by lethal overdose. The most severe inflamed ankle joint of each rat was taken for histopatological analysis. The tissues were removed and fixed in 10% formalin (VWR South Plainfield NJ) for three days, then immersed in decalcifying solution (VWR South Plainfield NJ) for 20 hours. Paraffin embedded ankle tissue was cut, fixed and stained with hematoxilin and eosin. The sections were graded in a 0-4+ scale, according to severity, for each one of the following parameters: a) inflammatory cell infiltration, b) synovial membrane hypertrophy, c) narrowing of the joint space, and d) cartilage destruction and regeneration, following the criteria recommended in (7). Accordingly, maximum damage registered as a histopathology index with this approach was 16.

### Anti-collagen antibodies

Twenty eight days after the first collagen injection, the rats were sacrificed and the serum obtained for anti-collagen antibodies determination. Frozen at -20°C, the samples were shipped on ice overnight to Dr. Griffiths' laboratory (University of Utah, School of Medicine). Antibodies to porcine type II collagen were determined with highly purified, ELISA grade, native porcine type II collagen. The positive control sera was a high-titer pool developed at the reference laboratory and standardized to 1000 Antibody Units/mL (AbyU/mL).

### Statistical analysis

Data were analyzed using SPSS v15.0 software. Statistical significance was considered when $p \leq 0.05$. Because of non-normal distribution of the data (Kolmogorov-Smirnov test), arthritis intensity (arthritic score) was presented as median (quartile). Incidence was reported in percentage. Arthritis incidence was evaluated by Wilcoxon test. Anti-collagen antibody titer, as well as, arthritic score differences among groups were compared by Kruskall-Wallis test. Correlation between arthritis incidence and arthritis intensity with anti-collagen antibody titers were performed by $Chi^2$ (with Yates correction) and Spearman test, respectively. Because hystologic changes and weight differences showed normal distribution (Kolmogorov-Smirnov test), they were analyzed by two-tailed *t*-test.

## RESULTS

Negative control group or naïve group did not developed arthritis at any time; the weight increase was the expected for that strain of rats, no anti-type II collagen antibodies were found, and none histopathological abnormality was noticed.

- *Arthritis incidence.* Although much later than expected, signs of induced arthritis started to appear suddenly in experimental animals, overnig-

**Table 1.** Incidence and intensity of arthritis.

| Group (number) | Incidence day19th / day23rd (%) | Intensity day19th / day23rd (%) | | |
|---|---|---|---|---|
| | | Mild | Moderate | Severe |
| Placebo (2) | 100/100 | 28/14 | 14/28 | 58/42 |
| Dh-100 (3) | 71/86 | 0/0 | 60/33 | 40/67 |
| Dh-1000 (4) | 71/71 | 40/20 | 60/40 | 0*/40 |
| Be-100 (5) | 71/71 | 0/0 | 80/80 | 20/20 |
| Be-1000 (6) | 71/100 | 40/0 | 40/86 | 20/14 |

*At day 19 post collagen-IFA injection, when the arthritis intensity of the placebo group reached its maximum, all treatment groups showed a non statically significant anti-inflammatory activity ($p > 0.05$). At day 23, only Dh-1000 and Be-100 groups maintained the effect; however, the intensity of the arthritis seems to be lower in the former. No statistical significance was found in arthritis incidence. $p > 0.05$ (Wilcoxon test). Difference in arthritis intensity against placebo group. $p < 0.05$ (Fisher exact test).*

ht, 16 days after the injection of collagen-IFA suspension. The reaction was first noticed by the swelling and redness of the forepaw fingers, followed by inflammation of the ankle joint, the metatarsal region, the fingers of the hind paws, and finally of the metacarpal region as the disease progressed. The arthritis signs were always bilateral, albeit asymmetric. That is, although observed in both sides, the inflammation scores of the right and the left sides of a given animal were different.

In group 2, without any enzyme treatment, all the animals showed a well developed arthritis syndrome (100% incidence) by day 19$^{th}$ (Table 1). In contrast, at the same interval only 71% of the animals from any treatment group developed arthritis, yet this difference was not significant ($p > 0.05$). Differences in dose-response derived from the nature of the enzyme source were less clear from day 23$^{rd}$, and until the end of the experiment, animals from group 5 showed an incidence of 71%, while group 6 had a 100% incidence ($p > 0.05$). In agreement with previous reports, we therefore confirm in this experiment that high doses of Be-SOD not only render the enzyme less effective as an anti-inflammatory drug, but also can be deleterious or inappropriate for handling inflammation pathologies. The groups of animals treated with Dh-SOD at different doses, in contrast, showed an arthritis incidence of 86% and 71% for groups 3 and 4, respectively. Thus, it appears that, at least within the range of doses tested, the protection provided by Dh-SOD shows a dose-related response, being more effective at the highest dose tested. Again, as previously reported and with another inflammation model, Dh-SOD appears to provide protection against inflammation in the arthritis collagen-induced model. It is also quite remarkable that with high dose of Dh-SOD and low dose of Be-SOD treatment at least 29% of the animals showed no arthritis symptoms at all.

- *Arthritis intensity*. he intensity of the induced arthritis varied within the different groups according to the treatment applied. At day 19, for example, 58% of rats in group 2 showed a severe arthritis score, while only 20% in the groups 5 and 6 had that arthritis intensity. Group 4, in contrast, did not show severe arthritis, while 40% of the animals of the group 3 they did. From day 23, and until the end of the experiment, the percentage of severe arthritis intensity increased in all instances, but in group 6, being always more notorious among the animals treated with Dh-SOD.
- *Arthritic score*. According to the criteria employed to grade the severity of induced arthritis in this model, a maximum score of 34 is feasible. From the day at which appearance of arthritis signs became evident (day 16), and until the end of the experiment (day 28), the median arthritic score group 2 was 21.5 (10-29). All groups showed, but group 3 with a median arthritic score of 22 (2-28), significant difference when compared to placebo group ($p < 0.05$). Group 4 was the most effective by reducing the total score down to 9 (0-21), albeit the difference among the other effective treatments was not significant ($p > 0.05$). Groups 5 and 6 median arthritic score were 13 (0-22) and 16 (5-22), respectively.

  Time course arthritic score is exposed in figure 1. At day 16$^{th}$ all treatments, except group 5, showed anti-inflammatory activity ($p < 0.05$). By day 21$^{st}$ only group 4 was different from placebo ($p < 0.05$). Since day 23$^{rd}$ and until the end of the experiment all the animals that had developed arthritis showed a severe ankylosis irrespective of the treatment applied.
- *Rat weight*. Healthy rats in the control group showed an average weight increase of 8.15% ± 3.9 from the beginning to the end of the experiment. Quite remarkably, the placebo group

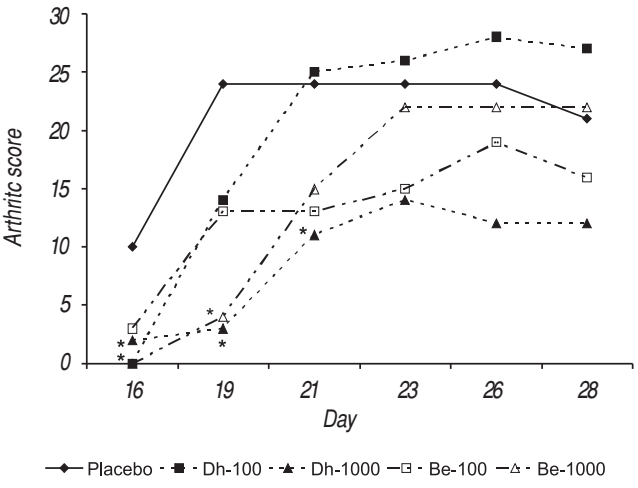


***Figure 1.*** *Arthritic score. Collagen injection was done at day 0. At day 16, when arthritis first appeared, all treatments but Be-100, had anti-inflammatory effect. At day 19, both treatments Dh-1000 and Be-1000 were effective. By day 21 only Dh-1000 group was different from placebo. Since day 23 and until the end of the experiment there was no difference among treatments (* = p < 0.05).*

showed instead a weight reduction of 5.3% ± 1.1 from the beginning of the experiment, which was statistically different from all treatment groups ($p < 0.05$). In all treatments an increase in body weight during experiment was registered. At the end, the total body weight gain was most important in group 4 (7.44% ± 1.9) and group 5 (5.48% ± 0.66), meanwhile values for group 3 and group 6 were 2.85 % ± 0.1 and 2.21% ± 0.7, respectively.

- *Anti-type II collagen antibodies*. No antibodies in the naive group were found. The rats from the placebo group showed a high antibody titer (25.7 ± 2.2 AbyU/mL), which was significant different ($p < 0.05$) only from group 4 (17.9 ± 2.8 AbyU/mL). The other groups approach the antibody titer of the placebo group. The values for groups 3, 5, and 6 were 22.34 ± 6.3, 32.13 ± 20.2, and 30.5 ± 12.2; respectively. Even the animals from treatment groups showing no arthritis, or a very mild one, had very low titer of antibodies and animals with moderate to severe arthritis had higher titers. No correlation appears to derive from arthritis intensity and antibody titer ($r^2 = 0.39$) ($p > 0.05$). Among SOD treatment groups, 55% of the rats with arthritis had antibodies, while only 30% of the rats without arthritis did ($p < 0.05$) (Table 2).
- *Histopathological analysis*. According to the criteria adopted in this study, maximum score for tissue damage was 16. No clear differences among the afflicted tissues of the groups showing arthritis were observed. The tissues showed an intense infiltration of mononuclear and polymorphonucelar cells with mild synovial, cell hyperplasia, and active destruction of cartilage.

**Table 2.** Positivity of anti-collagen antibodies.

| | SOD Treated Rats | |
|---|---|---|
| | With arthritis (%) | Without arthritis (%) |
| With antibodies | 55 | 30 |
| Without antibodies | 5 | 10 |

*All rats from placebo group had antibodies. SOD treated rats showed inhibition of antibodies production in both, with and without arthritis; though it was greater in the second ($p < 0.05$). Diagnostic test results were: Positive predictive value = 67%; Negative predictive value = 75%; Sensibility = 94%; Specificity = 27%. $p < 0.05$ $Chi^2$ (Yates correctioin). All rats from placebo group had antibodies.*

## DISCUSSION

Since the initial finding by Trentham[4] that intradermal injection of either heterologous or homologous type II collagen in the back of rats induces arthritis, this model has been considered as the one that more closely resembles human rheumatoid arthritis.[17] In the original description, Trenthan observed that approximately 40% of rats of several strains developed arthritis. Because of this, and in order to find a higher incidence of disease, several animal models were developed. Courtenay[18] got a higher incidence of arthritis after collagen injection in DBA/1 mice; however, it was only 60% and the antigen had to be mixed with Complete Freund Adjuvant instead of the incomplete form used in the rat model. In an experiment conducted by Griffiths,[7] she observed that arthritis was easily induced and with an incidence of more than 95% in some inbred rat strains, such as Dark Augui (DA) rats. She concluded that the predisposition for the development of the disease is controlled at least in part by genes whiting or closely linked to the rat major histocompatibility complex-RT1. The development of arthritis after type-II collagen injection in DA rats requires both humoral and cellular immunity,[5] with nitric oxide participation[6] and increased levels of some pro-inflammatory cytokines, like tumor necrosis factor alfa (TNF-α), interleukin 1 (IL-1), and transforming growth factor beta (TGF-β).[19]

The clinical and histological manifestations of CIA in DA rats are characteristic. The disease starts suddenly by the second week after collagen injection with maximum arthritis seen at day 21 and then slowly subsides in a couple of weeks.[20] At the late stages of the disease there is fibrous fusion of the joint with final total destruction of cartilage and new bone formation. The major histological changes include synovial hyperplasia, infiltration of the synovial tissue with inflammatory cells, exudation of cells into the joint space, marginal erosions, periostitis, fragmentation of cartilage surface, and formation of bone. These lesions are similar to those found in human rheumatoid arthritis, except for new bone formation which does not occur in human.[21]

Different anti-inflammatory and immunomodulatory drugs have been evaluated using CIA in rats[8] and mice.[22] The anti-inflammatory and immunologic effects of superoxide dismutase (SOD) has been evaluated in mice CIA;[15] however, only a small report of the enzyme regarding its anti-inflammatory an histological effects in rats has been reported.[16] In this

work, we intended to evaluate the anti-inflammatory, immune response, and histological findings of SOD's obtained from two different sources in the CIA in DA rats.

We found that both Dh-SOD and Be-SOD decreased the incidence of arthritis. By day 23 (maximum arthritis incidence and intensity in placebo group), the incidence in Be-100 and Dh-1,000 groups was 71%. This finding suggests that when administrated as a preventive treatment at some specific dose both SOD's used in this experiment can inhibit the development of arthritis in the CIA rat model. In the work of Kakimoto *et al*.[15] neither native nor pyran human SOD had an effect in preventing development of arthritis in mice CIA, while gelatin-conjugated SOD treatment group showed an incidence of only 75%. The absence of a protective effect of polyethylene glycol SOD was noticed in Beauchamp.[16] Accordingly, it appears that the source of SOD may indeed influence the activity and properties of this enzyme, and thus, its potential use in preventing arthritis and as an anti-inflammatory drug. In the present work we observed that arthritis severity varied with the different treatments. The group treated with Dh-1000 showed the mildest form of disease evolution. The group treated with Be-100 showed less severe damage than the placebo group, but somehow the effect was less protective than with the Dh-1,000 treatment. Again, different sources and doses of SOD seem to possess different biological activities.

The biological effects of SOD in the CIA model have been tentatively explained in different ways. According to Sato et al.,[23] these forms of SOD have only a mild inactivation of the protease inhibitor ($a_1$-PI) which is a very important anti-inflammatory protein. This protease is the main elastase inhibitor which is produced by polymorphonuclear cells and is essential for the initial damage of cartilage surface. This initial damage of the cartilage is a necessary step for the reactivity of antibodies against the type-II collagen found in the cartilage.[24] In addition, treatment of arthritis with SOD can also reduce cartilage damage by direct removal of ROS and, indirectly, by modulation of the immune response[25] in the inflicted tissue. The ROS are known to promote immunoglobulin aggregation, proteglycan and hyaluronic acid degradation, endothelial cell damage, and decreased lymphocyte blast transformation.[26] Also, the anti-inflammatory action of SOD may also derive from the inhibition of the ROS-mediated degradation of protein-X, which is produced by endothelial cells and is thought to play a steroid-like anti-inflammatory action.[27] Finally, by inhibition of activation of the hypoxia-inducible factor-1a and nuclear factor-kB, two key transcription factors that are regulated by changes in cellular oxygenation and cytokine stimulation, and that in turn orchestrate the expression of a spectrum of genes critical to the persistence of synovitis.[28]

Once the arthritis score started to be apparent at day 16 after type II collagen injection, it increased rapidly in all groups. The administration of SOD produced lower arthritis score than the one found in the placebo group; however, only Dh-1,000, Be-100, and Be-1,000 had significant value. The Dh-1,000 treatment group showed the lower arthritis score during the whole course of the experiment. In a previous experiment, PEG-SOD[16] showed no difference from placebo in the arthritis score of rats with CIA. When human SOD, either native or conjugated, was tested in CIA in mice,[15] no difference in the arthritis score was observed.

When different anti-inflammatory and anti-rheumatic drugs were evaluated in the CIA rat model,[8] it was evident that the non-steroideal and steroideal anti-inflammatory drugs showed prevention in the development of inflammation, this effect was assigned to the inhibition of the prostaglandin synthesis. On the other hand, some immunosupresive drugs, such as D-penicillamine, gold or levamisole, were not able to suppress edema formation. In another experiment[29] it was evident that the antirheumatic drug, bucillamine, was effective in preventing inflammation in this same model, authors concluded that this effect could be related to the inhibition of synovial cell proliferation which produce many pro-inflammatory substances like cytokines and prostaglandins. Our finding that SOD showed an anti-inflammatory effect suggests that the superoxide scavenger action of this enzyme can inhibit prostaglandin as well as cytokine synthesis.[30]

The weight in the SOD groups was not different from that of healthy rats. On the other hand, the placebo group showed an important weight decrease. Even the number of studied rats is small in order to evaluate side effects; the CIA model described here did not yield evidence of weigh loss, which is a non-desirable effect when anti-inflammatory drugs are evaluated. Quite remarkably, in contrast to those of the placebo group, animals in all SOD-treated groups maintained their tendency to gain weight, a sign interpreted to reflect healthy conditions in these groups.

The production of antibodies against type II collagen was not affect by most of the SOD treatments, but by SOD Dh-1000. These antibodies have been shown to be essential for the development of the disease in the CIA rats[5] and also have been shown to

be important in the pathogenesis of human RA.[21] The anti-inflammatory effect of some anti-rheumatic drugs, like cyclophosphamyde and prednisolone, is mediated through the inhibition of antibody synthesis (afferent pathway of immune response).[8] Our findings suggest that SOD effects its anti-inflammatory action primarily by regulating the efferent pathway of the immune response,[15] which could be related to its superoxide scavenger activity and influence of the enzyme over the production and action of soluble mediators of the inflammatory reaction.[30] However, because some non-arthritic rats had negative antibody titers, a secondary anti-inflammatory effect may be mediated through the inhibition of antibody synthesis (afferent pathway of immune response). As expected, histological changes were not different among the animals at the end of the experiment. Animals from both placebo and SOD treatments showed the same degree of damage, although synovial proliferation seemed less intense in animals treated with the enzyme.

In conclusion, in spite of being effective as an anti-inflammatory compound, SOD cannot prevent the tissue destruction induced by type II collagen injection in the CIA model. This is, so far, the most common observation with most anti-rheumatic drugs tested with this procedure. It would be interesting to test, therefore, if D-penicillamine, a drug that has been shown to be ineffective as an anti-inflammatory drug but efficient in preventing joint destruction and promoting bone formation in CIA model,[8] together with SOD could act synergistically and become useful anti-inflammatory and anti-rheumatic combined treatments.

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