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Reduced Level of Serum Thiols in Patients with a Diagnosis of Active Disease

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ABSTRACT

Oxidative stress, or the production of oxygen-centered free radicals, has been hypothesized as the major source of DNA damage that in turn can lead to altered genetic expression, disease, and aging of humans. Serum protein thiol levels in blood are a direct measure of the in vivo reduction/oxidation (redox) status in humans, because thiols react readily with oxygen-containing free radicals to form disulfides. Moreover, serum thiols also reflect DNA repair capacity and the possible eventual accumulation of genetic damage, since a key DNA repair enzyme, poly ADP-ribose polymerase (PARP), is thiol/disulfide redox regulated. This study tests the hypothesis that serum protein thiols can be used to estimate individual aging status by comparing the levels of apparently healthy subjects ($n = 90$) to those of individuals ($n = 306$) with an active disease diagnosis. Nine categories of human disorders all showed highly significant reductions in serum protein thiols from 46 to 91 nM cysteine/200 μL serum (mean ± S.D. = 59 ± 40) compared to a control mean of 128 ± 39 nM cysteine/200 μL serum ($p < .001$). These data strongly confirm an important role of oxidative stress in human disease development, and identify serum thiol status as a potential biochemical endpoint useful in the assessment of aging.

INTRODUCTION

THE ORIGINS OF AGING and age-related human disease are uncertain, but current data and logical argument suggest oxidative stress as a major culprit.[1] Oxidative stress can be defined as the generation of oxygen-centered free radicals from physiologic responses to environmental and metabolic exposure.[2] Moreover, many human responses to oxidative stress are under direct genetic control including the efficiency of DNA repair enzymes and oxygen-centered radical scavenger enzymes such as superoxide dismutase (SOD) and catalase.[3–5] By far the largest sources of oxidative stress, however, are environmental: diet, lifestyle, smoking, and other hazardous exposures. In this regard age is probably the single most important factor since it reflects not only the genetic factors but their combined interaction with the environment at any particular point during our lifetime. No matter the origin of oxidative stress, there is only one known biochemical outcome to reactive oxygen species (ROS) elevation: DNA damage and the consequent alteration of genetic expression. Hence, elevated oxidative stress and its impact on DNA repair are believed to be responsible for the progression of many human disorders including aging, autoimmune disorders, cancer, diabetes, drug resistance, HIV/AIDS, and impaired immune function.[1,6,7]

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Direct links between oxidative stress, ROS production, DNA damage, and altered gene expression have been well documented.[1–9] Estimating individual health consequences from oxidative stress through intermediate biochemical endpoints has proved difficult and has been evolving over the past half century.[2,5] The primary limitation has been deciding which cell types to sample and optimal noninvasive procedures of human specimen collection, in order to be both clinically reproducible and biochemically valid as indicators of health risk. Chromosomal aberrations, sister chromatid exchanges, DNA adducts, point mutations, enzyme markers (e.g., cytochrome P-450 isozymes, glutathione transferase, catalase, SOD), macro/micro nutrients, and epigenetic gene expression (e.g., DNA methylation) in blood cells and serum have met with some success, but are limited by cost and the need to isolate viable specimens in a reproducible, controlled, and timely manner.[1,2,5,6,9,10]

To circumvent such limitations, the scientific focus has switched from measuring biological factors that induce DNA damage to measuring DNA repair. Underlying this approach is the observation that DNA repair can be measured in millions of white blood cells, requires only a few milliliters of whole blood, and the sample takes only minutes to isolate and purify. Moreover, when humans are compared with other mammals there is a striking correlation with lifespan, in turn supporting the argument that DNA repair should be considered a vital clinical parameter when estimating the detrimental effects of age and disease.[11–13]

Several procedures have been used to estimate DNA repair capacity, including unscheduled DNA synthesis, nucleoid sedimentation, DNA strand breaks by alkaline elution, and PARP (poly ADP-ribose polymerase) activity. All are effective and their use in human populations shows that DNA repair capacity can explain hazardous occupational exposures, genetic predisposition to cancer, drug resistance, aging, immune dysfunction, and even immune enhancement.[6,11] Nonetheless, methodological limitations to obtaining viable peripheral blood leucocytes from human subjects remain.

Our laboratory has proposed a surrogate estimate of DNA repair requiring only a few microliters of serum or plasma, obtainable by venous puncture or finger prick sampling. The serum protein thiol test is based on the thiol content of ammonium sulfate precipitated serum proteins. The theory behind the test is that the sulfhydryl/disulfide content of blood proteins is in redox balance with the levels of individual endogenous oxidative stress. Because the key DNA repair enzyme, PARP, contains three cysteine residues in the DNA-binding zinc-finger amino acid sequence, cysteine oxidation to sulfide prevents DNA binding, thus inhibiting PARP from participating in DNA repair.[14] Our lab has demonstrated that elevated serum thiols are associated with both longevity and successful nutritional anti-oxidant intervention, in turn supporting the thiol test as a surrogate predictor of DNA repair.[12,15] Here we report further validation of the thiol test performed in a semi-automated manner in microtiter plates, and its successful application in detecting active clinical disease.

MATERIALS AND METHODS

Materials

Ammonium sulfate, DTNB (5,5′-dithiobis [2-nitrobenzoic acid]), EDTA, potassium phosphate, and sodium chloride were all supplied by Sigma.

Blood sample collection

5 mL SST vacutainer tubes for blood serum collection were used to process about 3–5 mL venous blood by venal puncture. Resulting blood specimens were centrifuged at 2000G to separate the serum from the rest of the blood components. Serum specimens were then stored at 4°C and shipped at room temperature.

Non-symptomatic control subjects

Apparently disease free normal individuals of both sexes ($n = 90$), reporting no symptoms or health disorders, with ages ranging from 37 to 70 years were recruited into this study. Subjects were drawn from laboratory technical staff at Biomedical Diagnostic Research and the University of Lund. Healthcare workers also

recruited healthy volunteers from various clinics for routine clinical analyses including serum protein thiol levels. Only data on age, sex, and blood tests were used.

Active disease patients

A reference laboratory (Solon Medical Laboratories, Solon, Ohio) performing routine clinical chemistry analyses supplied serum samples from 306 patients of both sexes between the ages of 34 and 100 years who had submitted blood samples for routine tests. Serum samples were sent on only after other routine analyses were completed, usually within two days of the original sample collection. The clinical diagnosis of each (anonymous) patient was supplied for studying the relationship of serum thiols to active disease. Only age, sex, and blood sampling data were available for statistical analysis. Sera were stored at 4°C and analyzed within 10 days of collection of the samples from Solon Medical Laboratories.

Serum protein thiol analytical procedure

Thiols present in serum proteins were precipitated with 80% saturated ammonium sulfate and were determined as previously described with minor modifications to accommodate a microtiter plate scale analysis.[12] Briefly, 50 μL aliquots of serum were precipitated with 200 μL saturated ammonium sulfate. The resulting suspensions were vortexed, and incubated for 15 minutes at room temperature, and the precipitated protein harvested by centrifugation at 12,000G. The supernatant was discarded and the pellet redissolved in 150 μL physiologic saline for 30 minutes. Two 50 μL aliquots of the redissolved protein pellet were prepared as follows: 50 μL sample + 200 μL physiologic saline, 50 μL sample + 200 μL DTNB reagent, 50 μL physiologic saline + 200 μL DTNB reagent. The DTNB reagent contained 9.5 mg/mL in 0.1M K_2HPO_4, 17.5 mM EDTA adjusted to pH 7.5, and diluted 1:50 with saline before use. The assay was carried out in 96–well microtiter flat bottom plates and the absorbance read at 412nm. Results were calculated by subtracting the saline blank absorbance and DTNB blank absorbance from the absorbance of saline + DTNB + sample. The 80% ammonium sulfate precipitated serum protein thiol samples were estimated as nM cysteine per 200 μL aliquot of serum standardized against a 0–124 nM cysteine concentration dose range. Serum samples were assayed in duplicate.

TABLE 1. INTRA-INDIVIDUAL REPRODUCIBILITY OF SERUM PROTEIN THIOLS USING MICROTITER PLATE SEMI-AUTOMATED TECHNOLOGY AND DUPLICATE DETERMINATIONS

Patient identity n = 15	*Serum protein thiols*[a]			
	Result 1	*Result 2*	*Mean ± S.D.*	*Coefficient of variation (%)*
314101	105	105	105 ± 0	0.0
313501	179	187	183 ± 6	3.3
313502	187	179	183 ± 6	3.3
313503	152	179	166 ± 19	11.4
315304	170	177	174 ± 5	2.9
313505	182	180	181 ± 1	0.6
313506	145	148	147 ± 2	1.4
312602	146	153	150 ± 5	3.4
312601	145	140	143 ± 4	2.8
309801	116	112	114 ± 3	2.6
309802	147	137	142 ± 7	4.9
310101	160	152	156 ± 6	3.8
311201	146	152	149 ± 4	2.7
311901	150	155	153 ± 4	2.6
309101	148	142	145 ± 4	2.8
Average				3.3

[a]Nanomolar cysteine in 200 μL serum precipitated with 80% saturated ammonium sulfate.

Reproducibility studies

Serum protein thiols were performed on a microscale in standard microtiter plates beginning with 50 μL aliquots of serum samples (final reaction volume = 250 μL) instead of the previous published procedure[12] that required 200 μL serum aliquots and final reaction volumes of 1 mL. Although reproducibility data has been presented on the original macro method, none has yet been reported for the new microscale assay. Consequently, we analyzed the reproducibility of the serum protein thiol test and, specifically, the micro procedure. For this purpose, our reference lab evaluated inter- and intrasubject variability using duplicate determinations as well as temporal and laboratory (i.e., lab site or technician) variability.

Statistics

The non-paired *t*-test was used to compare control and active diseased subjects for the influence of sex and age on serum protein thiol levels. Linear regression analyses were used to evaluate inter-individual variation over time.

RESULTS

Intra-individual reproducibility

Fifteen asymptomatic, apparently healthy volunteers provided serum samples for assessment of reproducibility of duplicate serum protein thiol tests performed using the micro-scale method. The data 1 indicate that duplicate determinations are consistent, with average coefficients of variation of only 3.3% (Table 1). These data suggest that intra-individual variation in the micro-scale assay procedure is minimal and individual serum protein thiol levels are reliable.

Inter-individual reproducibility

Reliability of individual determinations over a 12-day period was analysed (Table 2). Indi-

TABLE 2. INTER-INDIVIDUAL REPRODUCIBILITY OF SERUM PROTEIN THIOLS USING THE MICROTITER PLATE SEMI-AUTOMATED TECHNOLOGY AND DUPLICATE DETERMINATIONS ANALYZED OVER A 12-DAY PERIOD

Patient identity n = 16	*Serum protein thiols*[a]				
	Day 0 analysis	*Day 5 analysis*	*Day 12 analysis*	*Mean ± SD*	*Coefficient of variation (%)*
1	150	124	135	136 ± 13	9.6
3	128	93	113	111 ± 17	15.3
5	150	130	145	142 ± 10	7.0
7	117	90	98	102 ± 14	13.7
9	88	63	76	76 ± 13	17.1
11	123	104	114	114 ± 9	7.9
13	143	111	120	125 ± 17	13.5
15	132	109	112	118 ± 12	10.2
17	89	68	76	78 ± 11	14.1
19	107	90	108	102 ± 10	9.8
21	114	81	115	104 ± 19	18.3
23	137	127	143	135 ± 8	5.9
25	137	141	138	139 ± 2	1.4
27	116	98	103	106 ± 9	8.5
29	122	101	103	109 ± 12	11.0
31	135	118	117	123 ± 10	8.1
Average *t*-test	124 ± 19	103 ± 22	114 ± 20	113 ± 12	10.7

[a]Nanomolar cysteine in 200 μL serum precipitated with 80% saturated ammonium sulfate.
Day 0 vs. day 5 $p < 0.001$
Day 0 vs. day 12 N.S.
Linear Regression (n = 32)
Day 0 vs. day 5 $r = 0.91$, $p < 0.001$
Day 0 vs. day 12 $r = 0.90$, $p < 0.001$

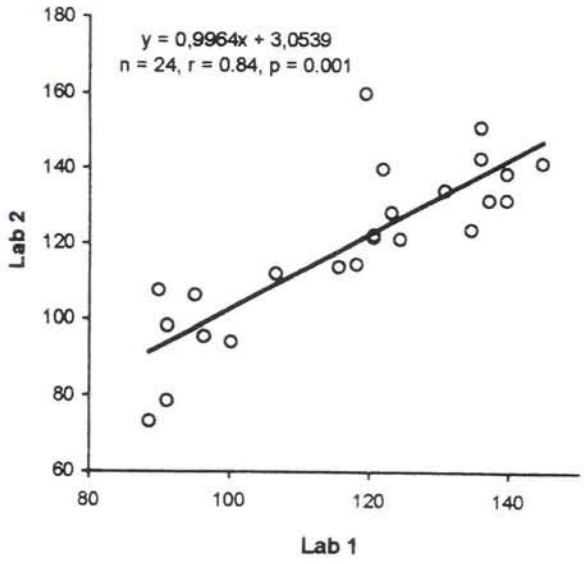

FIG. 1. Inter-laboratory reproducibility of serum protein thiols using microtiter plate semi-automated technology and duplicate determinations.

vidual values were stable when stored at 4°C and analyzed on day 0, 5, and 12 for serum protein thiols. Each of the three individual values varied with a mean of only 10.7% over the twelve-day period. Mean values were not significantly different between the 0 and 12 day data, showing no obvious decay over time. Furthermore, linear regression analysis comparing day 0 and day 3 values versus day 0 and day 12 values yielded highly significant correlation coefficients of ≥0.9. Hence, not only did the individual serum protein thiol values not decay with storage, but the inter-individual variation observed between them at day 0 (baseline) was almost the same as was observed on days 3 and 12. In conclusion, micro-scale determination of serum protein thiols is reliable and can be used to assess thiol variation in selected human populations.

Inter-laboratory reproducibility

Reliability was also tested by comparing different technicians carrying out the same assay procedure on the same set of serum samples, but at different locations and with different equipment. Twenty four serum samples from apparently healthy human subjects were analyzed for serum protein thiols using the micro-scale procedure, transferred to a second laboratory (within three days), stored at 4°C, and re-analyzed within nine additional days (Fig. 1). Comparing laboratories, serum protein thiols yielded a highly significant correlation coefficient, $r = 0.84$, $p = 0.001$), indicating that inter-individual variability between sites of performance and technicians were small.

Serum protein thiols in patients with active disease

Three hundred and six donated blood samples were used. After the original physician-requested clinical tests, aliquots were provided for analysis of serum protein thiol content using the micro-scale technique.

Compared to normal controls, active disease subjects showed a dramatic and highly significant reduction in serum protein thiol levels ex-

TABLE 3. THE EFFECT OF SEX ON SERUM PROTEIN THIOLS IN PERIPHERAL BLOOD SAMPLES OF SUBJECTS WITH ACTIVE DISEASE DIAGNOSIS AND COMPARED TO ASYMPTOMATIC CONTROLS

	n	Sex	Age (years) Mean ± S.D.	Serum thiols Mean ± S.D.
All controls (asymptomatic)	90	F + M	52 ± 11	128 ± 39
	52	F	52 ± 9	122 ± 36
	38	M	53 ± 13	136 ± 43
All active disease categories	306	F + M	76 ± 16	59 ± 40
	180	F	78 ± 17	56 ± 34
	126	M	73 ± 15	65 ± 47
Non-paired t-test:				
within control groups			N.S.	N.S.
within disease groups			N.S.	N.S.
group 1 vs. group 2			$p < 0.001$	$p < 0.001$

Serum thiols = nanomolar cysteine protein in 200 μL 80% saturated ammonium sulfate precipitated fraction of serum.

pressed as nM cysteine/200 μL 80% ammonium sulfate precipitated serum: controls = 128 ± 39, active disease = 59 ± 40, $p < 0.001$. There was no influence of sex on thiol levels whether analyzed within or between groups (Table 3).

On the other hand, subject age was statistically different between the control and active disease groups, requiring a more detailed analysis to accommodate the age-related selection bias (Table 4). Sample size was large enough so that two subgroups could be created, based on ages above and below mean group age, and then comparing each for changes in their serum protein thiol levels. Comparing control ($n = 38$, 63 ± 7) to active disease groups ($n = 125$, 60 ± 14), when corrected for age and yielding no significant differences, there still remained a highly significant difference ($p < 0.003$) in serum protein thiols (131 ± 35 and 67 ± 47, respectively). This suggests that serum protein thiols are a dominant independent variable, not explained solely by age. In addition, there were nine separate clinical diagnoses in our sample.

TABLE 4. THE EFFECT OF AGE ON SERUM PROTEIN THIOLS IN PERIPHERAL BLOOD SAMPLES OF SUBJECTS WITH ACTIVE DISEASE DIAGNOSES AND COMPARED TO ASYMPTOMATIC CONTROLS. EACH ACTIVE DISEASE GROUP AND THE CONTROLS WERE DIVIDED INTO TWO GROUPS: ONE GROUP COMPRISING ALL SUBJECTS BELOW THE MEAN OF AGE AND THE OTHER, ALL SUBJECTS ABOVE THE MEAN OF AGE

Group	n	*Age (years) Mean ± S.D.*	*Serum thiols Mean ± S.D.*
Controls	90	52 ± 11	128 ± 39
Below age mean	52	45 ± 5	125 ± 42
Above age mean	38	63 ± 7[a]	131 ± 35
All diseases	306	76 ± 16	59 ± 40[c]
Below age mean	125	60 ± 14[a]	67 ± 47[c]
Above age mean	181	87 ± 5	54 ± 33[c]
Blood disorders	35	76 ± 17	72 ± 45[c]
Below age mean	15	60 ± 15a	93 ± 50[c]
Above age mean	20	88 ± 6	57 ± 28[c]
Cancer	12	77 ± 13	54 ± 68[c]
Below age mean	5	65 ± 11[a]	60 ± 91[c]
Above age mean	7	85 ± 5	50 ± 54[c]
Cardiovascular disease	69	82 ± 12	49 ± 36[c]
Below age mean	26	72 ± 14[b]	56 ± 29[c]
Above age mean	43	88 ± 3	45 ± 40[c]
Diabetes:	26	56 ± 29[b]	68 ± 44[c]
Below age mean	9	50 ± 12[b]	74 ± 63[c]
Above age mean	17	83 ± 7	65 ± 31[c]
Infectious/Inflammatory disease	29	79 ± 12	46 ± 33[c]
Below age mean	16	72 ± 10[b]	46 ± 46[c]
Above age mean	13	88 ± 4	46 ± 29[c]
Kidney disease	26	79 ± 17	53 ± 29[c]
Below age mean	8	59 ± 17[a]	37 ± 20[c]
Above age mean	18	88 ± 5	57 ± 30[c]
Metabolic disease	35	79 ± 14	58 ± 27[c]
Below age mean	13	64 ± 10[a]	62 ± 39[c]
Above age mean	22	87 ± 5	56 ± 18[c]
Neurologic disease	61	66 ± 18[a]	65 ± 38[c]
Below age mean	31	50 ± 7[b]	76 ± 45[c]
Above age mean	30	83 ± 8	53 ± 26[c]
Thyroid disease	13	69 ± 19[a]	91 ± 49[c]
Below age mean	6	51 ± 8[b]	112 ± 50
Above age mean	7	87 ± 6	73 ± 45[c]

Non-paired *t*-test.
Controls vs. disease (age matched): [a]N.S.; [b]$p < 0.001$
Controls vs. disease (all thiol groups): [c]$p < 0.003$
N.S. = not significant, serum thiols nM cysteine protein in 200 μL 80% saturated ammonium sulfate precipitated fraction of serum.

Six of the nine disease categories, when corrected for age differences, still had serum protein thiols significantly lower than those in the controls (Table 4). Together, these data suggest that serum protein thiols can indicate basic redox imbalances that in turn predict clinical disease.

DISCUSSION

Aging is almost impossible to define because of multiple genetic and lifestyle factors interacting in individuals that together determine the risk of age-related human disease. What may be tolerated and overcome in one individual is life threatening in another. To date, it has been difficult to identify a practical biochemical marker useful in the diagnosis, prevention, and prognosis of aging disorders. Oxidative stress is a unifying concept common to most clinical disorders, that hypothesizes reactive oxygen species as the major source of DNA damage that, if not repaired, results in dysfunctional gene expression followed by age-related disease.[1,2,5,8,9] Consequently, measures of in vivo oxidative stress could be predictive of age-associated pathology.

This study tests such a hypothesis and provides evidence that in vivo oxidative stress (measured as the depletion of serum protein thiols) correlates with clinical disease in humans. Remarkably, nine separate disease categories (blood, neoplastic, cardiovascular, diabetic, infectious/inflammatory, kidney, metabolic, neurological, and thyroid diseases) displayed significant reductions in serum thiols, supporting the suggestion that this parameter could be used to detect general manifestations of human aging (Fig. 2).

Given the correlation between oxidative stress and disease, the most inclusive mechanism is probably inhibition of DNA repair. This topic has been reviewed by Pero[6]: oxidative stress effects on ADP-ribosylation (an important mechanism in DNA repair) is down-regulated in aging, autoimmunity, cancer, diabetes, drug resistance, HIV/AIDS, and immune function. Serum protein thiols are a useful surrogate indicator of DNA repair,[8] correlating well

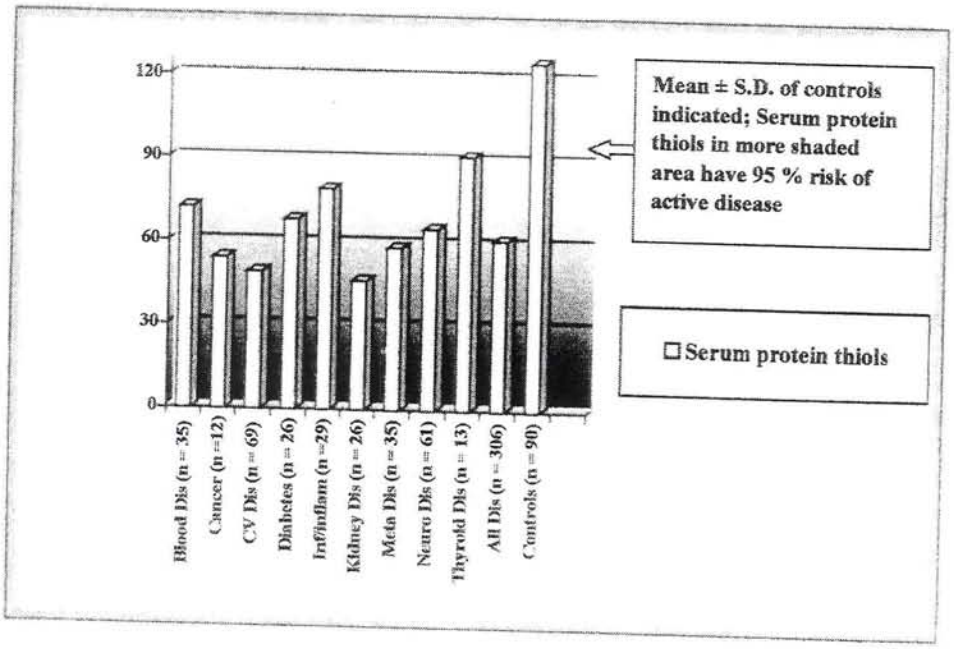

FIG. 2. Serum protein thiols in 306 patients with a diagnosis of active disease compared to 90 apparently healthy subjects. Microscale determinations of serum protein thiols were performed in duplicate by procedures yielding high reproducibility. Non-paired *t*-test statistics were used to determine significance between groups. Blood Dis = blood diseases, Cancer = neoplastic diseases, CV Dis = cardiovascular diseases, Diabetes = diabetic related disorders, Inf/Inflam = infectious/inflammatory diseases, Kidney Dis = kidney diseases, Meta Dis = metabolic diseases, Neuro Dis = neurological diseases, Thyroid Dis = thyroid diseases, All Dis = including all sub-groups analyzed together.

with both longevity[12] and antioxidant intervention.[15] Our data confirm down regulation of DNA repair measured as reduced serum protein thiols in many individuals with active clinical disease. With its broad application to many human diseases, this test might have general applicability in evaluating aging populations.

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REFERENCES

1. Cross CE, Halliwell B, Borisch ET et al. Oxygen radicals and human disease. *Ann Intern Med* 1987; 107:526–545.
2. Kohen R, Nyska A. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 2002;30:620–650.
3. Hansen MF, Cavene WK. Genetics and cancer predisposition. *Cancer Res* 1987;47:5518–5527.
4. Setlow RB. Repair deficient disorders and cancer. *Nature* 1978;271:713–717.
5. Harris CC. Chemical and physical carcinogenesis: advances and perspectives for the 1990s. *Cancer Res* 1991;51:5023S–5044S.
6. Pero RW, Olsson A, Sheng Y et al. Progress in identifying clinical relevance of inhibition, stimulation, and measurements of poly ADP-ribosylation. *Biochimie* 1995;77:385–393.
7. Baruchel S, Wainberg M. The role of oxidative stress in disease progression in individuals infected by the human immunodeficiency virus. *J Leuco Biol* 1992;52: 111–114.
8. Cerutti P. Prooxidant states and tumor promotion. *Science* 1978;271:713–717.
9. Bohr V, Anson RM, Mazur S, Dianov G. Oxidative DNA damage processing with aging. *Toxicol Lett* 1998; 102/103:47–52.
10. Verma M, Dunn BK, Ross S, et al. Early detection and risk assessment: proceedings and recommendations from the workshop on epigenetics in cancer prevention. *Ann NY Acad Sci* 2003;983:298–319.
11. Pero RW, Holmgren K, Persson L. Gamma-radiation induced ADP-ribosyl transferase activity and mammalian longevity. *Mut Res* 1985;142:69–73.
12. Pero RW, Hoppe C, Sheng Y. Serum thiols as a surrogate estimate of DNA repair correlates to mammalian life span. *J Antiaging Med* 2000;3:241–248.
13. Grube K, Burkle A. Poly(ADP-ribose)polymerase activity in mononuclear leukocytes of 13 mammalian species correlates with specific life span. *Proc Nat Acad Sci U S A* 1992;89:11759–11763.
14. Mazen A, Demurcia JM, Molinete M et al. Poly(ADP-ribosyl) polymerase: a novel finger protein. *Nucleic Acids* 1989;17:4689–4698.
15. Pero RW, Giampapa V, Vojdani A. Comparison of a broad spectrum anti-aging nutritional supplement with and without the addition of a DNA repair enhancing cat's claw extract. *J Antiaging Med* 2002;5: 345–353.

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