Original Research Article

Levels of folic acid, vitamin B12 and Malondialdehyde in stroke patients

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Abstract

Background: Stroke is a major health problem in elderly population, however there remains a significant subset of younger patients with ischemic stroke in whom conventional vascular risk factors play a smaller role. Folic acid and vitamin B12 deficiency may cause increased homocysteine levels in blood which in turn increase the risk of atherosclerosis of cerebral arteries at young age. Oxidative stress characterized by increased generation of oxygen free radicals results in generation of lipid peroxidation product like malondialdehyde [MDA] and it is potential contributor to acute ischemic stroke. **Aims and Objectives:** To determine the association of levels of serum folic acid, vitamin B12 and MDA in patients of stroke in young with age and sex matched controls. **Methodology:** Present study was conducted at Government Medical College, Aurangabad. we studied 50 cases of stroke in young who were free from conventional vascular risk factors like diabetes mellitus, hypertension, hypercholesterolemia and cigarette smoking with 50 age and sex matched controls were selected. Their fasting serum folic acid, vitamin B12 levels by chemiluminescence method and serum Malondialdehyde level by Thiobarbituric acid method were determined. **Results:** In this study the levels of serum folic acid and vitamin B12 was significantly decreased in patients of stroke in young compared to controls with p-value of <0.0307 and <0.0232 respectively. The serum MDA levels was very highly significant [p<0.001] in patients of stroke in young than in controls. **Conclusion:** Hence it is concluded that serum folic acid and/or serum vitamin B12 deficiency play as an important independent risk factor for stroke in young patients in the absence of conventional risk factor. The serum MDA was significantly raised in all the patients of stroke in young, hence it is an indicator of lipid peroxidation in the pathophysiology of stroke.

Key Words: Ischemic stroke, folic acid, vitamin B12, vegetarians, oxidative stress, MDA.

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INTRODUCTION

Stroke remains a major cause of mortality and morbidity worldwide. The burden of stroke arises largely from the elderly population. However, there remains a small but significant subset of younger patients with ischemic stroke, in whom conventional vascular risk factors play a smaller role[1].Folic acid and vitamin B12 deficiency may cause increased homocysteine levels in blood. Plasma homocysteine levels above 12 µmol/L are known to increase the risk of cerebrovascular and coronary artery diseases at younger age[2-4]. Brain is very rich in polyunsaturated fatty acids which are highly susceptible to free radical attack increasing the product of lipid peroxidation, the Malondialdehyde [MDA]. Lipid peroxidation arising from the reaction of free radicals with lipids is considered a prevalent, important feature of the cellular injury brought about by free radical attack[5,6]. In the present study we compared the serum folic acid, vitamin B12 and MDA levels in patients of stroke in young with different age groups.

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MATERIALS AND METHODS

Source of data: A study of serum folic acid, vitamin B12 and malondialdehyde levels in patients of stroke in young was conducted in patients admitted to Government Medical College, Aurangabad, Maharashtra. Patients under study were predignosed and each gave informed consent. Whereas controls were normal healthy age and sex matched individuals were included.

MATERIALS Method of sample collection: Selected subject's blood samples were collected with all aseptic precautions. 10 ml of blood was collected from a median cubital vein. It was allowed to clot for 30 minutes in a clean dry test tube and was subjected to centrifugation for 20 minutes to separate the serum. The serum samples were stored at -70^{o}C till they were analyzed.

Sample analysis: The separated serum was used to estimate

- Serum folic acid by chemiluminescence method.
- Serum vitamin B12 by chemiluminescence method.
- Serum MDA by Thiobarbituric acid [TBA] method.

METHODS Estimation of folic acid[Chemiluminescence method].

A quantitative measurement of serum folic acid was done using immulite Folic acid kit in IMMULITE 1000 system.

Principle: Analysis of folic acid is done by competitive immunoassay method. Immulitefolic acid is a competitive, liquid-phase, ligand-labeled, protein binding chemiluminescent enzyme immunoassay with in situ immobilization and with an antiliganddetection system. The solid phase, a polystyrene bead enclosed within an Immulite Test Unit, is coated with a murine monoclonal antibody specific for folic acid binding protein. After the sample preparation procedure, the patient sample, ligand-labeled folic. acid analog and folic acid binding protein are simultaneously introduced into the Test Unit and incubated for approximately 30 minutes at 37^{o}C with intermittent agitation. During this time, folic acid in the sample competes with the ligand-labeled folic acid analog for a limited amount of folic acid binding protein and the folic acid binding protein is captured by the antibody on the bead. Incubation cycles: 2 x 30min.

Procedure: Samples being tested for folic acid must be pretreated with the working solution.

Preparation of working solution

- Borate-KCN buffer solution - 1000 microL/test
- Ligand-labeled folate - 20 microL/test
- Dithiothreitol - 20 microL/test

Sample pretreatment

1. Pipette 200 microL of each adjustor, control or patient sample-serum-into the tubes prepared.
2. Add 1000 microL of the working solution to all tubes.
3. Loosely cap all the tubes and place them in a covered, boiling water bath [100^{o} C] for 15-20 minutes.
4. Remove the tubes from the boiling water bath and cool them in an ambient water bath for 5 minutes.
5. Pipette at least 350 microL of the treated sample to an Immulite 1000 sample cup.

Estimation of vitamin B12 [Chemiluminescence method]: A quantitative measurement of serum vitamin B12 was done using immulitevitamin B12 kit in IMMULITE 1000 system.

Principle: Analysis of vitamin B12 is done by competitive immunoassay method. Immulitevitamin B12 is a solid-phase, competitive chemiluminescent enzyme immunoassay. Immulite 1000 vitamin B12 involves a preliminary heat denaturation step. Vitamin B12 in the patient sample is released from the carrier proteins by incubation at 100^{o} C in the presence of dithiothreitol and potassium cyanide to inactivate vitamin B12-binding proteins, even at extreme levels, as well as antibodies to intrinsic factor. After the heat denaturation test, the treated patient sample and hog intrinsic factor are simultaneously introduced into an Immulite 1000 Test Unit containing a polystyrene bead coated with a B12 analog and incubated for approximately 30 minutes at 37^{o} C with intermittent agitation. During this incubation, vitamin B12 in the treated sample competes with the B12 analog on the solid phase for a limited number of vitamin B12 binding siteson the purified intrinsic factor. Alkaline phosphatase-labeled anti-hog intrinsic factor is introduced and the Test Unit is incubated for another 30-minute cycle.

Procedure: Samples being tested for vitamin B12 must be pretreated with the working solution.

Preparation of working solution

- Borate-KCN buffer solution - 1000 microL/test
- Dithiothreitol - 20 microL/test

Sample pretreatment

1. Pipette 200 microL of each adjustor, control, or patient sample-serum-into the tubes prepared.
2. Add 1000 microL of the working solution to all tubes. Vortex.
3. Loosely cap all the tubes and place them in a covered, boiling water bath [100^{o}C] for 15-20 minutes.
4. Remove the tubes from the boiling water bath, and cool them in an ambient water bath for 5 minutes.
5. Pipette at least 350 microL of the treated sample to an Immulite 1000 sample cup.

Chemiluminescence reaction: First, the alkaline phosphatase conjugate (reagent) is bound to the bead (within the test unit) during the immunological reaction. The amount of alkaline phosphatase captured is inversely proportional to the concentration of vitamin B12 in the patient sample. Once the test unit is washed, a luminogenic substrate is added to the test unit and is moved onto the luminometerchain. Ten minutes later the test unit arrives in front of the photomultiplier tube, where the light generated by the luminogenic reaction is measured. Unlike, chemiluminescentreactions involving acridium esters the enzyme amplified reaction in the immulite 1000 system produces a prolonged glow. In the luminogenic reaction, the substrate (an adamantyldioxetane phosphate64) is dephosphorylated into an unstable anion intermediate by the alkaline phosphatase conjugate captured on the bead. The unstable intermediate emits a photon uponde composition. The amount of light emitted is directly proportional to the amount of bound alkaline phosphatase and in turn to the amount of vitamin B12 in the sample.

Estimation of serum Malondialdehyde

Principle: Auto-oxidation of unsaturated fatty acids involves the formation of semi-stable peroxides, which then undergo a series of reactions to form short chain aldehydes like Malondialdehyde (MDA). One molecule of MDA reacts with two molecules of TBA with the elimination of two molecules of water to yield pink crystalline pigment with absorption maximum at 535 nm.

Reagent: TBA reagent: 75 mg of TBA was dissolved in 15% Trichloroacetic acid [TCA], to this 2.08ml of 0.2N hydrochloric acid [Hcl] was added, the volume was made up to 100ml using 15% TCA.

Procedure: Test :0.75ml of serum was taken and 3.0 ml of TBA reagent was added.0.75ml of distilled water was taken and 3.0 ml of TBA reagent was added. Both the test tubes were kept in boiling water bath for 15 minutes. They were cooled and centrifuged for 10 minutes at 3,000 rpm. Absorbance of the supernatant was read against the blank at 535nm.

Calculation: Concentration of serum MDA in nmols/ml = Absorbance of test **x** Volume of the solution in cuvette**X**100

Molar extinction coefficient **x** Volume of serum = Absorbance of Test **x** 109**x** 3.75**X** 100

1.56 **x** 105**x** 1000 **x** 0.75 = Absorbance of Test x 3205.1

=.. ..... n mol/dl **x** 0.01

= ……n mol/ml

RESULTS

The case-control study involved 100 subjects out of which 50 were cases and 50 were healthy controls. The results were tabulated in master chart and statistically analyzed with the help of SPSS software. Mean, standard deviation (SD) and p value of all three parameters were calculated.

Age groups distribution

Table 1: Comparison of controls and patients according to broad age groups

Age Groups (Years)	Controls		Patients	
	No	%	No	%
21-30	16	32	16	32
31-40	16	32	16	32
41-50	18	36	18	36

Folic acid: The serum folic acid levels in 50 controls ranged from 4.0 to 20.0 ng/ml, while in the patients of stroke in young it ranged from 2.19 to 12.3ng/ml. The results are shown in table 2.

Table 2: Comparison of serum FOLIC ACID levels among controls and patients

Folic Acid Ng/Ml	Controls		Patients	
	No	%	No	%
<3	0	0.00	15	37
3+	50	100	35	70
Total	**50**	**100**	**50**	**100**

The mean and standard deviation [SD] of serum folic acid in the controls were 9.1and 8.66 and in the patients of stroke in young the mean and SD of serum folic acid were 6.4 and 4.22 respectively. The levels of serum folic acid was significantly decreased in patients of stroke in young compared to controls [p<0.021].

Vitamin B12: The serum vitamin B12 levels in the 50 controls ranged from 184 to 912pg/ml and in 50 patients of stroke in young it ranged from 162 to 764pg/ml.

Table 3: Comparison of serum VITAMIN B12 among controls and patients

Vit. B 12 pg/ml	Controls		Patients	
	No	%	No	%
<174	0	0.00	21	42
174+	50	100	29	58
Total	**50**	**100**	**50**	**100**

The mean and SD of serum vitamin B12 in the controls were 334 and 12.9respectively and the mean and SD in the patients of stroke in young were 251.3 and 11.1 respectively. The levels of serum vitamin B12 was significantly decreased in patients of stroke in young compared to controls [p<0.036].

Serum Malondialdehyde: The serum Malondialdehyde levels in 50 controls ranged from 4.19 to 6.9nmol/ml, while in the patients of stroke in young it ranged from 8.42 to 17.34nmol/ml.

Table 4: Comparison of serum MDA among controls and patients

Malondialdehydenmol/Ml	Controls		Patients	
	No	%	No	%
<6.50	18	36	5	10
6.51-9.99	32	64	8	16
10.00+	0	0	37	74
Total	**50**	**100**	**50**	**100**

The mean and SD in the controls were 5.9 and 2.13 respectively and the mean and SD in the patients were 13.2 and 4.7 respectively. The levels of serum malondialdehyde was very highly significantly increased in patients of stroke in young compared to controls (P<0.001).

Table 5: Mean, standard deviation (SD) and p-value along with the results of test of significance for the three parameters in this study

Parameters	Controls		Patients		p
	Mean	SD	Mean	SD	value
Folic Acid	9.1	8.6	6.4	4.22	0.021
Vit.B12	334	12.9	251.3	11.2	0.036
Malondialdehyde	5.9	2.13	13.2	4.7	0.001

DISCUSSION

The current study is a case-control study in which the serum folic acid, vitamin B12 and Malondialdehyde levels in 50 patients of stroke in young were compared with 50 healthy age and sex matched controls. The results were tabulated and statistically analyzed. All the 50 patients of stroke in young were estimated for serum folic acid and compared with controls. Serum folic acid was significantly decreased in patients of stroke in young compared with controls [p<0.021]. This study goes hand in hand with Wayne. H. Giles *et al*[7] where patients with the serum folate concentration <9.2 nmols/L were at increased risk for ischemic stroke and the study done by Lu-chen Weng *et al*[8] shows low folate intake was significantly and independently associated with ischemic stroke. Study done by Ka He *et al*[9] shows increased intake of folate was associated with a significant lower risk of ischemic stroke. Killian Robinson *et al* study shows that serum folate levels was decreased in cases than in controls [p<0.005]. The levels of serum vitamin B12 was significantly decreased in patients of stroke in young compared to controls [p<0.036]. Our study resembles study done by Nigel ChoonKait Tan *et al*[1] where the mean vitamin B12 was significantly lower in ischemic stroke patients than controls with p-value less than 0.001. Weikert. C *et al*[10] and M.G.H.vanOijen[11] studies shows decreased levels of vitamin B12 is a risk factor for cerebral ischemia and independent risk factor for ischemic heart disease. vitamin B12 deficiency leads to raised serum homocysteine levels which is common in India and is a major risk factor for stroke, the important predisposing factor is vegetarian diet. Similar finding was found in the study by Wadia *et al*[12] who observed that mean serum vitamin B12 levels in vegetarian with stroke was 142.8±158.6 and in nonvegetarians 273.0±194.5 [p<0.001]. With these findings they concluded that vitamin B12 deficiency leads to raised serum homocysteine levels which is common in India and is a major risk factor for stroke, the important predisposing factor is vegetarian diet, alsothe study done by Refsum *et al*[13] shows that vitamin B12 levels was low in 75% of total group [diabetes, IHD, diabetes with IHD, and patients with neither diabetes nor IHD] with associated raised homocysteine. They found serum vitamin B12 lower in vegetarian than in nonvegetarian. J.Kalita *et al*[14] study also shows low vitamin B12 levels in vegetarian [p<0.003] cases with ischemic stroke. Plasma levels of folate and vitamin B12 were directly correlated with homocysteine as their metabolism is linked. Folate was inversely correlated with homocysteine but not with vitamin B12. As folate is heat labile and largely destroyed by prolonged cooking at high temperature with Indian dishes such as curries, inadequate intake of fresh fruits and vegetables and hence low folate levels is seen Indian diet. Frying of vegetables which can destroy 90% of the folate content of vegetables[15-21].Our study mimics the study done by Giuseppe Salemi *et al*[22] where plasma homocysteine were increased with significantly decreased serum folic acid and vitamin B12 in patients than in controls group in acute atherothrombotic stroke and study by Jayantee Kalita *et al*[23]found that serum Hcy was elevated in 60.6% stroke patients, which is related to low serum vitamin B12 [25.7% patients] and folic acid [42.1% patients]. There is growing evidence that high homocysteine levels contribute to the pathogenesis of ischemic stroke. Homocysteine is believed to cause atherogenesis and thrombogenesis via endothelial damage, vascular smooth muscle proliferation, and coagulation abnormalities. High homocysteine levels are associated with increased risk of cardiovascular and cerebrovascular disease, although there are studies that show no increase in risk, and there is still debate as to the strength and validity of the association. Links between homocysteine, low vitamin concentrations and vascular disease were seen. However concentrations of homocysteine rise as the levels of folate, vitaminB12 and vitamin B6 fall and high homocysteine concentrations are often seen with deficiency of these vitamins[24,25]. Deficiency of B-vitamins, folate and vitamin B12 was associated with elevated plasma homocysteine concentrations. Thus, diminished availability of essential cofactors in the homocysteine-methionine-metabolism appears to be responsible for elevated homocysteine concentrations. Usually insufficient dietary intake is considered as the cause for the deficiency of these

vitamins. There seems to exist a link between oxidative stress involved in the pathogenesis of stroke and the depletion of B-vitamins. Since activation of immunocompetent cells like T-lymphocytes and macrophages is associated with overwhelming production of oxidizing compounds, immune activation is a major cause of oxidative stress. Oxidativestress in scope with chronic immune activation could therefore lead to the depletion of antioxidants including oxidation-sensitive vitamins like folate and vitamin B12. Methyltetrahydrofolate and cobalamine are important cofactors in the biochemical conversion of homocysteine and both are readily oxidized. Oxidative stress resulting from immune activation indeed could represent the cause for moderate hyperhomocysteinemia[26]. In the present study the serum MDA was significantly increased in stroke in young patient compared to controls [p< 0.001]. Since MDA is a specific marker of lipid peroxidation these data suggested that free radical production was increased in brain during ischemia**.** This study is in agreement with studies done by Jaspreet Kaur *et al* [27]who showed a significant increase in serum MDA [p<0.05] in stroke and TIA patients as compared to control group. Benedicta D'Souza *et al* 5study shows increase in lipid peroxidesin ischemic stroke patients. Significant higher levels [p<0.001] of MDA were seen in ischemic stroke patients when compared to control subjects. Increased lipid peroxides and oxidative stress has been implicated as a potential contributor to the pathogenesis of active CNS injury. It involves the generation of oxygen free radicals either as a cause or result of disease progression. Our study was also in accordance with M Beg *et al*[28],Levent SinanBir *et al* [29],Purnima DeySarkar *et al* [30] where serum MDA was significantly increased in stroke patients compared to controls with p value <0.001 in all the three studies. Recep Aygul *et al* [31] observed that the plasma MDA, NO and Hcylevels were significantly higher in the stroke patient compare to controls [p<0.01]. In this present study, deficiencies of folic acid and vitamin B12 have been confirmed as a risk factor which can cause increase in homocysteine level. These in turn lead to free radical production which causes oxidative stress in Ischemic stroke. The antioxidant capacity of the body is combated to balance the free radicals. Therefore Supplementation of folic acid and vitamin B12 has been demonstrated to be efficient in lowering mildly elevated plasma homocysteine levels and in reversing homocysteineinducedimpairment of endothelium-dependent vasoreactivity. Certainly, creatine, folic acid and vitamin B6, by scavenging superoxide radicals, are essential for cardiovascular health[32].

CONCLUSION

Our data suggest that low serum vitamin B12 levels and/or low folate levels increase the risk of stroke in young and nutritional deficiency may play a dominant role. The important predisposing factor of low vitamin B12 could be due to vegetarian diet. A regular supplement or fortification of food with vitamin B12 and folate could be a worthwhile preventive measure for stroke in young in this country. The serum MDA is raised in patients with acute stroke and hence it is an indicator of involvement of lipid peroxidation in the pathophysiology of the stroke in young patients.

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