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CHANGES IN ANTIOXIDANT ENZYME LEVELS AND DNA DAMAGE DURING AGING

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ABSTRACT

Multiple mechanisms underlie the human aging process, but interest continues in the role that free radicals and antioxidants may play. The concentrations of lymphocyte free radical generation ($O_2^{\cdot-}$ & H_2O_2), DNA damage and antioxidant enzyme levels (glutathione S-transferase, superoxide dismutase and catalase) were evaluated in 110 healthy individuals with an age range of 20-80 years. The antioxidant enzyme levels were significantly less in very old age when compared to young. Moreover, the levels of free radical concentration and DNA damage were increased in the same age group with respect to younger group. Cigarette smoking had a positive relation with free radicals and DNA damage, and inverse relation with antioxidants. On the other hand, body mass was found to have positive relation with free radical generation only. The data indicate that depletion of antioxidant enzyme levels would render the older people more susceptible to free radical stress and DNA damage.

KEY WORDS : Free radicals, DNA damage, Antioxidants, Aging

INTRODUCTION

Reactive oxygen species are formed continuously as a consequence of biochemical reactions as well as external factors, which are proposed to be important in aging as well as a number of age-related degenerative diseases (1). The possible role of free radicals in the aging process has been the subject of considerable attention recently (2). The studies on insects and mammals show that the rate of superoxide anion and H_2O_2 formation increased in later part of life (3). The oxidative damage products such as protein carbonyls, lipofuscin, n-pentane exhalation and lipid peroxidation products were also elevated with advancement of age (4,5). The aging organisms are more susceptible to prooxidation of intracellular redox state (6). Free radicals can attack any structural components. However, with regard to aging, DNA is considered to be prime target (7), thus causing DNA-protein cross-links, sugar moiety damage and modifications in purine and pyrimidine bases (8). Oxidation of sugar moiety induces base release and strand breaks, whereas, oxidative base modifications result in mutations (9). Antioxidants are believed to decrease attacks on DNA by free radicals and thus to protect against mutations that cause disease status (10).

Apart from aging, it has also been postulated that variation in environment (industrial and urban) and life-style measures act as stimulants of free radical generation, DNA damage and reduced antioxigenic potential (11,12). Although data are available on free radical generation and oxidative DNA damage in various disease conditions as well as on toxicity, data on aging human populations is lacking, especially in India. The present study is aimed to evaluate the concentrations of lymphocyte antioxidant enzymes i.e., glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and DNA damage in relation to life style and obesity.

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MATERIALS AND METHODS

The study population consisted of 110 healthy volunteer sample in the defined electoral area (suburbs of Tirupati, India) aged 20-80 years. The study subjects were neither compelled to participate in the survey nor subjected to any kind of risk. Information on age, habits of smoking and anthropometric measurements like height, weight, circumferences of the waist and hip were collected from each subject. The body mass index (BMI) was calculated as BMI = weight in kg/height in meters^2 (Kgm^{-2}). Waist hip ratio (WHR) was calculated from the circumferences of waist and hip.

Venous blood (10 ml) was collected in the morning from all the subjects into disposable vials containing EDTA. Lymphocytes were separated from the whole blood by dextran sedimentation technique (13).

Techniques

a. Free radicals

1. **Superoxide anion:** Superoxide anion can reduce nitroblue tetrazolium (NBT) to the insoluble formazan (14). The ability to reduce NBT was assayed by incubating lymphocytes with 0.1% NBT dissolved in phosphate buffer saline for 20 min at 37°C. The assay was terminated by adding 0.6 ml of glacial acetic acid and the extracted NBT dye was read at 560 nm.

2. **Hydrogen Peroxide:** Hydrogen peroxide released by lymphocytes was estimated by the horse-radish peroxidase method (15). To the lymphocytes 0.5 ml of phenol red solution (1%) containing peroxidase enzyme (3.75 units/assay) was added and incubated at 37°C for 20 min. The reaction was terminated by adding catalase (150 units/assay) and the optical density readings were taken at 610 nm. $1x10^6$ lymphocyte cells/assay were taken for both superoxide anion and H_2O_2 assay.

b. Lymphocyte antioxidants

1. Glutathione-S-transferase: GST activity was measured following the increase in absorbance at 340 nm using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate as described by Habig et al (16). To 3ml cuvette were added 1.0 ml phosphate buffer, 0.1ml CDNB (30mM) and 0.1 ml enzyme, and the volume was adjusted to 2.9 ml with distilled water. The reaction mixture was pre incubated at 37° C for 5 min. Reaction was started by adding 0.1 ml of 30 mM glutathione and monitored spectrophotometrically by the increase in absorbance at 340nm.

2. **Superoxide dismutase:** SOD activity was assayed according to the method of Misra and Fridovich (17). The assay medium contained 50 mM sodium carbonate, bicarbonate buffer (pH 9.8), 0.1 mM EDTA, 0.6 mM adrenaline in a total volume of 3 ml. Adrenaline was the last component to be added and the adrenochrome formed in four minutes was recorded at 470 nm. One unit of SOD activity was defined as the amount of enzyme needed to cause 50% inhibition of adrenaline autoxidation at pH 9.8.

3. **Catalase:** Catalase assay was carried out by the method of Beer and Sizer (18). The decomposition of H_2O_2 was followed directly by measuring the decrease in absorbance at 240 nm.

c. DNA damage

DNA was extracted from lymphocytes as per the procedure specified by Hoar et. al. (13).

1. TBA assay for DNA damage: Sugar fragments consist of compounds that carry one or several carbonyl functions, for example 2 - deoxyguanosine - 5 - aldehyde which have been mistaken for malondialdehyde since they give a very similar 2-thiobarbituric acid reaction (19). One aliquot of the DNA solution was mixed with one aliquot of 0.6% 2-thiobarbituric acid. The contents were heated at 90°C for 20 min and the red color developed was measured at 537 nm. The values were expressed as nmol MDA equivalents/mg DNA.

Data were processed for statistical analysis

including multiple regession analysis and partial correlations. p values below 0.05 were regarded as statistical significance.

RESULTS AND DISCUSSION

The study subjects were divided into four groups by age: young (under 40 years); middle-age (40-54 years); old (55-69 years); very old (70 & above). Mean values of anthropometry, free radical generation, DNA damage and antioxidant enzyme levels in different ages were tested by one-way analysis of variance and presented in tables 1&2 respectively. Anthropometric measurements did not show significant variation within the age groups, except waist circumference and WHR . Free radical generation, DNA damage and antioxidant enzymes such as GST and SOD were found to have significant association with age. With regard to the age patterns, height was nearly constant across the entire age span. Weight, circumferences of waist and hip, BMI and WHR increased from age 20 to 54 and then dropped. Generation of free radicals i.e., superoxide anion and H_2O_2 shows a progressive increase from younger age group to very old, while the increase in DNA damage is upto 69 years only. On the other hand antioxidant enzyme levels show a gradual decrease from younger to very old age.

Partial correlation coefficients were calculated for free radical generation, DNA damage and antioxidants with body mass index, waist hip ratio and cigarette smoking and the results were presented in table 3. Cigarette smoking had a statistically significant positive correlation with free radicals, DNA damage and inverse correlation with antioxidants. Body mass index had significant positive correlation with $O_2^{\cdot-}$ and H_2O_2, but no relation with DNA damage and antioxidants, while, WHR was found to have insignificant relationship with free radicals, DNA damage and antioxidants.

Regression equations may be used as predictions of free radicals, DNA damage and antioxidant enzyme levels that takes age, body mass index, waist hip ratio and habit of smoking

Table 1. Values (Mean ± SD) for anthropometry in different age groups

Variable	Young (n=30) 20-39 yrs	Middle (n=30) 40-54 yrs	Old (n=30) 55-69 yrs	Very old (n=20) 70 yrs	F-value
Age (yrs)	26.67 ±5.84	46.60 ±4.30	61.43 ±3.63	75.36 ±3.64	—
Height cm	165.30 ±6.13	164.50 ±6.84	163.10 ±5.68	166.30 ±3.77	0.84
Weight kg	57.13 ±7.64	64.27 ±13.71	57.50 ±12.13	55.00 ±6.08	0.73
Body mass index kgm^{-2}	20.91 ±2.36	23.68 ±4.27	21.55 ±3.95	19.85 ±1.46	0.25
Waist circum-ference cm	74.73 ±4.67	87.20 ±7.80	84.68 ±7.19	81.45 ±2.06	2.27*
Hip circum-ference cm	88.00 ±3.21	93.07 ±7.00	89.93 ±6.93	87.64 ±3.07	0.54
WHR	0.85 ±0.03	0.93 ±0.03	0.94 ±0.03	0.92 ±0.02	16.09*

F - Values were calculated by one-way analysis of variance
*Significant at 5% level.

Table 2. Values (Mean ± SD) for free radical generation, DNA damage (MDA of Leucocytes) and antioxidant enzyme levels in different age groups

Variable	Young (n=30) 20-39 yrs	Middle(n=30) 40-54 yrs	Old(n=30) 55-69 yrs	Very old(n=20) 70 yrs +	F-value
Superoxide anion μ moles/1x10^6 cells	2.14 ±1.17	3.95 ±1.77	5.16 ±3.09	5.71 ±2.86	1.98*
H_2O_2 μ moles/1x10^6 cells	1.23 ±0.86	2.64 ±1.44	3.61 ±2.43	4.05 ±2.59	6.86*
MDA equiv nmol/mg DNA (DNA damage)	1.78 ±1.59	3.78 ±1.99	5.07 ±3.06	4.89 ±2.32	3.0*
GST μ moles/mg protein	92.20 ±25.90	75.40 ±17.13	73.86 ±19.88	59.82 ±14.97	3.23*
SOD U/mg protein	64.40 ±22.99	51.13 ±17.52	47.57 ±14.45	37.82 ±12.49	2.44*
Catalase U/mg protein	45.93 ±19.25	40.47 ±14.17	32.07 ±13.20	22.45 ±9.61	1.15*

F-Values were calculated by one-way analysis of variance. * Significant at 5% levels

Table 3. Partial correlation coefficients for free radical generation, DNA damage and antioxidants with cigarette smoking, BMI and WHR controlled for age.

Variable	Smoking	BMI	WHR
$O_2^{\cdot-}$	.6970*	.2038*	.0069
H_2O_2	.6456*	.1964*	.0166
DNA damage	.7478*	.1271	-.0114
GST	-.7348*	-.1452	.1021
SOD	-.6900*	-.1029	.0929
CAT	-.6526*	.0564	.1582

* Significant at 5% level

into account (Table 4). Cigarette smoking and age account for a high percentage of variation in free radicals and DNA damage (positive) and antioxidants (negative), whereas, body mass index serving as a moderate predictor of free radicals, DNA damage and antioxidants.

Results of the present study indicate a two fold increase of lymphocyte free radical generation ($O_2^{\cdot-}$ and H_2O_2) and DNA damage (measured in the form of nmol MDA equivalents/mg DNA) in older than in younger age groups. Oxidant byproducts are major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular diseases and cataracts (20). Significant increase in free radical concentration and MDA levels in older age groups is a strong indication of DNA damage (7, 8). This indicates that old people were more susceptible to oxidative stress and our results are consistent with the general definition of aging as the progressive accumulation of changes responsible for the decreased ability of the organism to maintain the homeostatic balance and to adapt for various environmental stimulii.

The intra and extracellular antioxidants check in time the formation of free radical generation. The concentrations of antioxidants in normal persons vary depending on age and sex (21) and the risk of peroxide stress is compensated by the increase of certain antioxidants. Due to metabolic disturbances, especially with age, the antioxidant levels would presumably diminish and in the presence of increased peroxidative stress, the

Table 4. Predictive equations for free radical generation, DNA damage and antioxidants

Dependent Variable	Intercept	Co-efficients				Multiple r^2
		Age	Smoking	BMI	WHR	
$O_2^{\cdot-}$	-.2847 (-.06)	.0653 (3.61)*	3.2833 (7.60)*	.2069 (2.85)*	-5.4119 (-.76)	69.23
H_2O_2	-.0450 (-.01)	.0551 (3.45)*	2.4849 (6.49)*	.1675 (2.60)*	-5.2282 (-.83)	63.60
DNA damage	-.0157 (-.01)	.0623 (3.87)*	3.5366 (9.18)*	.2334 (3.60)*	-6.6646 (-1.05)	75.04
GST	72.753 (1.70)	-.6065 (-4.01)*	-29.841 (-8.26)*	-1.7937 (-2.95)*	95.828 (1.61)	71.21
SOD	52.823 (1.21)	-.4528 (-2.95)*	-24.178 (-6.60)*	-1.2327 (-2.0)	65.160 (1.08)	60.33
CAT	-9.9148 (-.26)	-.4863 (-3.66)*	-19.127 (-.603)	-.6379 (-1.20)	102.71 (1.97)	59.44

Values within paranthesis are t-values * Significant at 5% level

lower levels of antioxidant defense system may be inadequate for scavenging the free radicals that arise. Epidemiological and experimental studies have also shown that reduced antioxigenic potential leads to free radical mediated oxidative stress (22). Antioxidant functions are associated with lesser DNA damage, in vitro cell damage and as indicated by epidemiological studies with lowered incidence of degenerative diseases in the process of aging (23).

Smoking had a strong positive correlation with free radical concentrations, DNA damage and inverse correlation with antioxidant enzymes. Tobacco smoking and several of its constituents, such as hydroquinone and catechol, have been shown to generate free radicals and to induce oxidative damage (24). Epidemiological studies reveal that smokers initially showed significantly more oxidative damage to bases in DNA than non-smokers (10). The results of the present study are in good agreement with the above findings indicating that increased free radical generation and reduced antioxidant potential form a link between cigarette smoking and oxidative stress represented by antioxidant imbalance (Table 3, 4). Bodymass index had a positive relation with oxidative stress but antioxidant levels did not vary with body mass. The findings of Loft et. al., have (25) shown the body mass as a significant predictor of DNA damage. The high risk for several chronic diseases caused by smoking and body mass is probably increased more by a low antioxidant status.

In conclusion, the antioxidant levels show a general tendency to decrease with age, which would render the older people more susceptible to free radical stress and DNA damage. The study demonstrates that strong correlates of free radicals, DNA damage and antioxidants are cigarette smoking and age, with body mass index serving as a moderate predictor.

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