JOURNAL OF APPLIED NUTRITION, VOLUME 49, NUMBERS 1 & 2, 1997 ORIGINAL REPORT

THE EFFECT OF ORAL SUPPLEMENTATION WITH LEGUME DERIVED SUPEROXIDE DISMUTASE ON ERYTHROCYTE SUPEROXIDE DISMUTASE IN HEALTHY VOLUNTEERS

Mario Introna[1], D.C., Jeffrey Moss[2], D. D. S. and Robert A. Ronzio[3]*, Ph.D.

Abstract: The effect of orally administered superoxide dismutase (SOD) on erthyrocyte SOD (ESOD) was studied in healthy volunteers using a double blind, placebo controlled protocol. This study employed Dismuzyme Plus™, a coated, copper-zinc SOD preparation from cultured non-soy legumes. An initial experiment demonstrated that 73% of the SOD activity of Dismuzyme Plus could be recovered after incubation in 0.5 percent pancreatin. Control subjects (n = 4) received a glucose placebo. The study group (n = 6) received 10 g of Dismuzyme Plus. Blood samples were collected before supplementation, and again 1, 2 and 4 hours after supplementation. ESOD activity was then determined. The ratio of the highest ESOD activity occurring at any time after baseline ($ESOD_{max}$) divided by the initial level ($ESOD_i$) was calculated for each subject. For controls the ratio of $ESOD_{max}/ESOD_i$ was 1.01; for the study group, the ratio was 1.09, a statistically significant increase ($p < .05$). Possible mechanisms underlying the increase in ESOD activity are discussed.

Key Words: superoxide dismutase, erythrocytes, legume oral supplementation

INTRODUCTION

Free radicals and reactive oxygen species (ROS) are linked to a wide range of chronic diseases and conditions, including cardiovascular disease, stroke and heart attack, neurodegenerative diseases, cancer, autoimmune disease and chronic inflammation, cataract and macular degeneration (1-5). Cumulative oxidative damage could impair cell and tissue function when ROS production outstrips the ability to block ROS and to repair radical-induced damage. Harmon proposed such a model to explain aging (6). Among ROS produced in the body, superoxide represents a prevalent free radical: It is produced by activated phagocytic cells, as well as by mitochondria and cytochrome P450 oxidation (7-9). Excessive superoxide and transition metal ions could lead to the formation of hydroxyl radicals through Fenton chemistry. Hydroxyl radicals avidly attack unsaturated fatty acids of membrane lipids to produce lipid peroxides that perturb cellular function. In addition, superoxide can react with nitric oxide to create peroxynitrite, a potent oxidant and free radical generator (10).

[1] Chiro-Med, 3705 Richmond Avenue S1, NY
[2] Moss Nutrition, 2011 Riverdale St. W. Springfield MA
[3] Address correspondence and reprint requests to:
Robert A. Ronzio, Ph.D.
Biotics Research Corporation,
4850 Wright Road,
Suite 150, Stafford, Texas 77477
Phone: 281/240-8010
FAX: 281/240-2304

This research was supported by Biotics Research Corporation

The defensive enzyme, superoxide dismutase (SOD), efficiently disposes of superoxide by catalyzing its conversion to hydrogen peroxide Three forms of SOD have been characterized in humans and other mammals: manganese SOD, localized in the mitochondrial matrix; copper/zinc SOD, associated with the cytoplasm; and extracellular copper/zinc SOD (ECSOD) ECSOD occurs in body fluids, though this enzyme in the blood is largely bound to endothelial cell membranes in the vasculature (11)

Recent research underscores the physiologic importance of superoxide dismutase (SOD) in decreasing free radical damage Induction of manganese-SOD by interleukin-1 alpha lowered the degree of injury due to myocardial reperfusion in the rat (12) Neurons from transgenic mice that over express copper/zinc SOD increased the survival of transplanted dopaminergic neurons (13) Transgenic experiments with fruit flies suggested that increased levels of endogenous SOD can minimize free radical-induced DNA damage (14) Furthermore, heparin is believed to reduce inflammation by releasing bound ECSOD into the blood (15)

Several strategies to increase SOD levels have been reported Diet can profoundly alter endogenous SOD activity Administering copper to copper-deficient rats raised ESOD levels (16) Rhesus macaques fed a marginal zinc diet had lower levels of ECSOD than animals fed a zinc-replete diet (17) Exogenous SOD can increase antioxidant activity For example, injectable SOD diminished inflammation associated with colitis and rheumatoid arthritis in humans (18) When administered topically or intravenously, SOD ameliorated tissue injury due to superoxide produced by inflammation, lung injury due to hyperoxia and reperfusion injury in lab animals (19, 20) SOD exhibited radioprotective effects when administered to experimental animals (21) SOD derivatives, including infused polyethylene glycol-SOD, have been used in hemorrhagic shock in lab animals (22)

Whether orally ingested SOD can improve antioxidant status remains an open question Even though pancreatic proteases, intestinal secretory IgA and tight junction integrity limit the uptake of luminal proteins, these mechanisms do not preclude enzyme absorption by the intestine, In principle, orally ingested SOD may be physiologically significant Because SOD possesses an extremely high turnover rate, even a modest increase in SOD activity could assist in restoring antioxidant balance

Two previous investigations of the effects of orally administered animal derived SOD on laboratory animals reported negative results One study detected an uptake of 10% of radioactivity from ingested ^{65}Zn labeled SOD isolated from bovine erythrocytes (23) The radioactive species taken up by tissues were not identified No change in the levels of SOD activity in plasma and liver was detected in animals supplemented with crude bovine SOD for 3 days (23) In a second study, mice were fed a diet supplemented with crude bovine SOD (nutritional supplement) for 7 days (24). No increase in SOD activity in kidney, liver, or intestine was detected. These studies demonstrateed several limitations. Enteric coated bovine SOD was not used, therefore uptake may have been limited by proteolytic degradation in the digestive tract. Furthermore, the specific activities of health food brands of SOD are highly variable and the specific activity such SOD preparations used in experiments is open to question unless verified by independent analysis (BA Klende, JC Stiles and WS Sparks, unpublished observations) Finally, consumption of SOD with food possibly diminishes its rate of absorption as compared to administration as a bolus on an empty stomach.

To overcome these limitations, we hypothesize that the effects of oral SOD can be best evaluated by employing coated, plant-derived SOD and assessing blood levels of SOD over time intervals of hours rather than days This assessment method is necessary in view of the reported rapid clearance of exogenous SOD (25) Therefore, the present study was undertaken to examine the ef-

fect of supplementation of SOD as Dismuzyme Plus™, a coated preparation from cultured legumes. Initial experiments were conducted to determine the stability of SOD activity in Dismuzyme Plus when incubated with pancreatic enzymes. The effect of Dismuzyme Plus on the activity of erthyrocyte SOD (ESOD) was then examined using a double blind, placebo-controlled design. As such, it represents the first report of a clinical study of orally ingested SOD on internal SOD activity

METHODS AND MATERIALS

Dismuzyme Plus™, from cultured non-soy legumes, was supplied by Biotics Research Corporation, Houston, TX. Dismuzyme Plus contains 150 µg SOD per gram, as measured according to the method of McCord and Fridovich (26). U S P. grade Pancreatin was obtained from American Laboratory, Inc., Omaha, Nebraska

Clinical study

Ten healthy volunteers (6 males, 4 females, ages 18-58) participated in this study. Informed consent was obtained from all participants and the study was approved by the Research Department of Palmer College of Chiropractic. Fasting blood samples were drawn (zero time), then the subjects were randomly selected to receive capsules containing either 10 g Dismuzyme Plus Granules (study group, n = 6) or 10 g glucose (control group n = 4). Blood samples were collected again 1, 2 and 4 hours after ingestion. Erythrocyte SOD activity was determined by Monroe Clinical Laboratory, Southfields, New York

Statistical Analysis

Initially statistical analysis was performed using a multivariate analysis of variance with repeated measures, using group as the between subjects variable and time as the within subjects variable. Then results were checked using one way analysis of variance with group one way being the dependent variable. The analyses were repeated using the nonparametric Wilcoxon test. $P < 05$ was taken as the criteria of significance

RESULTS

Stability of legume SOD activity

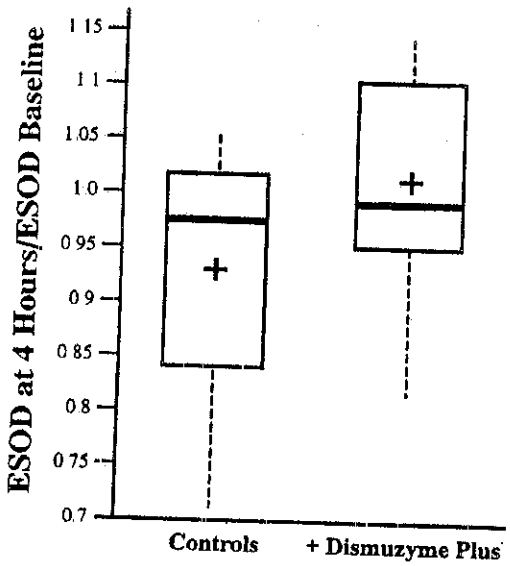
A preliminary experiment was carried out to assess the stability of SOD activity of Dismuzyme Plus under conditions approximating those occurring in the small intestine. Dismuzyme Plus (10 g) and pancreatin (500 mg) were suspended in 50 mL of 10 mM sodium phosphate buffer, pH 7 0, and incubated with stirring at 37°C. SOD activity was measured after 45 minutes. More than 73% of the initial activity was recovered after incubation with the mixed pancreatic proteases

Clinical study

Initially the ratios of ESOD activities measured 1, 2 and 4 hours after ingestion of capsules to zero time values were compared for control and supplemented subjects. The means for the 4 hour comparison shown in Figure 1 are representative. No significant difference between the subjects receiving the placebo and those receiving the SOD supplement was observed

Although none of the values exceeded the laboratory reference range for ESOD (9-13 units/mg hemoglobin), examination of the time course for each subject suggested that a slight increase in ESOD activity occurred at different times among those individuals who ingested Dismuzyme Plus. Therefore a new variable was defined as the maximum SOD activity observed at any time during the test period ($ESOD_{max}$) divided by the initial ESOD ($ESOD_i$). $ESOD_{max}/ESOD_i$ for each individual in the test group was compared with controls. Assuming a directional hypothesis, a significant difference in the direction on this variable could be demonstrated ($F(1,9) = 4,05$; p, 05, one tailed). As shown in Figure 2, the mean $ESOD_{max}/ESOD_i$ for the control group was 1 01, while this ratio for the study group supplemented with Dismuzyme Plus was 1 09. The difference represents a significant 8 percent increase in SOD activity within 4 hours

Figure 1. Comparison of ESOD activity 4 hours after supplementation with legume-derived superoxide dismutase Controls received a placebo (10 g glucose) and test subjects received 10 g Dismuzyme Plus as described in Methods and Materials Crosses denote means; boxes indicate interquartile range; and cross bars represent 50th percentiles.



of supplementation ($p < 0.05$) Because the data were skewed, the results were checked using a nonparamentric Wilcoxon test and essentially the same results were obtained.

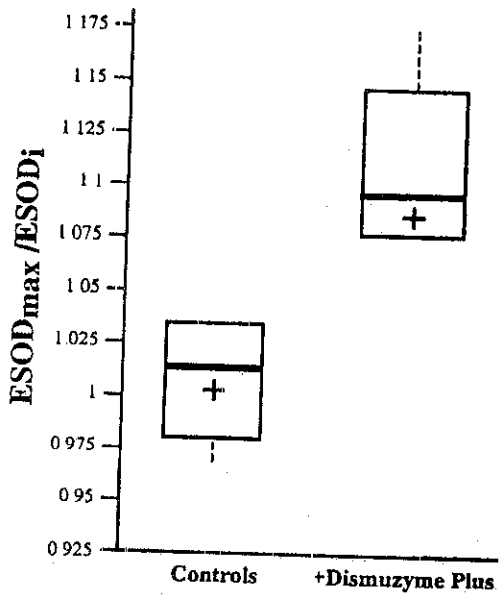
DISCUSSION

The results of control experiments indicated that less than 25% of the activity was lost through degradation by pancreatic proteases under mild conditions These data suggests that a substantial portion of legume SOD activity as Dismuzyme Plus could survive transit through the gastrointestinal tract

Anecdotal reports from physicians and veterinarians have suggested that supplemental SOD as Dismuzyme Plus may enhance antioxidant status and reduce oxidative stress Using a typical level of supplementation, 10 g of Dismuzyme Plus, an increase in ESOD was observed within 4 hours in the present study The basis for the apparent short term increase in ESOD activity is not known An 8 percent increase in ESOD activity is equivalent to approximately 1 9 μmole ESOD (27) The increase in SOD activity was greater than the amount ingested.

Erythrocytes do not participate in protein synthesis and the lifespan of human erythrocytes is 120 days Consequently, the relatively rapid increase in ESOD activity was independent of *de novo* synthesis This fact would seem to rule out

Figure 2. Comparison of maximal ESOD activity during 4 hour test period Control subjects received a placebo and the supplemented group received Dismuzyme Plus, as described in Figure 1 The maximal ESOD activity was divided by base line values for each subject Symbols are as presented in Figure 1



adaptation which is known to occur with either oxidative stress, thyroid hormone or other factors that involve protein synthesis and require time spans of weeks (29)

Among the several possible explanations of the apparent stimulation of ESOD by Dismuzyme Plus supplementation are the binding of existing SOD to erythrocytes or the activation of an apoenzyme. *In vitro* experiments demonstrated that negligible ECSOD binds to erythrocytes (11), therefore it seems unlikely that the binding of ECSOD to erythrocytes led to increased ESOD activity.

Recent evidences suggests that SOD is secreted by human cell lines (30) Whether this mechanism can increase blood ESOD levels *in vivo* is not clear Dismuzyme Plus is a crude enzyme preparation and therefore contains many substances in addition to SOD, including copper and zinc up to 10 µg per gram. If both transition metals in Dismuzyme Plus were efficiently absorbed, they could potentially activate SOD Depending upon copper status, exogenous copper can activate apoenzyme SOD in rat erythrocytes within hours of supplementation (16) ESOD in bovine erythrocytes can often be activated by additional copper *in vitro* (RT Coffey, personal communication). The typical U S diet supplies less than Estimated Safe and Adequate Daily Dietary Intake of copper, and the copper status for certain groups is suboptimal (31). On the other hand, human studies in which SOD activity was lower due to copper deprivation; recovery of SOD activity did not occur when ≤ 2.6 mg of copper per day was supplemented for up to 42 days (32)

Among the subjects receiving the SOD supplement, $ESOD_{max}/ESOD_i$ values occurred after 1 hour in one subject; after 2 hours in three subjects; and after 4 hours in the remaining two subjects The reason for these differences may reflect individual variation in nutrient uptake. Several factors affect intestinal permeability and nutrient uptake, including alcohol, aspirin and other medications. These were not controlled in this study Ingestion of such substances may explain the individual variations observed.

Whatever the nature of the underlying mechanism of activation, these results suggest that 1) the stimulation of ESOD activity may be transient, and 2) there may be a benefit in supplementing at several different times during the day, rather than once The present study does not rule out the possibility that ingested plant SOD can be absorbed The absorption of intact enzymes by the small intestine has been amply demonstrated in the literature and orally ingested proteolytic enzymes have long been used as effective supplements (33). Whether a sustained increase in ESOD activity can be promoted by prolonged supplementation with a coated, legume derived SOD as Dismuzyme Plus remains to be determined

REFERENCES

1 Witztum JL The oxidation hypothesis of atherosclerosis *Lancet* Sept 17, 1994; 344: 793-795

2 Williams LR. Oxidative Stress, Age-Related Neurodegeneration, and the Potential for Neurotrophic Treatment *Cerebrovascular Brain Meta Reviews* 1995; 7: 55-73

3 Ames BN Endogenous oxidative damage, aging and cancer. *Free Rad Res Communs* 1989; 7: 121-128

4 Ribbons KA Xiao-Jing Z, Thompson JH, Greenberg SS, Moore WM, Kommeier CM, Currie MG, Lerche N Blanchard J, Clark DA and Miller MJS. Potential Role of Nitric Oxide in a Model of Chronic Colitis in Rhesus Macaques *Gastroenterology* 1995; 108: 705-711

5 West S, PHD Vitale S, MHS Hallfrisch J PHD, Munoz B MS, Muller D, MS, Bressler S, MD, Bressler NM MD Are Antioxidants or Supplements Protective for Age-Related Macular Degeneration? *Arch Ophthalmol* 1994; 112: 222-227

6 Harman D Free radical involvement in aging Pathophysiology and therapeutic implications *Drugs Aging* 1993: 3: 60-80

7 Hurst JK and Barrette WC, Jr Leukocytic oxygen activation and microbicidal oxidative toxins *Mol Biol* 1989; 24: 271-328

8 Chance B Seis H, Boveris A Hydroperoxide metabolism in mammalian organs *Physiol Rev* 1979: 59:527-603

9 Ames BN, Shigenga MK, Hagen TM Oxidants antioxidants and the degenerative diseases of aging *Proc Natl Acad Sci (USA)* 1993; 90: 7915-7922

10 Ma TT, Ischiropoulos H and Brass CA Endoxtoxin-Stimulated Nitric Oxide Production Increases Injury and Reduces Rat Liver Chemiluminescence During Reperfusion Gastroenterology 1995; 108: 463-469

11 Karlsson K and Marklund SL Binding of human extracellular-superoxide dismutase C to cultured cell lines and to blood cells *Lab Invest* May 1989; 60 (5): 659-666

12 Visner GA Dougall WC, Wilson JM, Burr IA, and Nick HS Regulation of manganese superoxide dismutase by lipopolysaccharide interleukin-1 and tumor necrosis factor *J Bio Chem* 1990; 265:2856-2864

13 Nakao N, Frodl EV, Widner H, Carlson E Eggerding, FA; Epstein CJ & Brundin P. Over expressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease *Nature Medicine* March 3 1995; 1 (3): 226-

14 Orr WC and Shohal RC Extension of life span by over expression of superoxide dismutase and catalase in Drosophila melanogaster *Science* 1994; 263:1128-1130

15 Karlsson K and Marklund SL. Heparin-induced release of extracellular superoxide dismutase to human blood plasma *Biochem J* 1987; 242 (1): 55-59

16 DiSilvestro RA Copper Activation of Superoxide Dismutase in Rat Erythrocytes *Arch Biochem Biophys* 1989; 274 (1): 298-303

17 Olin KL Golub MS Gershwin ME, Hendricks AG, Lonnerdale B and Keen CL Extracellular superoxide dismutase activity is affected by dietary zinc intake in nonhuman primate and rodent models *Am J Clin Nutr* 1995; 61: 1263-1267

18 Jadot G Vaille A, Maldenado J and Vanelle P Clinical pharmacokinetics and delivery of bovine superoxide dismutase *Clin Pharmacokinet* 1995; 28:17-25

19 Mikawa K, Nishina K, Maekawa N and Obara H Attenuation of hyperoxic lung injury in rabbits with superoxide dismutase: effects on inflammatory mediators *Acta Anaesthesiol Scand* 1995; 39:317-322

20 Mihara K, Okay Y, Sawai K, Takakura Y and Hashida M Improvement of therapeutic effects of human recombinant superoxide dismutase on ischemic acute renal failure in rat via cationization and conjugation with polyethylene glycol *J Drug Target* 1994; 2:317-321

21 Jones JB Cramer HM Inch WR and Lampe HB Radioprotective effect of free radical scavenging enzymes *J Otolaryngology* 1990; 19:299-306

22 Rhee P MD, Waxman K MD Clark L BA Tominaga G, MD and Soliman MH MD Superoxide Dismutase Polyethylene Glycol Improves Survival in Hemorrhagic Shock The Am Surgeon 1991; 57:747-750

23 Giri SN and Misra HP Fate of Superoxide Dismutase in Mice following oral route of administration *Med Biol* 1984; 62: 285-289

24 Zidenberg-Cherr S Keen CL Lonnerdale B and Hurley LS Dietary superoxide dismutase does not affect tissue levels *Am J Clin Nutr* 1983; 37: 5-7

25 Petkau A Chelack WS Kelly K, Barefoot C and Monastersk L. Tissue distribution of bovine ^{125}I-superoxide dismutase in mice *Res Commun Chem Path Pharmacol* 1976; 15:641-654

26 McCord JM and Freidovich I Superoxide dismutase: on enzymic function for erythrocuprein (hemocuprein) *J Biol Chem* 1969; 244:6049-6055

27 De Villano BC and Tischfield JA. A radio immune assay for human cypro-zinc superoxide dismutase and its application to erythrocytes *J Immunol Methods* 1979; 29:253-262

28 Sawada Y Ohyama T and Yamazaki I Preparation and physicochemical properties of green pea superoxide dismutase *Biochim Biophs Acta* 1971; 268:305-312

29 Saito T Kurasaki M and Saito K Increased erythrocyte superoxide dismutase and catalase activities in patients with hyperthyroidism *Hokkaido University Medical Library* 1994; 31:133-140

30 Mondola P Annella T Santillo M and Santangelo F Evidence for secretion of cytosolic CuZn superoxide dismutase by HepG2 cells and human fibroblasts *Int J Biochem Cell Biol* 1996; 28:677-681

31 Olivares M and Uauy R. Copper as an essential nutrient *Am J Clin Nutr* 1996; 63:791S-796S

32 Milne DB and Nielsen FN Effects of a diet low in copper on copper-status indicators in postmenopausal women *Am J Clin Nutr* 1996; 63:358-364

33 White RR Crawley EH Vellini M and Rovati LA Bioavailability of ^{125}I bromelain after oral administration to rats *Biopharm Drug Disposition* 1988; 9:397-403