Original Research Article

OXIDATIVE STRESS PARAMETERS AND ANTIOXIDANT STATUS IN MIDDLE AGED AMD ELDERLY SUBJECTS: AN AGE-RELATED COMPARATIVE STUDY

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Abstract: Several studies in the Western countries have demonstrated that oxidative stress accelerates aging, enhances incidence of oxidative diseases and there is a slow decline of antioxidant status in older subjects but there is lack of significant works in this field in developing countries like India. The objective of the present study was to compare age-related oxidative stress vis-à-vis antioxidant defence in middle aged and older subjects. Measurement of concentration of protein carbonylation which is an early determinant of oxidative stress and end product of lipid peroxidation Thiobarbituric acid reactive substances helps to identify the effect of age related oxidative stress in human. Quantification of SOD in plasma and serum alpha-tocopherol was done to estimate the antioxidant status of the study population. The present hospital based non-interventional cross sectional study was designed to evaluate age- related oxidative stress as well as its impact in 50 middle aged (35-55y) and 50 older subjects (>60y). On the basis of results obtained from this study it was evident that concentration of serum Thiobarbituric acid reactive substances and serum protein carbonylation were significantly higher in older subjects. It has been observed that enzymatic antioxidant serum Superoxide dismutase and free radical scavenging alfa-tocopherol were significantly lower in elderly age group than middle aged group. In conclusion, our findings do suggest that elderly subjects of the study population appear to be exposed to considerable amount of oxidative stress. A comparison between middle aged and older subjects suggests a decline of antioxidant status in elderly subjects

Key Words: Aging, Antioxidants, Elderly, Middle Aged, Oxidative Stress

INTRODUCTION

Oxidative stress is essentially an explicit manifestation of an imbalance between systemic production of reactive oxygen species (ROS) and a biological system's competence to readily detoxify the harmful reactive intermediates or its ability to repair the resulting damage[1]. From the biochemical point of view oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of anti-oxidant defences. Disbalance in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals which can cause damage to all components of cells such as proteins, lipids, DNA etc.[2] Oxidative stress also can cause disruption in normal mechanisms of cellular signaling as because some reactive oxygen species act as cellular messengers in redox signaling. There is no dearth of evidence to show that aging might be caused by deleterious and cumulative effects of reactive oxygen species generated throughout the life span. Oxidative degradation of lipids is referred to as lipid peroxidation. Lipid peroxidation is a process in which free radicals take away electrons from membrane lipid, resulting in cell membrane damage. Markers of lipid peroxidation have been verified in many diseases such as ischemic heart disease, diabetes and neurodegenerative disease.

End product of lipid peroxidation was estimated by measuring serum Thiobarbituric Acid Reactive Substances (TBARs). Protein carbonylation is a type of protein oxidation. Among the diseases in which high levels of protein carbonyl (CO) groups have been observed include Alzheimer's disease, rheumatoid arthritis, diabetes, sepsis, chronic renal failure and respiratory distress syndrome[3,4]. The quantification of protein carbonyl groups in peripheral blood is widely used to measure the extent of oxidative modification. Superoxide dismutase (SOD) is an important factor in limiting oxygen toxicity; it is one of the best-studied metalloenzymes in human biochemistry. Alfa-tocopherol is chemically active antioxidant substance that acts as scavenger. It is well-accepted fact that aging has a significant association with accumulation of free radicals and tocopherols are believed to delay the process of aging. The present hospital based non-interventional cross sectional study aims at finding out association between oxidative stresses with antioxidant defense in relation to age. Justification and relevance of our proposed research work is based on the fact that although studies in healthy elderly population in developed countries have shown that oxidative stress may lead to an accelerated aging and higher incidence of oxidative diseases but there is an earnest need of some meaningful research work in this area in developing countries like India specially in eastern region of the country.

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MATERIALS AND METHODS

Study Design

Hospital based, non-interventional cross sectional study. Random sampling was done and control was not required in this study. Clearance was obtained from the institutional ethical committee. Written informed consent as per local language was taken from individuals taking part in this study after explaining the details of the study.

Subjects

A total of 50 elderly but otherwise healthy volunteers aged >60 yrs attending the geriatric department of the Medical College and hospital, Kolkata for the counseling constituted the elderly Group and 50 middle aged volunteers (35-55y) constituted the middle aged group.

Exclusion Criteria

History of chronic diseases like hypertention, diabetes, rheumatoid arthritis, any neuropsychiatric disorder like Parkinsons disease, motor neuron disease, chronic depression, any endocrinal abnormality, malignancy, smokers, drinkers and patient under any nutritional supplement etc.

Materials Used

Trichloroacetic Acid (TCA) solution, 2.5 M HCl solution, 0.5% TBA solution, n-Butanol, Stock Standard Solution (1mM 1,1,3,3-TetraethoxyPropane), 2,4-DNPH (10 mmol/L), HCL (2 mol/L), Protein washing solution (ethanol: ethyl acetate=1:1), Protein dissolving solution (2 gm SDS & 50mg EDTA in 100ml of 80mmol/L phosphate buffer, pH-8), Sodium pyrophosphate buffer (0.025M, pH8.3), PMS (phenazine methosulfate-186 micro M) sol, NBT (nitroblue tetrazolium 300 micro M), NADH sol-780 micro M, Glacial acetic acid and other chemicals used in the study are of analytical grade and purchased from the Sigma Aldrich, MERCK and SRL.

Equipments

Automated precision pipette, Centrifuge, Water bath, Autoanalyzer (XL-600), Incubator and Spectrophotometer.

Analytical Metods

Collection of sample: 10 ml of venous blood was collected aseptically from the individuals. For accurate comparison, fasting normal samples were obtained.2ml blood collected in EDTA vial and rest was collected in container having no anticoagulant

Assay of Thiobarbituric acid reactive substances (TBARs): Serum level of TBARs was measured by method of Dahle *et al.,* [5] (1962). 0.5ml serum and 2.5 ml TCA were kept for 10 mins at room temperature.2.5 ml of 2.5 mol HCL was added with constant stirring and to this 3.5 ml of TBA was added and incubated in boiling water bath for 30mins. After cooling Butanol was added and vortexed for 1min. It was centrifuged at 3000 rpm for 10mins and supernatant (colored MDA-TBA complex) was measured at 532nm in a spectrophotometer.

Assay of serum protein carbonylation: Serum protein carbonylation was evaluated by Levine's method[6] (1990). Serum was treated with 10% TCA and 2, 4 dinitrophenylhydrazine (DNPH) was added to the precipated protein after removal of impurities. DNPH reacted with carbonylated protein and converted into 2, 4 dinitrophenylhydrazone which has specific colour that was measured spectrophotometrically at 370nm wave length. Concentration of carbonylated protein was calculated as per Levine's method.

Assay of Superoxide dismutase (SOD): Plasma SOD was measured by the method of Kakkar *et al.,* [7] (1989). 1.35ml of double distilled water, 50μl of plasma,1.2ml of sodium pyrophosphate buffer (pH8.3),0.1ml of PMS and .3ml of NBT was mixed. 0.2ml of NADH solution was added to it to initiate the reaction. After incubation at 39 degree for 90 seconds the reaction was terminated by adding 1ml of glacial acetic acid. 4ml of n-butanol was added and mixed vigorously by vortexing. The mixture was centrifuged at 4000 rpm for 10 minutes and the absorbance of upper butanol layer was measured at 560nm. For the comparison, corresponding blank was prepared in the same way except addition of plasma. One unit of SOD was defined as the amount of enzyme that inhibited the rate of reaction by 50% under specified conditions.

Assay of alfa-tocopherol: Serum tocopherol was estimated by Baker & Flank's method [8] (1968). Serum tocopherol was measured by their reduction of ferric to ferrous ion which then formed a colour complex with alfa-dipyridyl. Tocopherol being lipid soluble was first extracted in to xylene and reading of absorbance was taken at 460 nm against blank.

Statistical methods

Healthy human volunteers were selected according to preset inclusion and exclusion criteria. Total 100 person fulfilled the inclusion criteria. Data analysis was performed using SPSS (version17) and Statistica version 6 (Tulsa, Oklahoma: statsoft Inc, 20001). Values were expressed as Mean ± SEM. Statistically significant difference was determined with the student's Independent t-test (two-tailed). The P<0.05 was considered significant. Correlation study was done.

RESULTS

In this study we got 2 Group of population- Group A) Middle aged population (35-55y) & Group B) Elderly population (>60y). All variables are normally distributed by Kolmogorov-Smirnov goodness-of-fit test.

Table 1 results are displayed in the form of Mean ± Standard deviation and Standard error of mean. Table 1 shows that Serum protein carbonylation is significantly higher in elderly population (1.79 ±0.398), p value <0, 000) than middle aged (1.26±0.426). Serum TBARS is significantly higher (11.57±0.490, p value <0.000) in elderly than middle-aged population (7.83±0.467). Plasma SOD level is significantly lower (3.98±0.475, p value <0.000) in elderly than middle-aged population (4.42±0.431). Serum alfa tocopherol is significantly lower (5.46±0.464, p value <0.000) in elderly than middle aged (7.46±0.437).

Table 2 Test of significance (Independent t-test-2 tailed) of different parameters between middle aged and elderly. From table 2, It is evident that serum TBARS and serum protein carbonylation are significantly higher in elderly population than middle aged (p=0.000).

Table 3 demonstrates Correlations between numerical variables without categorizing age groups – Pearson's correlation coefficient r. Correlation analysis shows there is significant positive correlation(r=0.92) between age and serum TBARS concentration. There is good correlation (r=0.51) between age and serum protein carbonylation. Plasma SOD negatively correlated (r=-0.37) with age. Significant negative correlation (r=-0.81) also exists between serum tocopherol concentration and age.

DISCUSSION

Harman[1] described aging as the progressive accumulation of diverse changes in cells an tissues with advancing age that increase the risk of disease and death. Most of the theories claim that human body eventually yield to overpowering force of damage caused by variety of factors. Subsequently, it was discovered that reactive oxygen species (ROS), some of which are not free radicals (because they do not have an unpaired electron in their outer cell) also contribute to the accumulation of oxidative damage to the cellular constituents.

A common approach to estimate oxidative stress in vivo is to measure the end products of lipid peroxidation. The most widely used index is plasma malondialdehyde (MDA), which is measured by thiobarbituric acid reacting substances (TBARS) assay. TBARS are formed as a byproduct of lipid peroxidation (i.e. degradation effect), which can be detected by TBARS assay using thiobarbituric acid as reagent. The present study included measurement of the concentration of TBARS for quantification of the end products of lipid peroxidation. In so far as the present study is concerned it is clearly evident from the results obtained from our assessment that serum TBARS is significantly higher (p=0.000) in elderly population than middle-aged population and there is a positive correlation (r=0.92) between age and TBARS concentration. Chan et al.,[9] in their Saopaulo oxidative and aging study reported that plasma concentration of TBARS increased significantly in individuals over 50 yrs age as compared with younger group. Mezzetti et al.,[10] & Block et al.,[11] referred to increase in lipid peroxidation products but they too did not mention any direct correlation between age and TBARS level. Andrela-Sanchez et al.,[12] in their work on European population postulated that TBARS production is dependant on consumption of polyunsaturated fatty acid. So it may be presumed that variation in results obtained by different investigators belonging to different regions & parts of the world may have some relation to lifestyle and food habits of elderly population and age Group of enrolled participants of concerned study. Gill et al., [13] postulated that the balance of oxidant and antioxidant systems in plasma shifts in favour of accelerated oxidation of protein and lipid (carbonyl &MDA) during aging. Rizvi Sl and Maurya Rk[14] observed a higher oxidative stress (increased MDA) in Indian population compared to values reported for European subjects. Saaswati M et al., [15] demonstrated an increased level of oxidative stress marker and altered lipid profile in urban diabetics (type 2) and healthy controls corresponding to respective rural population suggesting the effect of urbanisation and impact of different life style.

It has been generally demonstrated by several studies that protein peroxidation increases with progression of age and protein carbonylation is an early determinant of oxidative stress. In this experimental study we have also noticed that serum protein carbonylation is significantly higher (p=0.000) in older subjects than middle aged, which signifies noticeable increase in oxidative stressor in older subjects than young adults. We have also noticed significant positive correlation (r=0.53) between age and protein carbonylation. In the opinion of Kaspoglu and Ozben protein oxidation is generally reported to increase during aging.[16] These findings are in agreement with the observation of Bureau et al., which he obtained from his study with women group[17]. Mezatti et al., [10] showed that plasma protein peroxidation products is higher in elderly than in

younger subjects. Saha A *et al.,*[18] observed an enhanced oxidative stress by an increased protein carbonyl content both in plasma and in hemolysate of the diseased samples in type 2 diabetes and diabetes associated cardiovascular disease patients in their Kolkata based studies, but according to Isabella-Dalle-Done *et al.,* [4] what relationship might be among high level of carbonyl (CO) groups, oxidative stress and disease are still uncertain.

The most important enzymatic antioxidant is superoxide dismutase (Cu-Zn SOD), which catalyzes conversion of superoxide anions into H_2O_2 which is deactivated to H_2O by catalase. In the present study it has been observed that Cu-Zn SOD activity is significantly lower (p=0.000) in older age Group and SOD has a negative correlation(r=-0.39) with age. Similar to our observation Anderson *et al.,* [19] observed an age related decrease in Cu-Zn SOD activity. Guemouri *et al.,* [20] noted that SOD activities appear rather stable in adults less than 65 years old but the decrease for most enzymes in the elderly. Marjini A[21] in their studies with regard to age related alterations of plasma lipid peroxidation and erythrocyte SOD in different age Group of Gorgan city of Iran observed that plasma lipid peroxidation (MDA) significantly increase with aging. They have also observed that erythrocyte SOD activity significantly decreased with aging. SOD may play an important role in determining individual risk of developing certain diseases such as cancer or atherosclerosis etc. Besides constitutional individual differences in gene expression, anti-oxidative enzyme activities apparently depend on variations in life style and environmental factor.

The principal micronutrient antioxidants are vitamin E, vitamin C and β-carotene. Some authors have stated that oxidative stress, aging & decline of vitamin E are interrelated. It is observed in our study that alfa-tocopherol activity is significantly lower (p=0.0000) in elderly age Group and alfa-Tocopherol has negative correlation (r=-0.87) with age. Mecocci *et al.,* showed plasma levels of vitamin E in elderly is lower than younger in humans[22]. The author concluded those vitamins E are of particular importance for longevity. Paolisso *et al.,* [23] also showed that plasma levels of vitamin E is lower in aged subjects than young adults. According to Junqueira,[24] data from some literature show that plasma alfa-tocopherol appears to be increased with age. Again few studies found no effect of age in alfa-tocopherol concentration [25] but Mino *et al.,*[26] noticed decrease in plasma alfa-Tocopherol concentration. Dietary habits, lack of proper nourishment and necessary vitamin supplement etc may have some role in low concentration of vitamin E among elderly population. Since fundamental mechanism of age related oxidative stress and role of antioxidants is not clearly understood, this subject still exists as a contentious issue among the scientists working on this subject matter. In addition, analytical differences between laboratories make it difficult to compare the results obtained in different studies. In our endeavor to study the effect of certain parameters of oxidative stress and antioxidant status between middle aged and elderly population, significant increase in oxidative stress factors and simultaneous decline in antioxidant status was found.

It is true that relatively small sample size is considered as potential limitation of the present study and further intensive investigation is needed encompassing larger sample size, other parameters of oxidative stress, dietary habit, genetic predisposition, per capita income and lifestyle of study population.

Table 1: Results are displayed in the form of Mean ± Standard deviation and Standard error of mean

Parameters	Group A (Middle Aged) No of cases (n) = 50	Group B (Elderly) No of cases (n)=50
Serum TBARS (µmol/L) Mean±SD (SEM)	7.830 ± 0.466 (0.0783)	11.569 ± 0.490 (0.0775)
Serum protein carbonylation (nmol/mg of serum protein) Mean ±SD (SEM)	1.267 ± 0.426 (0.0674)	1.797 ± 0.398 (0.0646)
Superoxide dismutase (unit/ml) Mean±SD (SEM)	4.417 ± 0.430 (0.0681)	3.976 ± 0.475 (.0751)
alpha-Tocopherol (mg/L) Mean±SD (SEM)	7.457 ± 0.4372 (.06914)	5.457 ± 0.4635 (.07329)

Table 2: Test of significance (Independent t- test-2 tailed) of different parameters between middle aged and elderly

	t-score	Significance (2-tailed)	95% confidence interval of difference	
			Upper	Lower
Serum TBARS	-4.930	0.000	-3.526	-3.952
Protein carbonylatin	-5.672	0.000	-.343	-.715
Serum alpha-tocopherol	4.350	0.000	2.201	1.799
Superoxide dismutase	19.854	0.000	.6428	.2391

Table 3: Correlations between numerical variables without categorizing age groups – Pearson's correlation coefficient r. Correlations in bold are significant at the level of *p* <0.05

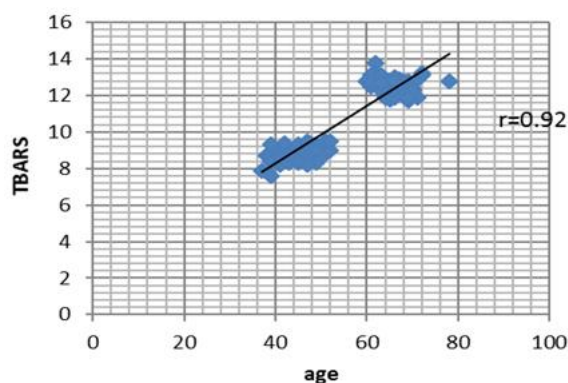| Age	TBARS	ProtCarbonyl	SOD	AlfaToco	
Age	1.00	**0.92**	**0.51**	**-0.37**	**-0.81**
TBARS	**0.92**	1.00	0.57	-0.40	-0.91
ProtCarbonyl	**0.51**	0.57	1.00	-0.29	-0.60
SOD	-0.37	-0.40	-0.29	1.00	0.43
AlfaToco	**-0.81**	-0.91	-0.60	0.43	1.00

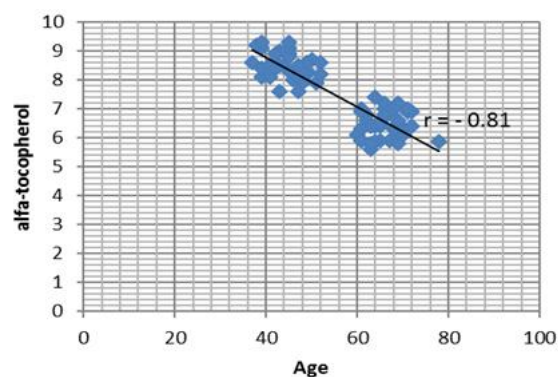**Figure 1:** scatter chart-showing correlation between age and TBARS

Figure 2: scatter chart-showing correlation between age and alpha-tocopherol

CONCLUSION

Oxidative stress, aging and decline in antioxidant status are interrelated. These findings highlight that free living healthy elderly subjects of Kolkata and suburbs are exposed to significant oxidative stress since we have noticed that oxidative stress parameters like serum TBARs and serum protein carbonylation are higher in elderly and antioxidants plasma SOD and alpha tocopherol are lower in old aged people. Whether life style factors have any role in compounding age related problems requires further study. However our study may open new avenues in the management of age related diseases if oxidative stress factors and antioxidant status of the elderly subjects are considered holistically.

ACKNOWLEDGEMENTS

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