Original Contribution

The induction of human superoxide dismutase and catalase in vivo: A fundamentally new approach to antioxidant therapy

Sally K. Nelson [a,b], Swapan K. Bose [a], Gary K. Grunwald [c], Paul Myhill [d], Joe M. McCord [a,b,d,*]

[a] Webb-Waring Institute for Cancer, Aging and Antioxidant Research, University of Colorado Denver Health Sciences Center, Denver, CO 80262, USA
[b] Department of Medicine, University of Colorado Denver Health Sciences Center, Denver, CO 80262, USA
[c] Department of Preventive Medicine and Biometrics, University of Colorado Denver Health Sciences Center, Denver, CO 80262, USA
[d] Lifeline Therapeutics, Denver, CO, USA

Abstract

A composition consisting of extracts of five widely studied medicinal plants (Protandim) was administered to healthy human subjects ranging in age from 20 to 78 years. Individual ingredients were selected on the basis of published findings of induction of superoxide dismutase (SOD) and/or catalase in rodents in vivo, combined with evidence of decreasing lipid peroxidation. Each ingredient was present at a dosage sufficiently low to avoid any accompanying unwanted pharmacological effects. Blood was analyzed before supplementation and after 30 and 120 days of supplementation (675 mg/day). Erythrocytes were assayed for SOD and catalase, and plasma was assayed for lipid peroxidation products as thiobarbituric acid-reacting substances (TBARS), as well as uric acid, C-reactive protein, and cholesterol (total, LDL, and HDL). Before supplementation, TBARS showed a strong age-dependent increase. After 30 days of supplementation, TBARS declined by an average of 40% ($p = 0.0001$) and the age-dependent increase was eliminated. By 120 days, erythrocyte SOD increased by 30% ($p < 0.01$) and catalase by 54% ($p < 0.002$). We conclude that modest induction of the catalytic antioxidants SOD and catalase may be a much more effective approach than supplementation with antioxidants (such as vitamins C and E) that can, at best, stoichiometrically scavenge a very small fraction of total oxidant production.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Superoxide dismutase; Catalase; Lipid peroxidation; TBARS; Antioxidant; Protandim; Free radicals

Oxidative stress is now recognized to be associated with more than 200 diseases, as well as with the normal aging process. In nearly all cases it is not clear whether the role is a causative one or whether the oxidative damage is simply a sequela of other types of tissue injury. The primary tools available to probe this question have been supplementation with exogenous antioxidants such as vitamins C and E, carotenoids, and a long list of other compounds capable of reacting stoichiometrically with reactive oxygen species such as superoxide and hydrogen peroxide. The results of studies with supplemental antioxidants have been quite disappointing overall. For example, a compelling amount of evidence has led to the "oxidative hypothesis" of atherosclerosis [1,2], yet randomized, double-blind, placebo-controlled studies such as the HOPE and HOPE-TOO trials have concluded that vitamin E supplementation does not prevent cancer or major cardiovascular events and may, in fact, increase the risk for heart failure [3,4]. A similar situation exists for diabetes, in that despite the undeniable presence of substantial oxidative stress, attempts to treat the disease by supplementation with antioxidants have failed to produce any significant improvement [5].

Because polyunsaturated fatty acids are easy targets for oxidants, and because the process of lipid peroxidation is, once initiated, a self-sustaining free radical chain process, the accumulation of lipid peroxidation products provides the most common biochemical marker of oxidative stress. There is strong correlation between thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation and products that reflect oxidative damage to DNA [6]. However, in normal

* Corresponding author. Webb-Waring Institute for Cancer, Aging and Antioxidant Research, University of Colorado Denver Health Sciences Center, Denver, CO 80262, USA. Fax: +1 303 315 8541.
E-mail address: joe.mccord@uchsc.edu (J.M. McCord).

healthy men who have low intake of fruits and vegetables, and who might be further stressed by smoking, and who have measurable levels of oxidative stress, a moderate supplement of vitamins E, C, and folic acid produced no alteration in measures of oxidant damage [7]. Similarly, studies that have used supplementation with a concentrate of fruits and vegetables [8] or the daily intake of 600 g of fruits and vegetables [9] have produced no effects on markers of oxidative damage to lipids or DNA. Thus, reasonable intakes of exogenous stoichiometric scavengers of oxidants fail to inhibit lipid peroxidation significantly.

This study has taken a different approach: the induction of endogenous antioxidant enzymes. The antioxidant enzymes superoxide dismutase (SOD) and catalase, by virtue of their ability to catalyze the disproportionation reactions of their substrates superoxide radical and hydrogen peroxide, respectively, have an enormous theoretical advantage over exogenous antioxidants that are stoichiometrically consumed. There are published reports of at least 30 different botanical extracts or purified phytochemicals that when ingested by mammals result in increased activities of SOD and catalase, with concomitant decreases in plasma TBARS indicative of decreased lipid peroxidation, which has come to be synonymous with decreased oxidative stress. It is assumed that these substances act primarily by *direct* induction of SOD and catalase and that this results in decreased oxidative stress.

In contrast, there are other substances that also result in elevated activities of SOD and catalase, but with concomitantly *higher* levels of lipid peroxidation. Among this class of substances are stanozolol [10], an extract of *Terminalia arjuna* [11], retinol [12], malathion [13], and cocaine [14]. Here, it is assumed that the substances act primarily by increasing the production of $O_2^{\bullet-}$ and H_2O_2, increasing oxidative stress levels. Any increased production of SOD and catalase is presumed to be secondary in these cases, reflecting an attempt to compensate partially for the increased oxidative stress. Interestingly, there is evidence that even this second method of inducing antioxidant enzymes in response to an oxidative insult can be protective as a way of developing "tolerance" to a subsequent larger insult [15]. Obviously, however, it would be more desirable to be able to induce the antioxidant enzymes without first inflicting oxidative damage.

The ingredients used in this study are derived from five botanical sources [*Bacopa monniera, Silybum marianum* (milk thistle), *Withania somnifera* (Ashwagandha), *Camellia sinensis* (green tea), and *Curcuma longa* (turmeric)]. They were selected on the basis of meeting several criteria. Each has substantial human experience in traditional medicine, establishing safety. At the doses selected, none was expected to produce unwanted pharmacological effects. Where mild side effects have been reported at much higher doses (e.g., a mild tranquilizing effect with *W. somnifera* and a mild stimulant effect with green tea extract) ingredients were selected to have offsetting effects. Most importantly, each is reported to have the ability to increase the activities of SOD and catalase while decreasing plasma TBARS [16–24]. Thus, we hoped that the desired effects would be additive in this five-ingredient composition, while individual dosages were maintained at levels sufficiently low to avoid any unwanted effects.

Materials and methods

This study involving human subjects was approved by the Colorado Multiple Institution Review Board (COMIRB 04-0556). The dietary supplement Protandim (Lifeline Therapeutics, Inc., Denver, CO, USA) was provided as a once daily capsule of 675 mg, consisting of the following: *B. monniera* (45% bacosides), 150 mg; *S. marianum* (70–80% silymarin), 225 mg; *W. somnifera* powder, 150 mg; green tea, 98% polyphenols and 45% (−)-epigallocatechin-3-gallate, 75 mg; and turmeric (95% curcumin), 75 mg. These five standardized plant extracts were supplied by the Chemins Co. (Colorado Springs, CO, USA).

Twenty-nine healthy volunteer subjects of both genders ranged in age from 20 to 78 years. Subjects were enrolled regardless of whether they supplemented with exogenous stoichiometric antioxidants (e.g., vitamins E and C) and were advised to continue their normal pattern, but were excluded if they were taking a supplement containing one or more of the five botanical extracts in Protandim. Blood samples were analyzed from these 29 subjects before any supplementation with Protandim to establish the age-related increase in lipid peroxidation. Subsequently, subjects were assigned to either of two groups. Group 1 consisted of 20 subjects who received the full supplement of 675 mg in a single daily capsule for 30 days. Twelve subjects continued the supplement for 120 days. At 0, 30, and 120 days, blood was taken by venipuncture for analysis. Group 2 consisted of 4 additional participants who received one-half as much Protandim, or 338 mg in a single daily capsule for 30 days. Blood was taken from Group 2 subjects at 0, 5, 12, and 30 days.

All blood samples were collected in heparinized tubes. Erythrocytes and plasma were separated by centrifugation. Packed erythrocytes were hemolyzed by a 10-fold dilution with deionized water. This hemolysate was analyzed for SOD by the standard assay of McCord and Fridovich [25] and for catalase by the method of Beers and Sizer [26]. Plasma was assayed for TBARS by the method of Ohkawa et al. [27] using 1,1,3,3-tetramethoxypropane (Sigma) as a standard.

In addition, plasma was analyzed by the clinical chemistry laboratory (University Hospital, Denver, CO, USA) for uric acid, high sensitivity C-reactive protein (CRP), and cholesterol (total cholesterol, LDL, and HDL). The Beckman Coulter Synchron LX System (Beckman Coulter, Inc., Fullerton, CA, USA) was used for the quantitative determination of these parameters. Uric acid was measured by a timed-endpoint method in which uric acid is oxidized by uricase to produce allantoin and hydrogen peroxide [28]. The hydrogen peroxide reacts with 4-aminoantipyrine and 3,5-dicloro-2-hydroxybenzene sulfonate in a reaction catalyzed by horseradish peroxidase to produce a colored product monitored at 520 nm. High sensitivity CRP was measured using a method based on the highly sensitive near-infrared particle immunoassay rate. An

anti-CRP antibody-coated particle binds to CRP in the sample, resulting in the formation of insoluble aggregates causing turbidity. Change in absorbance is monitored at 940 nm. Cholesterol was determined using cholesterol esterase to hydrolyze cholesterol esters to free cholesterol and fatty acids. Free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyzes the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a colored quinoneimine product read at 520 nm [29]. The HDL cholesterol method depends on a unique detergent, which solubilizes only the HDL lipoprotein particles and releases HDL cholesterol to react with cholesterol esterase and cholesterol oxidase in the presence of chromogens, to produce a colored product, which is read at 560 nm.

Data are presented as means ± standard error. Means of groups before and after Protandim supplementation are compared using paired *t* tests. Other comparisons (male vs female or vitamin E and C supplemented vs nonsupplemented) are by unpaired *t* test analysis. Pearson correlations are used to measure associations with age.

Results

Assessment of toxicity or side effects

All subjects were instructed to report any suspected adverse reaction or side effect (such as nausea, vomiting, headache, drowsiness, gastrointestinal discomfort, diarrhea, constipation, itching) to the investigators immediately and to discontinue use of the supplement. No such reactions or side effects were reported.

Effect on lipid peroxidation and TBARS

The primary objective of Protandim supplementation is to decrease oxidative stress. Our endpoint to assess oxidative stress in this study was TBARS, which measures a family of lipid peroxidation products (mostly lipid hydroperoxides) that break down during the analysis to yield malondialdehyde, which reacts with 2-thiobarbituric acid to yield a chromophore measured at 532 nm [27].

Fig. 1A illustrates the age-related increase in plasma TBARS in 29 healthy human subjects ranging in age from 20 to 78 years, before supplementation with Protandim. There is substantial scatter around the linear regression line and a clear correlation with age ($R^2 = 0.24$; $p = 0.007$), with the oldest individuals showing values up to threefold higher than the youngest individuals. There was no gender difference in this relationship (Fig. 1B; males, $n = 19$, average age 45.6, TBARS 1.99 ± 0.17 μM malondialdehyde equivalents; females, $n = 10$, average age 49, TBARS 2.04 ± 0.26; $p = 0.85$). There was a statistically significant difference between subjects who self-supplemented with vitamins C (usually 500 mg/day) and E (usually 400 IU/day) (Fig. 1C, $n = 13$, average age 47.5, TBARS 2.33 ± 0.22) versus those who took no supplemental vitamins ($n = 16$, average age 46.2, TBARS 1.75 ± 0.16, $p = 0.04$). Surprisingly, perhaps, those who did not supplement had lower TBARS than those who did. If we ignore the age
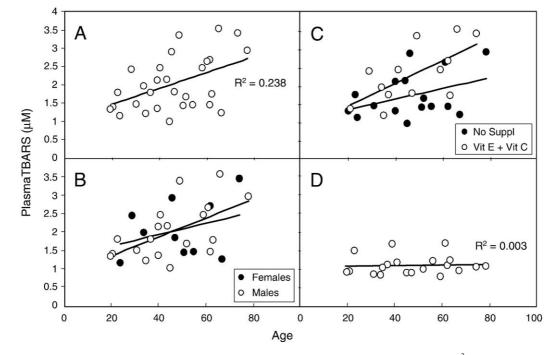

Fig. 1. (A) Normal subjects before supplementation with Protandim showed an age-dependent increase in TBARS ($n = 28$; $R^2 = 0.238$, $p = 0.007$). (B) Separation of these subjects by gender showed no significant differences. (C) The subjects represented by open circles ($n = 13$) self-reported supplementation with vitamin C and vitamin E; those represented by filled circles ($n = 16$) took no vitamin supplements. The subjects who took vitamins E and C showed significantly higher TBARS ($p = 0.04$) as well as a greater age relatedness. (D) The levels of TBARS dropped an average of 40% ($n = 20$; $p < 0.0001$) after 30 days of Protandim supplementation, and the age-related increase in TBARS disappeared ($R^2 = 0.003$, $p < $ ns).

relationship, the average pretreatment TBARS value for Group 1 subjects was 1.82 ± 0.15 ($n = 20$); this was significantly lowered by 40% to 1.10 ± 0.05 μM ($p = 0.0001$ by paired t test) after supplementation with Protandim for 30 days (675 mg/day). The scatter is remarkably less after Protandim (Fig. 1D), and the correlation of TBARS with age disappears ($R^2 = 0.003$; $p = 0.81$). Ten Group 1 individuals were assayed after 120 days of supplementation and showed no further change (0.93 ± 0.06 μM). All subjects but one (19/20) showed decreased TBARS after 30 days on Protandim. The age-related increase in lipid peroxidation products disappeared with Protandim supplementation. The changes were maintained at 120 days, with results indistinguishable from those at 30 days.

Plasma samples obtained before supplementation and after 30 days of supplementation were assayed with and without the inclusion of an internal standard, 1,1,3,3-tetramethoxypropane, to control for the possibility that Protandim components present in plasma might interfere with, or inhibit, the TBARS assay. The internal standard was fully recoverable (111%) in plasma, whether before or after supplementation.

Effect on SOD activity

Group 1 subjects supplemented with Protandim showed an average increase of $30 \pm 10\%$ in erythrocyte SOD activity after 120 days of supplementation, as seen in Fig. 2A. This increase was statistically significant ($n = 10$, $p < 0.01$ by paired t test). It should be noted that mature, circulating erythrocytes do not contain nuclei and therefore are not capable of inducing new synthesis of enzymes once they enter the circulation. Erythrocytes have a circulating life span of 120 days. Thus, during the 120-day course of the experiment 100% of the red cells would have been replaced by maturing reticulocytes from the bone marrow. This 30% increase should therefore represent a steady state that would be maintained if supplementation were extended indefinitely.

Effect on catalase activity

Group 1 subjects supplemented with Protandim showed a statistically significant increase of $54 \pm 14\%$ in erythrocyte catalase activity after 120 days of supplementation, as seen in Fig. 2B ($n = 10$, $p < 0.002$ by paired t test). The same considerations regarding turnover and replacement of erythrocytes apply to catalase as discussed above for SOD.

Effect on plasma uric acid

Subjects supplemented with Protandim showed an increase of $4.9 \pm 5\%$ in plasma uric acid concentration after 120 days of supplementation, but this increase did not achieve statistical significance. Because uric acid serves as an endogenous antioxidant, it was anticipated that uric acid levels might rise as a result of increased SOD activity, which would lead to lower levels of peroxynitrite production. Uric acid is thought to scavenge the oxidant peroxynitrite.

Effect on other blood parameters and lipid profile

No significant effects were seen in C-reactive protein levels overall. Only four subjects entered the study with elevated C-reactive protein levels. There was a nonsignificant trend toward reduction in three of these subjects, suggesting that further study of this parameter might be justified. No significant changes were seen in total cholesterol, LDL, HDL, or triglycerides.

Time course of lowering TBARS and effect of low-dose Protandim (338 mg/day)

To assess whether the suggested human supplement of 675 mg/day might be more than needed to achieve the desired reduction in oxidative stress, four subjects were given a lower dose of 338 mg/day for 30 days. Blood was drawn from these individuals on days 0, 5, 12, and 30 to provide additional information regarding the time required for the reduction in oxidative stress to manifest. Fig. 3 shows that the response of
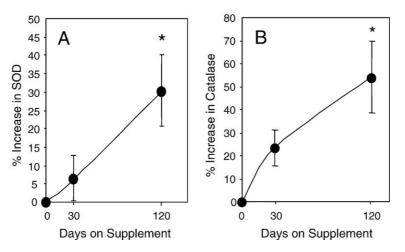

Fig. 2. (A) Group 1 subjects supplemented with Protandim for 120 days showed a significant increase (*) in erythrocyte SOD of $30 \pm 10\%$ ($n = 10$, $p < 0.01$). (B) Group 1 subjects supplemented with Protandim for 120 days showed a significant increase (*) in erythrocyte catalase of $54 \pm 15\%$ ($n = 10$, $p < 0.002$).
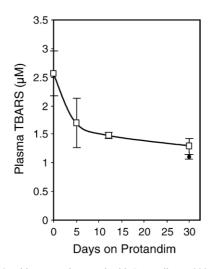
Fig. 3. Group 2 subjects supplemented with Protandim at 338 mg/day for 30 day showed a substantial decline in TBARS at 5 and 12 days (squares, $n = 4$). By 30 days, the levels of plasma TBARS dropped an average of 50% ($p < 0.03$). The closed circle shows the average TBARS of the Group 1 (675 mg/day) subjects after 30 days, which is not significantly different.

plasma TBARS is fairly rapid, with most of the change having occurred by 5 to 12 days. Fig. 3 also shows that the half-dose of Protandim was not quite as effective after 30 days as the full dose, lowering TBARS to an average value of 1.29 ± 0.14 μM ($n = 4$) versus 1.10 ± 0.05 μM ($n = 20$), although this difference is not significant. This, together with the nearly complete elimination of age-relatedness produced by the 675 mg/day dose, provides some assurance of the appropriateness of the 675 mg/day dose.

Discussion

No toxicity or evidence of other unwanted pharmacological effects of Protandim were noted at either level of supplementation. This, combined with the extensive human safety records of the individual botanical components of the composition and the relatively low doses used gives assurance that Protandim is a safe nutraceutical supplement.

The TBARS test is the most widely used in the literature to assess lipid peroxidation and was selected because it allows direct comparison with the largest number of studies from other laboratories. In particular, a recent study found plasma TBARS to be a predictor of cardiovascular events in patients with established heart disease, independent of traditional risk factors and inflammatory markers [2]. The TBARS assay has been somewhat controversial, criticized by some for lack of specificity because it yields higher values than gas chromatographic methods specific for malondialdehyde. Because of the high reactivity of malondialdehyde per se, its steady-state concentration may be very low and difficult to accurately assess. Thus, it is this ability of the TBARS test to collectively measure lipid peroxidation products, including precursors that will continue to break down to yield malondialdehyde, that we and others [30,31] regard as a strength of the assay. It is clear that native fatty acids do not undergo significant peroxidation during the acid-heating stage of the TBA test [30]. Rather, a

variety of species that are at or beyond the committed step (of lipid hydroperoxide formation) complete their breakdown to produce malondialdehyde during this stage.

Mature circulating erythrocytes do not contain nuclei and therefore do not have the capacity to induce new synthesis of enzymes once they enter circulation. Erythrocytes have a circulating life span of 120 days. Therefore, by the end of our experimental study we would expect that 100% of all erythrocytes have turned over, reflecting the new steady-state levels of SOD and CAT induced by Protandim. Stated another way, looking at erythrocyte levels of SOD and CAT gives a time-delayed snapshot of enzyme levels in recently produced cells. At 30 days only one-fourth of the erythrocytes display the new enzyme levels, so this dilution by the older pre-Protandim erythrocytes must be taken into account. The changes in the 30-day means seen in Fig. 2 are only about 25% of what they will become, and for this reason they are not yet significant. In humans, erythrocytes are the only tissue easily assayed. If liver and muscle were biopsied, we would expect to see a more rapid induction, as all cells in these tissues have nuclei and ongoing synthesis of new protein. We do not believe that the decrease in plasma TBARS reflects simply the SOD and CAT levels of erythrocytes, but rather those of all tissues.

The age-dependent increase in oxidative stress seen here in subjects before treatment (Fig. 1A) is very similar to that reported by others [32]. Remarkably, this age-dependent increase in TBARS was almost completely abolished by Protandim treatment (Fig. 1D), with an overall average reduction of the oxidative stress marker by 40%. This study met its objectives of establishing that the botanical composition defined herein is a safe and effective way of decreasing oxidative stress in healthy human subjects ranging in age from 20 to 78. The dosage defined (675 mg/day) seems well positioned for safety and efficacy. There was no evidence that the subjects showing the lowest initial levels of oxidative stress were in any way compromised by the modest elevations of SOD and catalase that were achieved—an outcome considered remote but theoretically possible due to our recognition that problems can result from too much SOD as well as from too little [33,34]. The results from this study show that subjects benefited to varying degrees from the Protandim-induced elevations of SOD and catalase activities. Even those with the lowest initial levels of lipid peroxidation saw modest declines, and in only one subject (1/20) was the post-Protandim value slightly higher that the initial value of TBARS. Serum TBARS values are known to fluctuate daily based on type, quantity, and timing of food ingested [35].

The effects of Protandim may go beyond direct induction of the SOD and/or catalase genes. The antioxidant enzymes form a system of mutual protection [36]: superoxide can inactivate both catalase [37] and glutathione peroxidase [38], whereas hydrogen peroxide can inactivate the cytosolic SOD [39]. Thus, in a system experiencing substantial oxidative stress the entire group of antioxidant enzymes may be subject to partial inactivation by the unscavenged concentrations of superoxide and hydrogen peroxide. If, under these conditions, SOD alone were induced, the concentration of superoxide would decrease, allowing partial

recovery of the activities of catalase and glutathione peroxidase as they escape from superoxide-mediated inactivation. Thus, it would seem that all three enzymes were induced if activities were measured. This might be expected only when starting under conditions of substantial oxidative stress. Under normal conditions, there may be little inactivation of catalase and glutathione peroxidase taking place, so the induction of SOD might have less effect on the activities of the other two enzymes. Another factor to consider is that 4-hydroxynonenal, a product of lipid peroxidation, serves to induce the synthesis of glutathione peroxidase [40]. If the induction of SOD results in a lowering of the rate of lipid peroxidation, then the concentration of 4-hydroxynonenal would fall and one might expect that less glutathione peroxidase would be synthesized, because less would be needed.

The possible effects of upregulation of antioxidant enzymes on longevity and life span should not go unmentioned. The so-called "free radical theory of aging" proposed first by Denham Harman in 1956 [41] may not account for all aspects of aging, but is nonetheless widely held. Mitochondrial metabolism is known to be a major source of superoxide generation, as well as of the non-free radical oxidant hydrogen peroxide, contributing to oxidative stress and aging [42]. The maximum life span of *Drosophila melanogaster* was significantly increased by the transgenic overexpression of both SOD and catalase [43], lending strong support to the Harman theory. Other work in mammals suggested a possible correlation between SOD and/or catalase expression and life span [44,45]. Very recently, Schriner et al. [46] have significantly extended both median and maximal life span in transgenic mice expressing a catalase targeted to the mitochondria. In view of this work, perhaps the "free radical theory of aging" should be renamed as the "oxidative stress theory of aging" to be inclusive of nonradical oxidants.

The significant difference seen in Fig. 1C between subjects who self-supplemented with vitamins C and E and those who took no antioxidant supplements was unexpected but interesting. The study was not designed to examine this point—there are no before- and -after or crossover data, so the result should be viewed with some reservation. It is possible, e.g., that subjects who have elevated oxidative stress and do not feel well may supplement more frequently; conversely, those who feel healthy may have lower levels of lipid peroxidation and may not feel the need to supplement. On the other hand, there are many studies documenting the pro-oxidant effect of vitamin C and its ability to increase TBARS production [47–49].

Given the lack of effectiveness in inhibiting lipid peroxidation in vivo by reasonable levels of supplementation with conventional stoichiometric antioxidants such as vitamins C and E [3–5,7,50,51] or by intake of fruits and vegetables [8,9], we believe the fundamentally different approach of safely and modestly inducing endogenous antioxidant enzymes may finally provide a powerful tool to study oxidative stress, the diseases associated with oxidative stress, and the aging process itself. Given the increasing awareness of TBARS as a useful clinical marker strongly and independently predictive, e.g., of cardiovascular events [2], we hope that the measurement and

control of oxidative stress may finally enter the arena of human health and medicine.

References

[1] Witztum, J. L.; Steinberg, D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? *Trends Cardiovasc. Med.* **11:**93–102; 2001.

[2] Walter, M. F.; Jacob, R. F.; Jeffers, B.; Ghadanfar, M. M.; Preston, G. M.; Buch, J.; Mason, R. P. Serum levels of thiobarbituric acid reactive substances predict cardiovascular events in patients with stable coronary artery disease: a longitudinal analysis of the PREVENT study. *J. Am. Coll. Cardiol.* **44:**1996–2002; 2004.

[3] Lonn, E.; Bosch, J.; Yusuf, S.; Sheridan, P.; Pogue, J.; Arnold, J. M.; Ross, C.; Arnold, A.; Sleight, P.; Probstfield, J.; Dagenais, G. R. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA* **293:**1338–1347; 2005.

[4] Brown, B. G.; Crowley, J. Is there any hope for vitamin E? *JAMA* **293:**1387–1390; 2005.

[5] Wiernsperger, N. F. Oxidative stress: the special case of diabetes. *Biofactors* **19:**11–18; 2003.

[6] Chen, H. J.; Wu, C. F.; Huang, J. L. Measurement of urinary excretion of 5-hydroxymethyluracil in human by GC/NICI/MS: correlation with cigarette smoking, urinary TBARS and etheno DNA adduct. *Toxicol. Lett.* **155:**403–410; 2005.

[7] Jacob, R. A.; Aiello, G. M.; Stephensen, C. B.; Blumberg, J. B.; Milbury, P. E.; Wallock, L. M.; Ames, B. N. Moderate antioxidant supplementation has no effect on biomarkers of oxidant damage in healthy men with low fruit and vegetable intakes. *J. Nutr.* **133:**740–743; 2003.

[8] van den, B. R.; van Vliet, T.; Broekmans, W. M.; Cnubben, N. H.; Vaes, W. H.; Roza, L.; Haenen, G. R.; Bast, A.; van den B., H. A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. *J. Nutr.* **131:**1714–1722; 2001.

[9] Moller, P.; Vogel, V.; Pedersen, A.; Dragsted, L. O.; Sandstrom, B.; Loft, S. No effect of 600 grams fruit and vegetables per day on oxidative DNA damage and repair in healthy nonsmokers. *Cancer Epidemiol. Biomarkers Prev.* **12:**1016–1022; 2003.

[10] Pey, A.; Saborido, A.; Blazquez, I.; Delgado, J.; Megias, A. Effects of prolonged stanozolol treatment on antioxidant enzyme activities, oxidative stress markers, and heat shock protein HSP72 levels in rat liver. *J. Steroid Biochem. Mol. Biol.* **87:**269–277; 2003.

[11] Karthikeyan, K.; Bai, B. R.; Gauthaman, K.; Sathish, K. S.; Devaraj, S. N. Cardioprotective effect of the alcoholic extract of *Terminalia arjuna* bark in an in vivo model of myocardial ischemic reperfusion injury. *Life Sci.* **73:**2727–2739; 2003.

[12] Dal Pizzol, F.; Klamt, F.; Benfato, M. S.; Bernard, E. A.; Moreira, J. C. Retinol supplementation induces oxidative stress and modulates antioxidant enzyme activities in rat Sertoli cells. *Free Radic. Res.* **34:**395–404; 2001.

[13] Ahmed, R. S.; Seth, V.; Pasha, S. T.; Banerjee, B. D. Influence of dietary ginger (*Zingiber officinales* Rosc) on oxidative stress induced by malathion in rats. *Food Chem. Toxicol.* **38:**443–450; 2000.

[14] Dietrich, J. B.; Mangeol, A.; Revel, M. O.; Burgun, C.; Aunis, D.; Zwiller, J. Acute or repeated cocaine administration generates reactive oxygen species and induces antioxidant enzyme activity in dopaminergic rat brain structures. *Neuropharmacology* **48:**965–974; 2005.

[15] Repine, J. E. Oxidant–antioxidant balance: some observations from studies of ischemia–reperfusion in isolated perfused rat hearts. *Am. J. Med.* **91:**45S–53S; 1991.

[16] Bhattacharya, S. K.; Bhattacharya, A.; Kumar, A.; Ghosal, S. Antioxidant activity of *Bacopa monniera* in rat frontal cortex, striatum and hippocampus. *Phytother. Res.* **14:**174–179; 2000.

[17] Sairam, K.; Rao, C. V.; Babu, M. D.; Goel, R. K. Prophylactic and curative effects of *Bacopa monniera* in gastric ulcer models. *Phytomedicine* **8:**423–430; 2001.

[18] Lang, I.; Deak, G.; Muzes, G.; Pronai, L.; Feher, J. Effect of the natural

bioflavonoid antioxidant silymarin on superoxide dismutase (SOD) activity and expression in vitro. *Biotechnol. Ther.* **4:**263–270; 1993.

[19] Soto, C.; Recoba, R.; Barron, H.; Alvarez, C.; Favari, L. Silymarin increases antioxidant enzymes in alloxan-induced diabetes in rat pancreas. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **136:** 205–212; 2003.

[20] Skottova, N.; Kazdova, L.; Oliyarnyk, O.; Vecera, R.; Sobolova, L.; Ulrichova, J. Phenolics-rich extracts from *Silybum marianum* and *Prunella vulgaris* reduce a high-sucrose diet induced oxidative stress in hereditary hypertriglyceridemic rats. *Pharmacol. Res.* **50:**123–130; 2004.

[21] Bhattacharya, S. K.; Satyan, K. S.; Ghosal, S. Antioxidant activity of glycowithanolides from *Withania somnifera*. *Indian J. Exp. Biol.* **35:**236–239; 1997.

[22] Panda, S.; Kar, A. Evidence for free radical scavenging activity of Ashwagandha root powder in mice. *Indian J. Physiol Pharmacol.* **41:**424–426; 1997.

[23] Luczaj, W.; Waszkiewicz, E.; Skrzydlewska, E.; Roszkowska-Jakimiec, W. Green tea protection against age-dependent ethanol-induced oxidative stress. *J. Toxicol. Environ. Health A* **67:**595–606; 2004.

[24] Rukkumani, R.; Aruna, K.; Varma, P. S.; Rajasekaran, K. N.; Menon, V. P. Comparative effects of curcumin and an analog of curcumin on alcohol and PUFA induced oxidative stress. *J. Pharm. Pharm. Sci.* **7:**274–283; 2004.

[25] McCord, J. M.; Fridovich, I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244:**6049–6055; 1969.

[26] Beers, R. F., Jr.; Sizer, I. W. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195:**133–140; 1952.

[27] Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95:**351–358; 1979.

[28] Fossati, P.; Prencipe, L.; Berti, G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. *Clin. Chem.* **26:**227–231; 1980.

[29] Allain, C. C.; Poon, L. S.; Chan, C. S.; Richmond, W.; Fu, P. C. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:**470–475; 1974.

[30] Gutteridge, J. M.; Quinlan, G. J. Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: the role of lipid radicals, iron salts, and metal chelators. *J. Appl. Biochem.* **5:**293–299; 1983.

[31] Liu, J.; Yeo, H. C.; Doniger, S. J.; Ames, B. N. Assay of aldehydes from lipid peroxidation: gas chromatography–mass spectrometry compared to thiobarbituric acid. *Anal. Biochem.* **245:**161–166; 1997.

[32] Junqueira, V. B.; Barros, S. B.; Chan, S. S.; Rodrigues, L.; Giavarotti, L.; Abud, R. L.; Deucher, G. P. Aging and oxidative stress. *Mol. Aspects Med.* **25:**5–16; 2004.

[33] Nelson, S. K.; Bose, S. K.; McCord, J. M. The toxicity of high-dose superoxide dismutase suggests that superoxide can both initiate and terminate lipid peroxidation in the reperfused heart. *Free Radic. Biol. Med.* **16:**195–200; 1994.

[34] McCord, J. M.; Edeas, M. A. SOD, oxidative stress and human pathologies: a brief history and a future vision. *Biomed. Pharmacother.* **59:**139–142; 2005.

[35] Sies, H.; Stahl, W.; Sevanian, A. Nutritional, dietary and postprandial oxidative stress. *J. Nutr.* **135:**969–972; 2005.

[36] McCord, J. M. Free radicals and myocardial ischemia: overview and outlook. *Free Radic. Biol. Med.* **4:**9–14; 1988.

[37] Kono, Y.; Fridovich, I. Superoxide radical inhibits catalase. *J. Biol. Chem.* **257:**5751–5754; 1982.

[38] Blum, J.; Fridovich, I. Inactivation of glutathione peroxidase by superoxide radical. *Arch. Biochem. Biophys.* **240:**500–508; 1985.

[39] Bray, R. C.; Cockle, S. H.; Fielden, E. M.; Roberts, P. B.; Rotilio, G.; Calabrese, L. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. *Biochem. J.* **139:**43–48; 1974.

[40] Larini, A.; Bianchi, L.; Bocci, V. Effect of 4-hydroxynonenal on antioxidant capacity and apoptosis induction in Jurkat T cells. *Free Radic. Res.* **38:**509–516; 2004.

[41] Harman, D. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11:**298–300; 1956.

[42] Cadenas, E.; Davies, K. J. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* **29:**222–230; 2000.

[43] Orr, W. C.; Sohal, R. S. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* **263:**1128–1130; 1994.

[44] Brown-Borg, H. M.; Rakoczy, S. G. Catalase expression in delayed and premature aging mouse models. *Exp. Gerontol.* **35:**199–212; 2000.

[45] Ku, H. H.; Brunk, U. T.; Sohal, R. S. Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radic. Biol. Med.* **15:**621–627; 1993.

[46] Schriner, S.E.; Linford, N.J.; Martin, G.M.; Treuting, P.; Ogburn, C.E.; Emond, M.; Coskun, P.E.; Ladiges, W.; Wolf, N.; Van Remmen, H.; Wallace, D.C.; Rabinovitch, P.S. Extension of murine lifespan by overexpression of catalase targeted to mitochondria. *Science* **308:**1909–1911; 2005.

[47] Song, J. H.; Shin, S. H.; Ross, G. M. Prooxidant effects of ascorbate in rat brain slices. *J. Neurosci. Res.* **58:**328–336; 1999.

[48] Song, J.H.; Simons, C.; Cao, L.; Shin, S.H.; Hong, M.; Chung, I.M. Rapid uptake of oxidized ascorbate induces loss of cellular glutathione and oxidative stress in liver slices. *Exp. Mol. Med.* **35:**67–75; 2003.

[49] Lachili, B.; Hininger, I.; Faure, H.; Arnaud, J.; Richard, M. J.; Favier, A.; Roussel, A. M. Increased lipid peroxidation in pregnant women after iron and vitamin C supplementation. *Biol. Trace Elem. Res.* **83:** 103–110; 2001.

[50] Meagher, E. A.; Barry, O. P.; Lawson, J. A.; Rokach, J.; FitzGerald, G. A. Effects of vitamin E on lipid peroxidation in healthy persons. *JAMA* **285:**1178–1182; 2001.

[51] Sumien, N.; Forster, M. J.; Sohal, R. S. Supplementation with vitamin E fails to attenuate oxidative damage in aged mice. *Exp. Gerontol.* **38:**699–704; 2003.