AGE AND METABOLISM: AN INSIGHT INTO LIPID PEROXIDATION AND OXIDATIVE STRESS STATUS.

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ABSTRACT

Oxygen and diet play a vital role in human life. A small fraction of the oxygen is diverted to form reactive oxygen species (ROS) either accidently or deliberately. Organisms are well protected against free radical (FR) damage by antioxidant enzymes (AOE) such as Superoxide dismutase (SOD) and Catalase (CAT) or by antioxidant vitamins such as alpha-tocopherol, ascorbic acid, carotenoids, polyphenols and glutathione. The current study focused on the correlation between nutrition, blood lipid profile and estrogen levels as a function of age in women. Here we compared two nutritional regimens: a vegetarian diet with predominance of plant food with consumption of dairy products and an omnivorous diet predominantly non vegetarian food.

Biochemical assessment of Oxidative Stress (OS) parameters such as lipid profile, blood glucose and methemoglobin levels along with the estrogen were done. From our study, methemoglobin levels showed 50% increase in adult non-vegetarians (ANV) when compared to young adult vegetarians (YAV), glucose concentration were also increased by 69% in young vegetarian (YV) group when compared to non-vegetarian group. Vegetarians irrespective of age showed higher triglycerides and lower high-density lipoprotein, probably suggesting a risk for developing cardio-vascular diseases. Antioxidant enzymes such as Superoxide dismutase (SOD) showed reduced activity in vegetarians irrespective of age with a increased Malondialdehyde content. The estrogen levels were significantly increased in young adults vegetarians when compared to adult vegetarians. Our result showed both the diet group are under the risk and was in contrast to the belief that vegetarian had a better health benefit because of their diet.

KEY WORDS - Oxidative stress (OS), Lipid peroxidation (LPO), Total cholesterol (TC), Super oxide dismutase (SOD), Radical Scavenging activity (RSA), Estrogen, Malondialdehyde (MDA).

INTRODUCTION

Diet is the sum of food consumed by a person or any other organisms and dietary habits are the habitual decisions of an individual or culture that makes choosing what food to eat. Although humans are omnivores, each culture and each person holds some food preferences may be due to personal tastes or ethical reasons. (Navneet Kumar *et al*, 2015)

Nutrients that we obtain through food have vital effects on physical growth and development, maintenance of normal body function, physical activity and health. Nutritious food is, thus needed to sustain life activity. Diet must provide all essential nutrients in the required amounts that vary with age, gender, physiological status and physical activity. Dietary intakes lower or higher than the body requirements can lead to under or over nutrition respectively. Eating too little food during certain significant periods of life such as infancy, childhood, adolescence, pregnancy and lactation and eating too much at any age can lead to harmful consequences. An adequate diet, providing all nutrients is needed throughout our lives, the nutrients must be

obtained through a judicious choice and combination of a variety of foodstuffs from different food groups. (Farzhaana *et al*, 2013)

While most traditional diets globally are centered on carbohydrates, particularly through cereals, Indian diets tend to have multiple staples like rice, wheat, potatoes, pulses coupled with high levels of sweets and sugars. With the advent of modernization, cereals now undergo processing, which robs them of protective nutrients and fiber. The high glycemic index (GI) of Indian diets a necessity for our lifestyles is not serving us well. (Vishwanathan *et al*, 2002)

Vegetarian diets are usually rich in carbohydrates, n-6 fatty acids, dietary fiber, carotenoids, folic acid, vitamin C, vitamin E, and magnesium. They are low in proteins, calories, saturated fat, long chain n-3 fatty acids, choline, retinol, vitamin D, vitamin B12, calcium, iron, zinc and iodine. The vegetarian diet in India includes a wide range of vegetables, fruits, cereals, pulses, spices, seasonings and cooking practices, (Timothy *et al*, 2006) and hence can have different levels of bio-availability and absorption for many nutrients.

Non-vegetarian (NV) foods have countless health benefits as they are excellent source of the high quality protein, healthy fat, vitamins and minerals including all the essential amino acids which body requires for important functioning. They contain lots of iron which is beneficial to maintain hemoglobin count and body stamina. They are rich in calcium, phosphorus and vitamin B12 which are very necessary for the bone and blood health. NV foods are mostly required for growing kids for their healthy growth and development of the muscles, bone and other body systems. In addition they are rich in zinc and selenium which helps in the tissue formation and metabolism whereas selenium helps in breaking down fat and other chemicals.

Meat eaters are not immune to nutrient deficiencies, but because meat is a valuable source of many essential nutrients, vegetarians are particularly susceptible to certain deficiencies. Vegetarians or vegans may have trouble getting enough protein, iron, zinc, calcium, and vitamin B-12 or essential fatty acids. Symptoms of deficiencies in those nutrients can range from mild fatigue to serious effects like severe depression, chronic sleep issues and slow recovery from illness or injury.

Oxygen and diet play a vital role in human life. Oxygen is vital to provide energy through numerous metabolic reactions. A small fraction of the oxygen is diverted to form reactive oxygen species (ROS) either accidentally or deliberately. Oxidative stress(OS) due to reactive oxygen species ROS is implicated in the pathogenesis of wide variety of diseases like cancer, cataract, diabetes mellitus, rheumatoid arthritis, atherosclerosis, viral autoimmune diseases and aging. Free radicals (FR) are produced in normal and or pathological cell metabolism. All organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase (SOD) and Catalase (CAT), or by chemicals such as a-tocopherol, ascorbic acid, carotenoids, polyphenols and glutathione. (Amit Kumar *et al*, 2014)

Understanding the health effects of non-vegetarian and vegan diets is quite good but many uncertainties remain. Therefore, much attention is currently focused on the beneficial effect of vegetarian versus non-vegetarian diet. The present study was planned to correlate the oxidative stress and antioxidant status in healthy vegetarians and non-vegetarians young and middle aged women. Estrogens or estrogens are a group of compounds named for their importance in both menstrual and estrous reproductive cycles. They are the primary female sex hormones. Natural estrogens are steroid hormones while some synthetic ones are non-steroidal. Estrogen, which is also secreted to a lesser extent by the adrenal glands, actually refers to a class of 18-carbon steroid hormones. (D'Eon 2002)

There is a wide debate as to whether it is better to be a vegetarian or non-vegetarian. Vegetarians usually consume more fruits and vegetables than non-vegetarians and because of their restricted consumption of animal sources of foods, they have lower intake of saturated fatty acids and increased intake of fiber and various kinds of anti-oxidants compared to those of non-vegetarian origin.

In view of the above mentioned facts the current study aimed at evaluating the antioxidant and nutritional status in the blood of females based on their age and dietary intake. The results of the study may reveal whether the lower intake of cholesterol, saturated fat and increase in cereals fruits and vegetables will contribute in lowering blood lipid concentrations in vegetarians. This study would be first of its kind to correlate the antioxidant status, age and dietary habit and hence would be efficient in deciding a balanced

diet for women during their early adulthood and middle age. The study can also provide an insight into the risk factors associated in developing age-related complications associated with diet. The study also focused on estrogen levels of the experimental group, estrogen provides a natural defense against many diseases in menstruating women.

MATERIALS AND METHODS

CHEMICALS

All the chemicals used were of laboratory/analytical grade- Acetic acid, Copper sulphate, Dextrose, Di-potassium hydrogen orthophosphate, EDTA, Ethanol, Ethyl alcohol, Glucose-6-phosphate, Glycine, Hexane, Magnesium chloride, Methanol, Orthophosphoric acid, Potassium dihydrogen orthophosphate, Sodium bicarbonate, Sodium carbonate, Sodium chloride, Sodium hydroxide, Sodium nitrate, Sodium tungsten, Sulphuric acid, Tartaric acid, Thiobarbutiric acid(TBA), Tricholoro acetic acid(TCA), Tris-HCl.

Fine chemicals were purchased from Sigma- Bovine Serum Albumin (BSA), Commassie Brilliant Blue R-250, Diphenyl-1-Picryl Hydrazyl (DPPH), Epinephrine, and Nicotinamide Adenine Dinucleotide Phosphate (NADP).

METHODOLOGY

The study included those candidates who willingly volunteered for blood donation. Blood was drawn with prior consent explaining the subjects about the objective of the project. Women from the Bangalore and surroundings were randomly selected and were divided into four groups:

1. Non vegetarian young women aged 18-26 years (n=15) - young adult- non vegetarians (YA-NV)
2. Vegetarian women aged 18-26 years (n=15),-young adults –vegetarians (YA-V)
3. Non vegetarian women aged from 27-35 years (n=15) -adult non vegetarians (A-NV)
4. Vegetarian women aged 27-35 years (n=15) - as adult vegetarians (A-V).

All had an approximately similar physical activity (no sports).

EXCLUSION FACTORS:

- Females during their menstrual periods were not considered.
- Eggetarians were excluded from the study.
- Subjects under any medical complication, medications and hormonal therapy were not considered.
- Subjects with mild or chronic diabetes, hypertension were avoided.

INCLUSION FACTORS:

- Candidates consuming milk products were included under vegetarians.

SAMPLE COLLECTION

- Blood was collected from individuals with their prior consent and under expert supervision.
- Prior to sample collection the height and weight of the candidates were noted. Blood was collected after 2hrs of food intake.
- 5ml of blood was collected with anti-coagulant (heparin/EDTA).

BIOCHEMICAL ANALYSIS

I. Erythrocyte-related parameters

a. Total hemoglobin content (Hb)

The total hemoglobin was estimated by laboratory kit method using hemoglobin reagent –Hemocor D. Hemocor D solution was used as standard. The Hb content was calculated by Abs of test/Abs of STD X 15.06 and expressed as gm/dl

b. Methemoglobin (Met-Hb)

The methemoglobin concentration was determined by the method of Kampen and Zijlstra, 1961. The methemoglobin concentration was calculated by MetHb conc/total Hb content X 100 and expressed as MetHb%.

II. Glucose concentration

Glucose concentration in blood samples were determined by the method of Folin-Wu, 1924. Absorbance was read at 420nm in a double bio-spectrophotometer expressed in µg/ml against reagent blank. Glucose concentration was then determined from standard curve with a working stock of 200µg/ml Standard Glucose stock. The results were expressed as µg/ml.

III. Total Protein content

Protein concentration in plasma was determined by the method of Bradford, 1976. The concentration of protein in sample was determined from standard curve of 200 µg/ml Bovine Serum Albumin (BSA) stock. The results were expressed as µg/ml.

IV. Lipid Profiles

a. Total cholesterol

The total cholesterol was estimated using the Cholesterol Test Kit method. (Charles C Allain *et al*, 1974) The absorbance was recorded spectrophotometrically at 505nm against the reagent blank. Standard absorbance was recorded with the provided reagents. The total cholesterol was calculated by Abs of test/Abs of STD X 200 and expressed as mg/dl.

b. HDL-Cholesterol

The HDL-Cholesterol was estimated using the Cholesterol Test Kit method. The absorbance was recorded spectrophotometrically at 505nm against the reagent blank. Standard absorbance was recorded with the provided reagents. The HDL-Cholesterol was calculated by Abs of test/Abs of STD X 50 X 2 and expressed as mg/dl.

c. LDL-Cholesterol

The LDL-Cholesterol was estimated by using the Friedewald's equation Total cholesterol-Triglycerides/5-HDL Cholesterol. The LDL-Cholesterol concentration was expressed as mg/dl.

d. VLDL-Cholesterol

The VLDL-Cholesterol was estimated by using the Friedewald's equation- Triglycerides/5. The VLDL concentration was expressed as mg/dl.

e. Triglycerides (TG)

The triglycerides were estimated using the Triglycerides Test Kit method. The total triglycerides was calculated by Abs of test/Abs of STD X 200 and expressed as mg/dl.

V. Antioxidant Enzymes (AOE)

a. Superoxide dismutase Activity (SOD)

SOD activity was determined by the method of Mishra and Fridovich (1972). Activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50%, which is equivalent to one unit and is expressed in terms of unit/mg protein.

b. Lipid Peroxidation (LPO)

Malondialdehyde (MDA)

Plasma LPO in terms of Malondialdehyde (MDA) was measured according to the method of Esterbauer and Cheeseman (1990), with slight modification. The absorbance of supernatant was read at 532 nm. The concentration of MDA in plasma was calculated using extinction coefficient (ε= 31,500) and is expressed as nmol-mL^{-1} of plasma.

c. Radical Scavenging Activity of Plasma (RSA)

This assay was performed according to the method proposed by Szabo *et al*., (2007) Absorbance was measured at 517nm with methanol as a blank. Values were compared for control (A0) and plasma (A) and percent radical scavenging activity (%RSA) was calculated by using 100 (A0 − A)/A0.

d. Estrogen analysis

The estrogen levels were estimated by laboratory kit method using Total Estrogen ELISA Kit. The estrogen level concentration was expressed as pg/ml.

RESULTS

STATISTICAL ANALYSIS: Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant

I. Erythrocyte-related parameters

a. Total hemoglobin content (Hb)

Significant changes were not noticeable in the Hb content among all the groups. But however, vegetarians showed relatively lesser levels of Hb content when compared to Non-vegetarian group irrespective of age. (Fig.1)

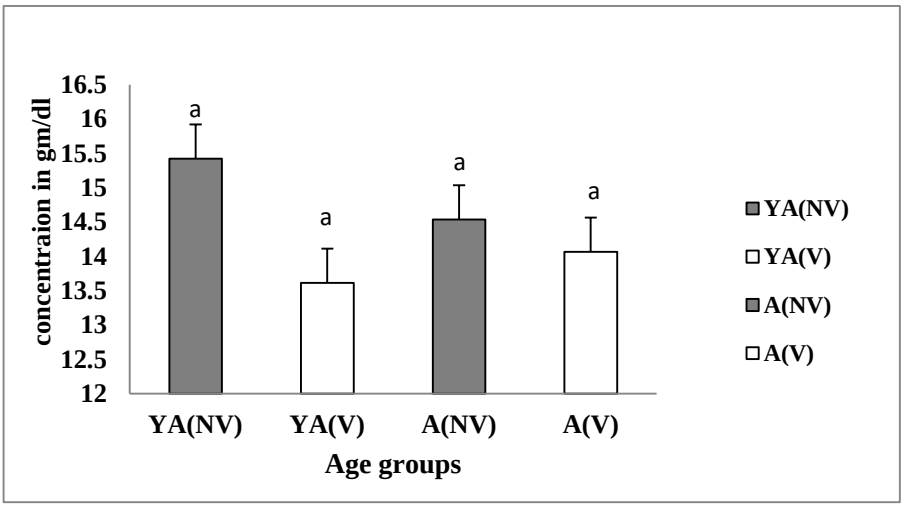

Fig.1

b. Methemoglobin (MetHb)

Young adults had lower levels of MetHb content ($p<0.001$) in comparison to the adult group irrespective of the diet. (Fig.2)

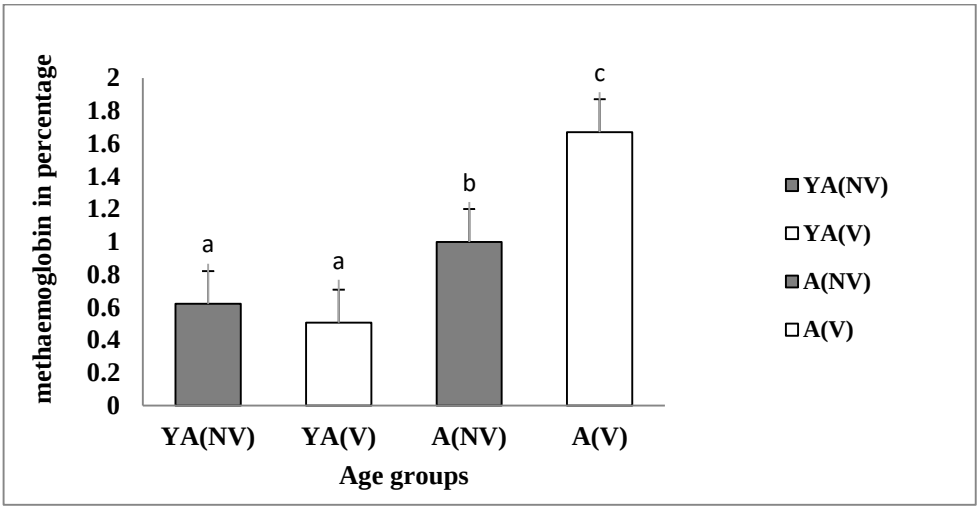

Fig.2

II. **Glucose concentration:**

Non-Vegetarians showed lower levels of glucose concentration ($p<0.001$) in comparison to the vegetarians group irrespective of age. (Fig.3)

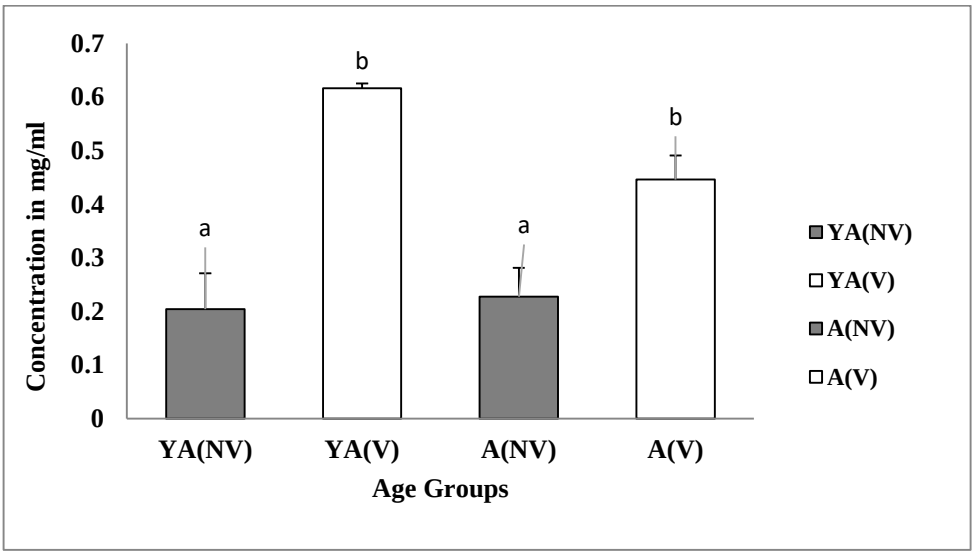

Fig.3

III. Total Protein content

Significant changes in protein content were not noticed in all the experimental groups. (Fig.4)

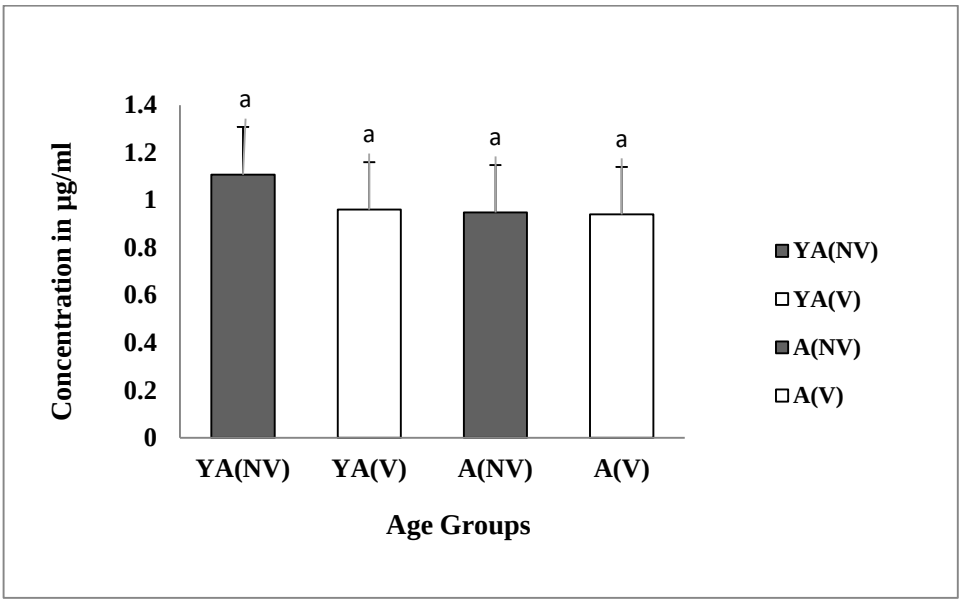

Fig.4

IV. Lipid Profiles.

a. Total Cholesterol (TC)

Young adults (YA) showed lower total cholesterol content when compared to the adults. Between adult non-vegetarian and adult vegetarians, Adult non-vegetarians had relatively higher total cholesterol content.(Fig.5)

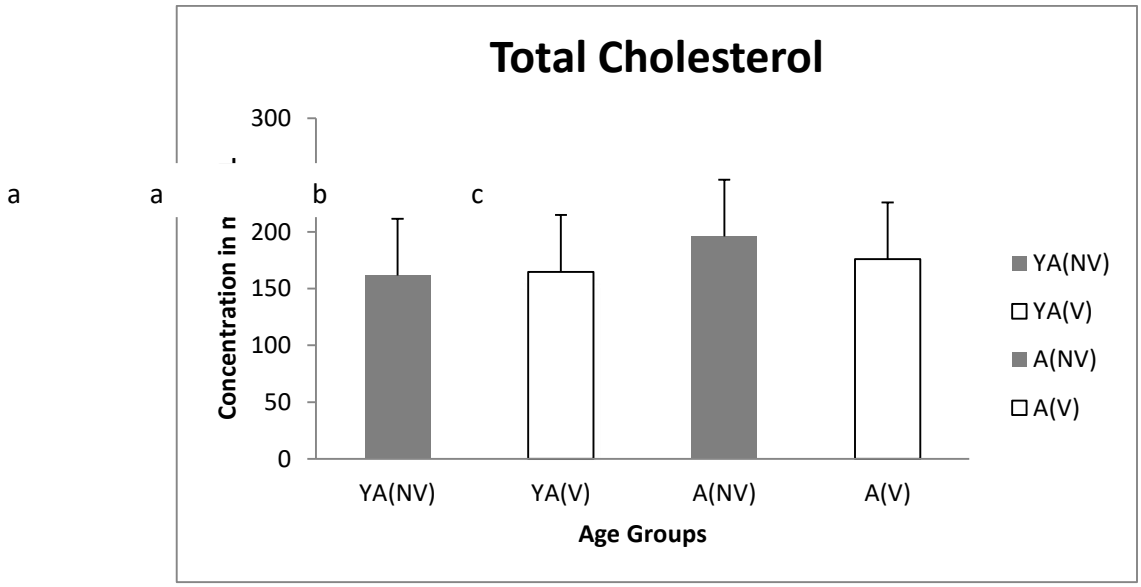

Fig.5

b. High density lipoproteins (HDL)

The present study results showed HDL values within the reference range of >60-70mg/dl in all the age groups. Irrespective of the age NV showed significantly (P< 0.001) higher levels of HDL content whereas among vegetarians adults revealed reduced (p<0.001) HDL content. (Fig 6)

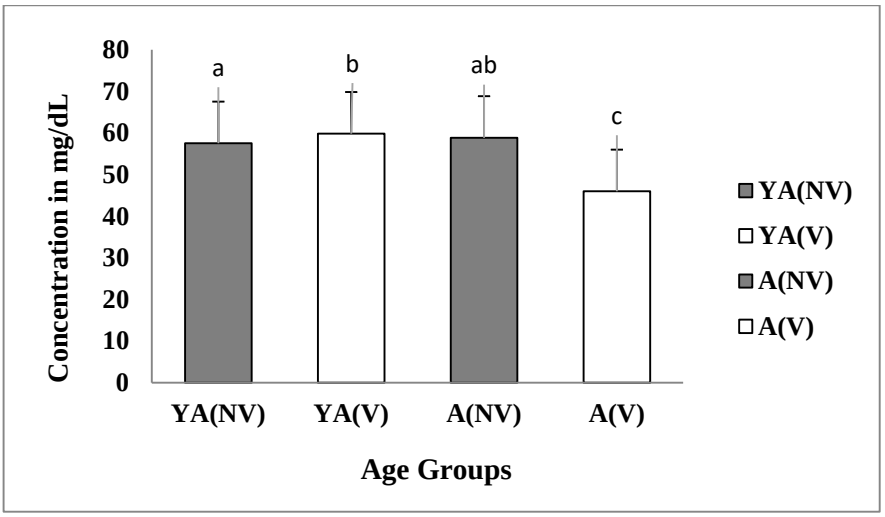

Fig.6

c. Low density lipoproteins (LDL)

In our study, LDL showed no changes within all the groups. (Fig 7)

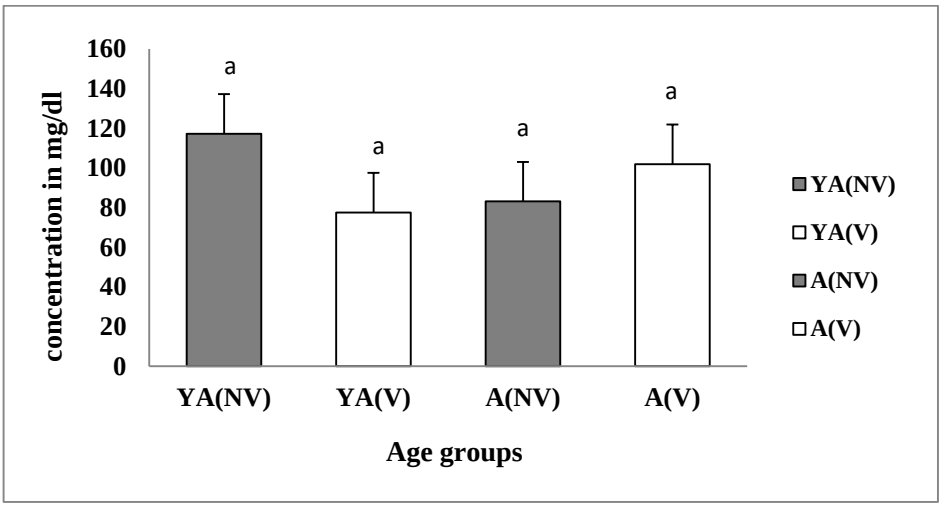

Fig.7

d. Very low density lipoproteins (VLDL)

The study results showed no significant changes in the VLDL level among all the groups. However the values were within the reference range (2-30 mg/dL) and reduced VLDL was observed in ANV when compared to other groups. (Fig 8)

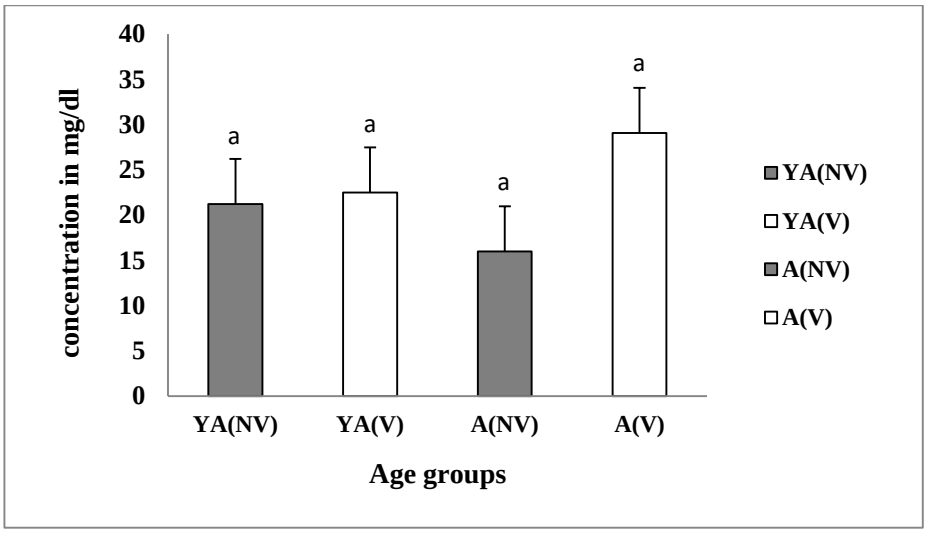

Fig.8

e. Triglycerides (TG)

From our results significant changes were noticeable in all the groups. Significantly ($p<0.001$) elevated levels of TG were seen in the ANV in comparison to other groups. Within the adults vegetarians showed reduced TG levels ($P< 0.001$) and within young adults NV showed remarkable reduction in TG level. (Fig 9)

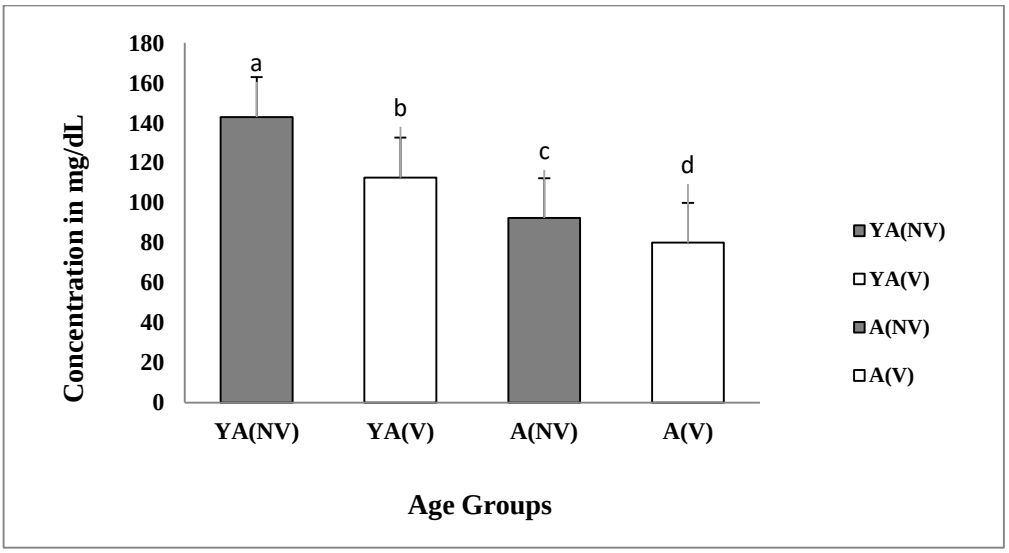

Fig.9

V. Antioxidant enzymes

a. Super oxide dismutase Activity (SOD)

SOD activity showed a significant increase (P< 0.001) in the A(NV) when compared to all other groups. However, Vegetarians had less SOD activity in comparison to non-vegetarians. (Fig 10).

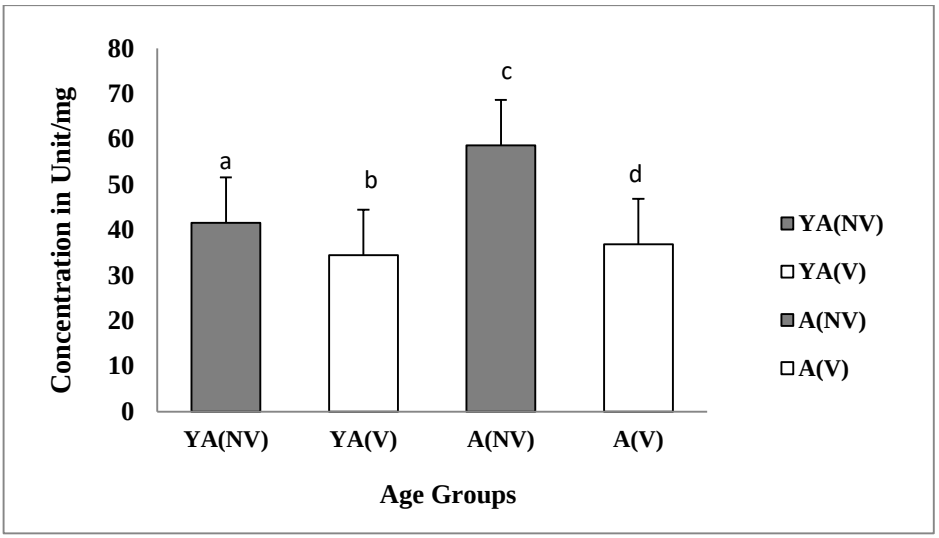

Fig.10

b. Lipid Peroxidation (LPO)

Malondialdehyde (MDA)

From our results no significant changes were noticed among the groups. (Fig.11)

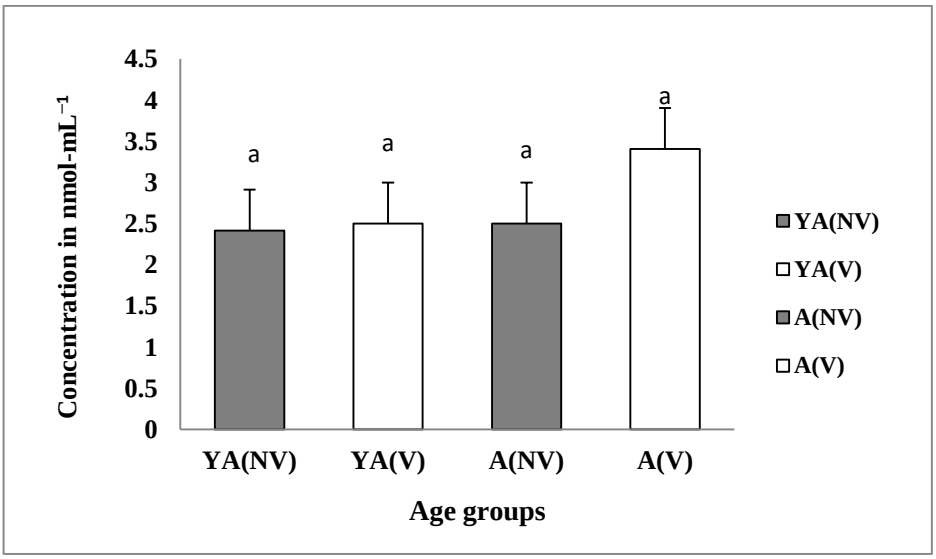

Fig.11

c. Radical Scavenging Activity of Plasma (RSA)

Radical Scavenging Activity of Plasma (RSA) showed no remarkable changes. However elevated activity was noticed in AV when compared to other groups. (Fig.12)

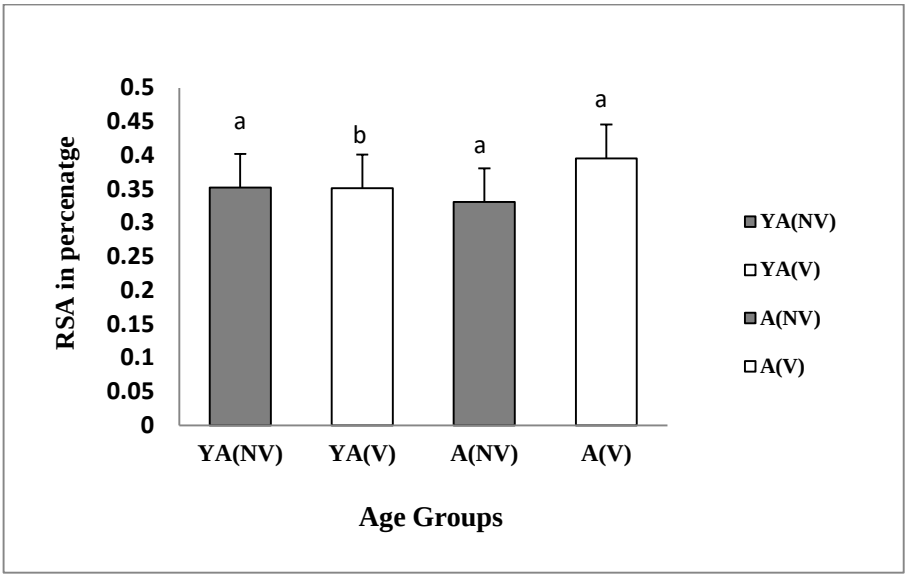

Fig.12

d. Estrogen analysis

Estrogen analysis was analyzed as a part of the hormonal interaction in metabolism. Our studies revealed a significantly ($P< 0.001$) higher estrogen levels in YAV when compared to AV. Within the NV groups there was no significant observed. All the samples analyzed had estrogen levels within the normal reference range (350pg/ml). (Fig.13)

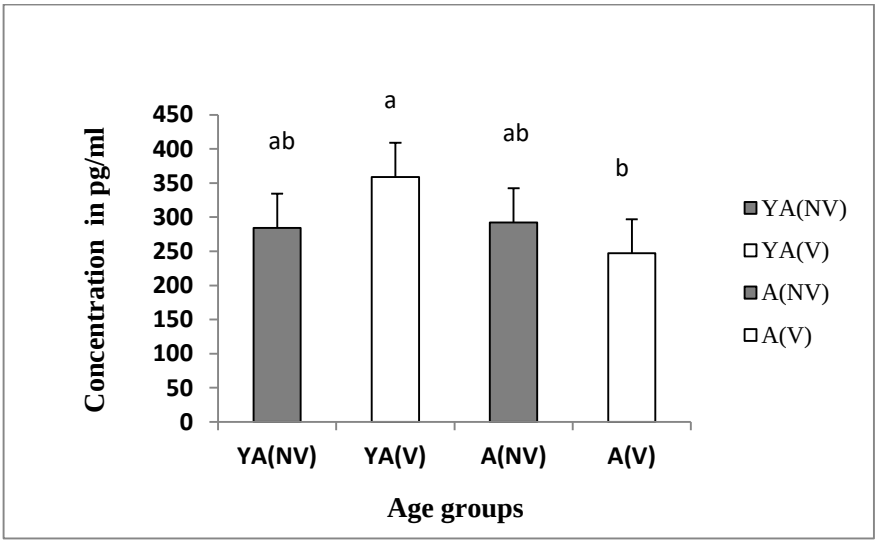

Fig.13

DISCUSSION

The nutritional profiles of vegetarian and non-vegetarian diet across various regions of India are not well documented. Studies have shown greater amounts of diet derived antioxidants such as vitamin C, A, E in Indian vegetarians that makes them less prone to oxidative stress and related diseases. Some studies on the nutritional profile of Indian vegetarian diet, demonstrate micro-nutrient deficiencies of zinc and iron that are primarily due to reduced absorption and vitamin B12 deficiency in rural and urban vegetarians. (Sridhar *et al*, 2014)

The indications of oxidative stress (OS) were correlated with less radical scavenging activity and significantly lower estrogen levels in the vegetarian group more so in the adults. Hence, a high risk group for oxidative stress related disorder. This observation is probably a clear indication of oxidative stress in the diet

group. But in contrast to this statement, in our study vegetarians irrespective of age had lesser Hb content, lower SOD activity, especially in adults and, higher MDA content.

Our result emphasizes on glucose and TC as a diet related parameter and age might have a lesser intervention. This is in accordance with the report that vegetarians had a favorable lipid profile than non-vegetarians. (Kumar *et al*, 2012)

Lipid profile showed no significant difference between the dietary groups in all age except for the triglycerides, where vegetarian had higher triglycerides content than NV. However the non-vegetarians showed increase HDL, probably a compensatory mechanism. This observation is an clear indication of the risk associated with non-vegetarian diet with advancing age to develop cardiovascular diseases.

Estrogen analysis also showed higher hormonal levels in the younger women, and a decreased amount was noticeable in the aged counterparts. This could be the hormonal alteration in the adults nearing the menopause.

In summary our study suggested that the diet and age played a tremendous role in the lipid and AO profile of women and the interplay was more or less independent of the hormonal influence. Both the dietary group had a risk of developing age-associated complications, contrast to the myth that vegetarians are favorable group. Hence a balanced diet in accordance with progression of age is recommended.

CONCLUSION

Cumulatively, our study reveals that MetHb levels were independent of diet and was function of age and probability of developing Methemoglobinemia is high with advancing age. There was an ambiguity in the lipid profile. Unlike expected, vegetarians showed levels of TG, LDL, VLDL, in par with NV. The variation in the levels of lipid profile among vegetarians and non-vegetarians were irrespective of age.

Estrogen analysis revealed slightly higher levels in the younger counter part, but the clear interplay with the antioxidant defense mechanism could not be established. Our study suggests that both diet and age had profound influence on the antioxidant status and lipid metabolism more or less in an independent way. This report is in contrast to the myth that vegetarians are the favorable group resisting lipid associated metabolic disorder.

ABBREVATIONS

ROS – Reactive oxygen species.

SOD – Superoxide Dismutase

CAT – Catalase

RSA – Radical scavenging activity

OS – Oxidative stress

FR – Free radical

HDL – High-density lipoprotein

LDL – Low-density lipoprotein

VLDL – Very low-density lipoprotein

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CONSENT FOR PUBLICATION

Not applicable.

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Legends for the figures.

Fig.1- Hemoglobin content. Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.2- Methemoglobin content. Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.3- Glucose concentration. Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.4- Protein content. Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant

Fig.5- Total Cholesterol. Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.6- HDL-Cholesterol. Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.7- LDL-Cholesterol. Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.8- VLDL Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.9- Triglycerides. Values are Mean ±SE, n=15. Results were analyzed by one-way Anova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant

Fig.10 - Superoxide dismutase. Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.11- Lipid peroxidation activity by MDA. Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.12- Radical Scavenging Activity (RSA) of plasma. Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.

Fig.13 - Estrogen analysis by ELISA. Values are Mean ±SE, n=15. Results were analyzed by one-way Annova and $p<0.05$ were considered significant. The graph bars sharing the similar alphabet are not significant.