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Research Article

Oxidative Stress in Vitamin B_{12} Deficient Female Subjects (30 – 50 years)

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ABSTRACT

Objective: *The present study was carried out to evaluate the oxidative stress in normal and vitamin B_{12} deficient females (age: 30 – 50 years).* ***Methods:*** *204 female subjects, aged 30 to 50 years were purposively selected from Vallabh Vidyanagar, Anand, Gujarat, India. Fasting venous blood sample was collected from all the subjects. Whole blood was estimated for Ascorbic Acid and Reduced Glutathione (GSH) levels while serum was used for the estimation of vitamin B_{12} and Total Antioxidant Capacity (TAC) using FRAP assay. The subjects having vitamin B_{12} levels >211 pg/ml were considered as normal subjects.* ***Results:*** *The female subjects were divided into two groups 30 – 40 years and 41 – 50 years. A significant difference ($P \leq 0.01$) was observed in vitamin B_{12} levels of normal and deficient female subjects belonging to both age groups. Female subjects (aged 41 – 50 years) who were having normal vitamin B_{12} levels were found to have significantly lower ($P \leq 0.05$) whole blood ascorbic levels (0.807 mg%) as compared to vitamin B_{12} deficient female subjects (1.031 mg%). Whole blood GSH levels of female subjects (30 – 40 years) who had a normal vitamin B_{12} levels were significantly higher ($P \leq 0.05$) compared to that of vitamin B_{12} deficient subjects. Serum TAC level was found to be significantly lower ($P \leq 0.01$) in vitamin B_{12} deficient female subjects compared to those having normal vitamin B_{12} levels as observed in both the groups.* ***Conclusion:*** *Vitamin B_{12} deficiency among 30 – 50 years female subjects was associated with oxidative stress as evident from a decreased level of whole blood glutathione levels and serum total antioxidant capacity.*

Key words: *Oxidative stress; Vitamin B_{12}; Reduced glutathione; Total antioxidant capacity*

INTRODUCTION

Free radicals plays an imperative role in cell's life and death. These are unstable/unpaired electrons in their outermost shell and may become highly reactive. Reactive oxygen species are produced from molecular oxygen/nitrogen through Electron Transport Chain, cytochrome P_{450}, and other cellular and sub-cellular functions. They affect beneficial metabolic and cellular processes and also play crucial role in pathological conditions of the body. It is normally balanced by endogenous antioxidant system. Imbalances in redox status may develop cellular oxidative stress.

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If the endogenous antioxidants fail to overcome the reactive metabolites production, then exogenous antioxidants would be necessary to balance redox status[1].

Vitamin B_{12} also called Cobalamin, is a water soluble vitamin with a significant role in the normal function of the brain and nervous system, in DNA synthesis and for the formation of blood. Vitamin B_{12} deficiency can lead to a wide spectrum of hematologic and neuropsychiatric disorders that can source hyper homocysteinemia and impaired immune system. Symptoms of vitamin B_{12} deficiency include fatigue, weakness, anorexia, paraesthesia, numbness and dizziness. Early diagnosis and prompt treatment of patients in the early stage of the disorder can often produce an improvement in their condition.

Vitamin B_{12} is absorbed from food after binding to intrinsic factor which is produced by the stomach. This vitamin B_{12}-intrinsic factor complex is essential for the absorption of vitamin B_{12} in the terminal ileum. Since the main sources of vitamin B_{12} for humans are meat and poultry, as well as dairy products and eggs suggesting that there has been less apprehension about B_{12} deficiency among vegetarians those who eat some animal based products[2].

In a cross-sectional study carried out amongst the higher socio-economic population of various age and sex in Bhuj City, Gujarat, India, total 866 patients were screened for serum vitamin B_{12} levels for 866 patients. They were 312 (36%) male and 554 (64%) female patients. Among these 866 patients, 352 (40.6%) were normal, 132 (15.2%) were borderline and 382 (44.2%) were deficient of vitamin B_{12}. Out of 382 deficient patients, 139 (36.39%) were males whereas 243 (63.61%) were females. Amongst these borderline and deficient 514 patients, 20 (4.0%) patients were between 10 – 21years, 218 (42.4%) patients were between 21 – 40years, 219(42.4%) patients were between 41 – 60 years and 57 (11.2%) patients were >60 years of age[3]. In a study conducted in civil hospital, Ahmedabad a total of 796 subject having age between 30 to 60 years had participated, out of which 313 (39.3%) had low vitamin B_{12} levels[2].

Vitamin B_{12} is an essential vitamin that plays a key role in many chemical reactions and affects many mechanisms in the body like nucleic acid metabolism, transference of methyl groups, synthesis and repair of myelin sheaths and formation of red blood cells. Vitamin B_{12} deficiency can lead to a great deal of dysfunction, mainly hematological and neuropsychic symptoms and hypothetically results in an effect that favors oxidant status in the oxidant/antioxidant ratio. Augmented levels of homocysteine, which has a pro-oxidant capacity, have been closely related to oxidative stress which generates reactive oxygen species, affecting the developing disulfide bonds. In addition, the trans-sulfation of homocysteine to glutathione, which is a major intracellular antioxidant, is an chief metabolic pathway related to homocysteine. Thus, a decline in glutathione levels is closely related to oxidative stress[4].

The study was conducted under the following objectives: (1) To study the prevalence of vitamin B_{12} deficiency in females (30 – 50 years) residing in Vallabh Vidyanagar. (2) To estimate whole blood ascorbic acid and reduced glutathione levels of females (30 – 50 years). (3) To analyse serum total antioxidant capacity (TAC) of females using FRAP assay (30 – 50 years).

MATERIAL AND METHODS

204 female subjects, aged 30 to 50 years were purposively selected from Vallabh Vidyanagar, Gujarat, India. The female subjects were divided into two age groups 30 – 40 years (N = 110) and 41 – 50 years (N = 94). Fasting venous blood sample was collected from all the subjects. Whole blood was estimated for Ascorbic Acid and Reduced Glutathione (GSH) levels while serum was used for the estimation of vitamin B_{12} and Total Antioxidant Capacity (TAC) using FRAP assay. The subjects having vitamin B_{12} levels >211 pg/ml were considered as normal subjects. Written consent was obtained from

all subjects after they were provided with a complete description of the study.

Serum Vitamin B_{12}

Serum Vitamin B_{12} was carried out by Chemiluminescence Immunoassay (CLIA) on Centaur.

Whole Blood Ascorbic Acid

Whole blood Ascorbic Acid was estimated by the method given by Roe and Kuether[5]. and Bessey *et. al*.[6], Exactly 0.5 ml of blood was mixed with 1 ml of chilled 5% TCA centrifuged at 5000 rpm for 10 minutes. 0.2 ml of supernatant was taken and volume was made up to 0.8 ml with 5% TCA and added 0.4 ml of 2,4 - Dinitrophenyl hydrazine (DNTP) /Thiourea/ Copper Sulphate (DTC) solution. The mixture was incubated at 37°C for 3 hours, 3 ml of 65% H_2SO_4 was added, mixed and allowed to stand at room temperature for an additional 30 minutes. Absorbance was determined at 520 nm using blank. The standard series of 4 to 16 µg was prepared in 0.8 ml of TCA and treated in the same way as the sample. Blank consisted of 0.8 ml 5% TCA and 0.4 ml DTC which was incubated for 3 hours, 3 ml of 65% H_2SO_4 added and mixed.

Whole Blood Glutathione

Whole blood Glutathione (GSH) was estimated by the method given by Ellman[7], 0.5 ml of blood sample was mixed with 1 ml of 5% TCA. The mixture was mixed by using cyclomixer followed by centrifugation at 5000 rpm for 10 minutes to obtain a protein free supernatant. After centrifugation, 0.1 ml of supernatant was taken in a plasma tube and added 3.9 ml of phosphate buffer (pH-8) and 0.2 ml of DTNB solution. Mixture was mixed and allowed to stand for 10 minutes at room temperature. The color developed was read at 412 nm in a double beam UV visible spectrophotometer. The standard series of 4 to 16 µg was prepared. The volume was made up to 4 ml with phosphate buffer (pH-8.0) and treated in the same way as sample. Blank was prepared by using 4 ml of phosphate buffer (pH-8.0) and treated in the same way as sample.

Serum Total Antioxidant Capacity

Total Antioxidant Capacity was estimated using FRAP. The procedure described by Benzie and Strain[8]. was used to evaluate the TAC. 0.02 ml of serum was taken in a test tube and volume was made up to 300 µl with the distilled water. 1.8 ml of FRAP reagent was added and allowed to incubate at 37°C for 10 minutes. The colored complex was measured at 593 nm using double beam U.V. spectrophotometer. Aliquot (50, 100, 150 & 200µl) of known concentration of Trolox was taken and volume was made up to 300µl with glass distilled water and treated in the same way as for sample. 300µl of distilled water was given the same treatment as for sample.

Statistical Analysis

The results are expressed as Mean ± SEM. The data was analysed by student's t-Test: Two-Sample Assuming Unequal Variances. A value of $P \leq 0.01$and $P \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

As shown in figure 1, out of 110 female subjects in the age group of 30 – 40 years, 66 female subjects had normal serum vitamin B_{12} levels (375.71 pg/ml) while, 44 female subjects in the same group were serum vitamin B_{12} deficient (158.75 pg/ml). Out of 94 female subjects in the age group of 41 – 50 years, it was found that 62 female subjects had normal serum vitamin B_{12} levels (441.42 pg/ml) whereas, 32 female subjects were found to have serum vitamin B_{12} deficiency (170.81 pg/ml). A significant difference ($P \leq 0.01$) was observed between the normal and vitamin B_{12} deficient female subjects of both the age groups. The present study indicates that 40% of subjects having age ranging from 30 – 40 years suffered from vitamin B_{12} deficiency while 34% of subjects were found to be vitamin B_{12} deficient in the age group of 41 – 50 years. Referring to a study reported by Issac, T. G., *et al*.[9], in India vitamin B_{12} deficiency is seen in about 3.8% of the population. About 75% of the subjects had metabolic signs of cobalamin deficiency, which was only partly explained by the

vegetarian diet. In a study, low serum levels of glutathione,

Ascorbic Acid present in the plasma forms a colored product on treatment with 2,4-dinitrophenylhydrazine in the presence of copper sulfate. The absorbance of the colored product was measured at 520 nm. As shown in Table 1, the whole blood ascorbic acid levels of normal female subjects having age group ranging from 30 – 40 years was found non-significantly lower (P≤0.05) (0.736 mg%) compared to vitamin B_{12} deficient female subjects (0.855 mg%). Normal female subjects having age group ranging from 41 – 50 years had whole blood ascorbic acid levels of 0.807 mg% which was significantly lower (P≤0.05) than the vitamin B_{12} deficient female subjects who had a level of (1.031 mg%). No significant difference was observed between the whole blood ascorbic acid levels of normal female subjects that fell in the age group of 30 – 40 years and 41 – 50 years. Similar trend was also observed in case of vitamin B_{12} deficient female subjects. These results indicate that at an advanced age vitamin B_{12} deficiency significantly affected the whole blood ascorbic acid levels. Kahn, S. B., Brodsky, I. and Fein, S. A.[10] reported low serum ascorbic acid levels in vitamin B_{12} deficient subjects. A high ascorbic acid levels in the blood of female subjects in the present study may be attributed to high consumption of seasonal fruits like amla as well as other foods viz. green leafy vegetables and sprouted pulses during the study period.

Glutathione (GSH) is a water soluble tripeptide composed of the amino acids glutamine, cysteine and glycine. The thiol is a potent reducing agent, rendering GSH the most abundant intracellular small molecule thiol, reaching millimolar concentrations in some tissues, as an important antioxidant. GSH plays a role in the detoxifications of a variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferases (GST) and glutathione peroxidase (GPs). The importance of GSH is evident by the wide spread utility in plants, mammals, fungi and some prokaryotic organisms[11]. As shown in Table 2, normal female subjects having age group ranging from 30 – 40 years had whole blood glutathione levels of 30.16 mg% of whole blood which was significantly higher (P≤0.05) than the vitamin B_{12} deficient female subjects who had a level of 25.14 mg%. The whole blood glutathione levels of normal female subjects having age group ranging from 41 – 50 years were non-significantly higher (P≤0.05) than that of vitamin B_{12} deficient female subjects (31.20 mg%). No significant difference was observed in whole blood glutathione levels of normal female subjects that fell in the age group of 30 – 40 years and 41 – 50 years. Similar trend was also observed in case of vitamin B_{12} deficient female subjects. There are two possible reasons for lower concentration of blood glutathione levels in vitamin B_{12} deficient subjects in both the age groups as reported by Niedzwiecki, M. M., *et al*.[12]. The first possible reason is: vitamin B_{12} is necessary in the synthesis of nucleic acid that make up DNA, the genetic material of our cells. White blood cells that are rapidly producing in the body use vitamin B_{12} at a higher rate. For the proper functioning of the immune system presence of adequate amounts of vitamin B_{12} is of great importance as these white blood cells are necessary for the destruction of cancerous cells, cellular debris as well as the pathogens identified by them. In vitamin B_{12} deficient state white blood cells are produced in inadequate amounts and the ones that are already existing in the body cannot function properly which leads to impaired immune function with increased susceptibility of the body to the infections. This results in the occurrence of pathogenic diseases, elevated oxidative stress and inflammation which drastically reduces the glutathione levels of the body. The second possible reason is: vitamin B_{12}, along with folate (B_9) and vitamin B_6 (all three are glutathione cofactors), participates in the methylation cycle (or methionine cycle) which transforms amino acid methionine to homocysteine and back to methionine, and the closely related trans-sulfuration cycle

(mediated by B_6) which converts homocysteine to cysteine and taurine. Cysteine, a by-product along the long chain of complex reactions of these cycles, is used by cells to synthesize glutathione. Approximately 50% of the cysteine used in the production of glutathione is derived from the conversion of homocysteine to cysteine through the trans-sulfuration pathway. Thus, vitamin B_{12} deficiency may lead to lack of cysteine produced from methionine which can impair glutathione synthesis considerably since cysteine is a limiting factor in glutathione production. In a study conducted on *Caenorhabditis elegans*, control worms possessed approximately 17.5 nmol of GSH per mg of worm proteins and no GSSG was detected. However, the GSH level was significantly reduced in the B_{12} deficient worms, which displayed a small amount of GSSG (approximately 0.6 nmol/mg protein)[13].

FRAP assay measures the change in absorbance at 593nm owing to the formation of blue color Fe^{11} tripyridyltriazine. Compound from colorless oxidized Fe^{11} from by the action of electron donating antioxidants. These include lipid-soluble antioxidant such as vitamin E, vitamin A and provitaminA (beta-carotene); and water soluble[14]. As shown in Table 3, the Total Antioxidant Capacity using FRAP assay of normal female subjects having age group ranging from 30 – 40 years was found to be significantly ($P \leq 0.01$) higher (9.61 mgTE/100ml of serum) compared to vitamin B_{12} deficient female subjects (8.42 mgTE/100ml). Normal female subjects having age group ranging from 41 – 50 years had serum total antioxidant capacity of 10.49 mgTE/100ml which was significantly higher ($P \leq 0.01$) than the vitamin B_{12} deficient female subjects who had a level of 8.34 mgTE/100ml of serum. No significant difference was observed between serum total antioxidant capacity of normal subjects that fell in the age group of 30 – 40 years and 41 – 50 years. Similar trend was also observed in case of vitamin B_{12} deficient female subjects. Many proteins under both physiological and pathological conditions are functionally regulated by glutathionylation, the finding of increased protein-bound glutathione further support the potential impact of glutathione pool changes in the pathogenesis of clinical signs in cblC (cobalamin deficiency type C) through different mechanisms: 1) lower availability of reduced glutathione; 2) glutathione depletion may amplify the oxidative damage, because of insufficient availability for glutathione dependent enzymes (GSH-peroxidase, GSH-transferase, xglutathione reductase). It is well known that glutathione depletion can occur because of a decreased synthesis, an increased utilization, or by the combination of both mechanisms[15]. After vitamin B_{12} is accumulated by living cells, it is converted into two coenzymes, 5′-deoxyadenosylcobalamin and methylcobalamin, which function as the coenzymes for methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) and methionine synthase (MS; EC 2.1.1.13), respectively. MCM catalyzes the conversion of L-methylmalonylCoA into succinyl-CoA in the catabolic pathway of certain amino acids, odd-numbered fatty acids, cholesterol, and thymine. MS catalyzes methionine synthesis from homocysteine (Hcy) with 5′-methyltetrahydrofolate. During vitamin B_{12} deficiency, the failure of vitamin B_{12} dependent methionine biosynthesis leads to the accumulation of Hcy, which has prooxidant activity and can activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate reactive oxygen species (ROS). These observations suggest that vitamin B_{12} deficiency disrupts cellular redox homeostasis to induce oxidative stress, which is implicated in various human diseases including atherosclerosis and neurodegenerative diseases[13]. These aforementioned mechanisms suggest a possible reason for the decreased anti-oxidant system of the body as evident from a lower level of serumn TAC in experimental subjects than their control counterparts. In a study conducted by Guney et al.[4], it was found that vegetarians had a reduced TAS and that vitamin B_{12} was positively correlated with TAS. The

researchers suggested that serum vitamin B_{12}, as a marker for functional vitamin B_{12} status, was the variable that influences TAS, and that functional vitamin B_{12} deficiency might contribute to hyperhomocysteinemia and decreased TAS in vegetarians. TAS (Total Antioxidant Status), and impaired antioxidant enzymatic activities were found to be associated with low serum levels of vitamin B_{12} in adults with type 2 diabetes. It was suggested that low vitamin B_{12} status with low folate was a potential triggering factor for developing hyperhomocysteinemia and oxidative stress in adults with type 2 diabetes[4].

As shown in figure 2, a negative and significant correlationship (R^2 = 0.018, P = 0.0010) was observed between whole blood glutathione levels and serum total antioxidant capacity of vitamin B_{12} deficient female subjects (30 – 40years). A negative and significant correlationship (R^2 = 0.120, P = 0.0032) was observed between whole blood ascorbic acid levels and serum total antioxidant capacity (figure 3) of vitamin B_{12} deficient female subjects (41 – 50years).

As discussed earlier, the results of the present study as well as the results reported by other researchers[4,12,15], the reduced glutathione levels was found to be significantly lower in vitamin B_{12} deficient subjects as compared to the normal subjects. Glutathione is considered as one of the potent antioxidants of whole blood which contributes to the Total Antioxidant Capacity of whole blood. Thus, in the present study a lower value of reduced glutathione among vitamin B_{12} deficient subjects compared to normal subjects may be attributed to a decrease in serum total antioxidant capacity.

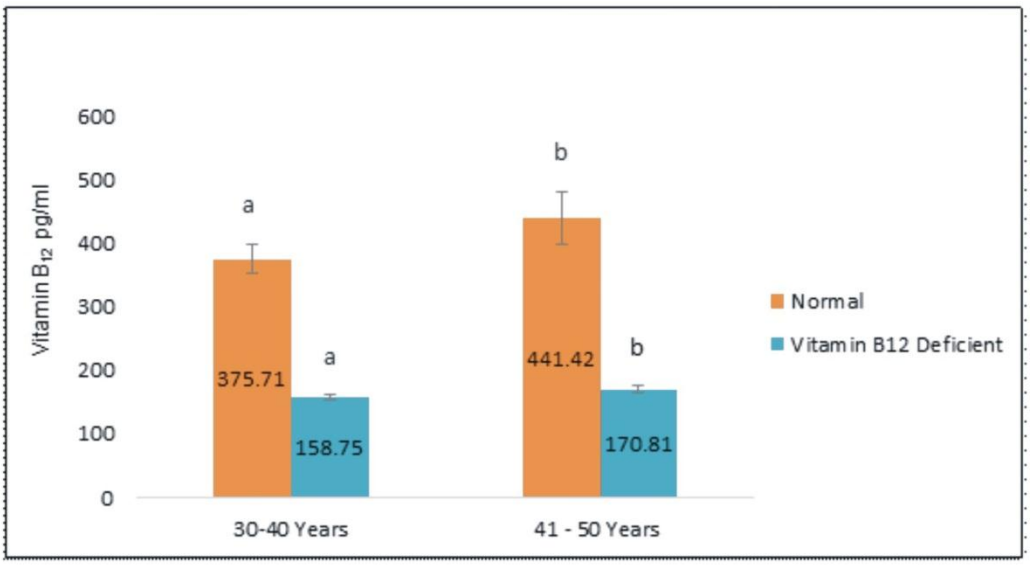

Fig. 1: Serum Vitamin B_{12} levels of Normal and Vitamin B_{12} deficient female subjects.
Bars sharing the same superscript within each set of two are significantly different (P≥0.01)

Table 1: Whole Blood Ascorbic Acid (mg%) levels of Normal and Vitamin B_{12} deficient female subjects

Age Group	Normal Subjects	Vitamin B_{12} Deficient Subjects	T-Value
30 – 40 Years	0.736 ± 0.061	0.855 ± 0.094	- 1.065^{NS}
41– 50 Years	0.807 ± 0.062	1.031 ± 0.099	- 1.768*
T-Value	-0.813^{NS}	- 1.151^{NS}	

Values are Mean ±SEM
* Indicates significant difference (P≤0.05)
^{NS} Indicates non-significant difference (P≥0.05)

Table 2: Whole Blood Glutathione (mg%) levels of Normal and Vitamin B_{12} deficient female subjects

Age Group	Normal Subjects	Vitamin B_{12} Deficient Subjects	T-Value
30 – 40 Years	30.16 ± 1.818	25.14 ± 2.112	1.799*
41– 50 Years	31.30 ± 1.951	31.20 ± 3.859	0.022NS
T-Value	- 0.429NS	- 1.377NS	

Values are Mean ±SEM
* Indicates significant difference (P≤0.05)
NS Indicates non-significant difference (P≥0.05)

Table 3: Serum Total Antioxidant Capacity (mgTE/100ml) levels of Normal and Vitamin B_{12} deficient female subjects

Age Group	Normal Subjects	Vitamin B_{12} Deficient Subjects	T-Value
30 – 40 Years	9.612 ± 0.301	8.421 ± 0.322	2.699*
41– 50 Years	10.49 ± 0.328	8.341 ± 0.446	3.877*
T-Value	- 1.970NS	- 0.145NS	

Values are Mean ±SEM
* Indicates significant difference (P≤0.01)
NS Indicates non-significant difference (P≥0.01)

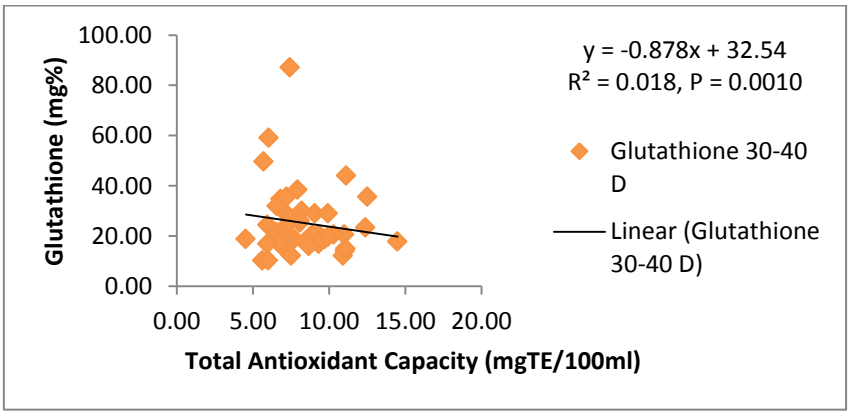

Fig. 2: Regression analysis between Total Antioxidant Capacity and Glutathione levels of Vitamin B_{12} deficient female subjects (30 – 40 years)

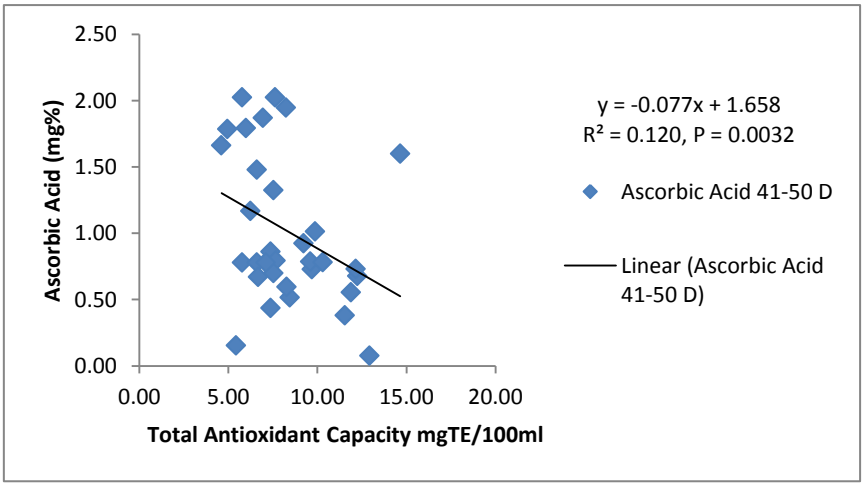

Fig. 3: Regression analysis between Total Antioxidant Capacity and Ascorbic Acid levels of Vitamin B_{12} deficient female subjects (41 – 50 years)

CONCLUSION

Vitamin B_{12} deficiency was associated with a reduction in whole blood GSH and serum TAC levels. This could be due to increase in homocysteine which has a pro-oxidant capacity that induces oxidative stress. The trans-sulfation of homocysteine to glutathione which is a major intracellular antioxidant, is an important metabolic pathway related to homocysteine. Thus, a decrease in glutathione levels is closely associated with oxidative stress.

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