May 2009

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ORIGINAL ARTICLE

Folic Acid and Vitamin B_6 Deficiencies Related Hyperhomocysteinemia in Apparently Healthy Pakistani Adults; Is Mass Micronutrient Supplementation Indicated in this Population?

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ABSTRACT

Objective: To determine the plasma/serum levels of homocysteine, and vitamins folate, B_6 and B_{12}, in Pakistani healthy adults.

Study Design: Cross-sectional study.

Place and Duration of Study: The Aga Khan University, from October 2006 to April 2008.

Methodology: Fasting levels of plasma/serum folic acid, pyridoxal phosphate (PLP), vitamin B_{12} and homocysteine were determined in 290 apparently healthy hospital personnel from institutions in two cities of Pakistan. Spearman correlation test and linear regression analysis was conducted.

Results: There were 219 males and 71 females with mean age of 46±10.5 years and mean body mass index of 23.5 ±3.8. Mean plasma homocysteine levels in Pakistani normal adults were found to be 17.95±8.4 μmol/l. Mean concentrations of plasma/serum folate, vitamin B_{12} and PLP were found to be 5±3.9 ng/ml, 522±296 pg/ml and 21.6±14 nmol/l, respectively. Serum/plasma levels of folate, vitamin B_{12} and PLP were negatively correlated with plasma homocysteine (rho coefficient=-0.367, p<0.001; -0.173, p=0.004; -0.185, p=0.002, respectively). Serum folate and plasma PLP levels were inversely related with plasma homocysteine, adjusted for gender, age, smoking and body mass index (p<0.001 and p=0.003, respectively). Percent deficiency values of folate, vitamin B_6 and vitamin B_{12} were 39.7%, 52.8% and 6.6% respectively.

Conclusion: The high levels of plasma homocysteine could indicate a reason for mass micronutrient supplementation to prevent the high incidence of cardiovascular disease observed in Pakistani population.

Key words: *Folate deficiency. Homocysteine. Hyperhomocysteinemia. Pyridoxal phosphate deficiency. Vitamin B_6 deficiency. Vitamin B_{12} deficiency.*

INTRODUCTION

Homocysteine is known to cause vascular endothelial dysfunction, which predisposes to atheroma formation, thrombo-embolism and coronary heart disease.[1] In a study carried out in the UK, plasma homocysteine concentrations were found to be elevated in South Asians living in Great Britain and it was suggested that it might be contributing to twice as many Coronary Heart Disease (CHD) deaths in this group compared with European whites.[2] The main determinant of the difference in homocysteine concentrations between these two groups was found to be reduced concentrations of folate and vitamin B_{12} in Great Britain's Asians indicating that nutritional deficiencies of B-complex vitamins might be contributing to their high rates of CHD. It has been pointed out that there is an epidemic of cardiovascular diseases in urban South Asians and it has also been advocated that preventive steps must be started very early in life.[3]

Pakistanis, being part of South Asian population, have very high rates of coronary artery disease. According to official estimates, Cardiovascular Disease (CVD) results in more than 100,000 deaths every year.[4] Endothelial dysfunction due to tissue accumulation of homocysteine could be another possibility because of deficiencies of folate and vitamin B_{12}. If deficiency of these micronutrients is widespread, this could provide a possible explanation to the high prevalence of CVD in Pakistanis and the concept of fetal origin of adult disease with new possibilities of intervention.[5] The present study was undertaken to determine the plasma/serum levels of homocysteine, folate, vitamin B_6 and vitamin B_{12} in healthy Pakistani adults.

METHODOLOGY

Two hundred and ninety normal healthy subjects were included in this study as representative of normal healthy people based on convenience sampling. They

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Received July 03, 2008; accepted January 26, 2009.

were selected from the personnel at the Aga Khan University, Civil Hospital and National Institute for Cardiovascular Diseases, Karachi, and at the Armed Forces Institute of Cardiology and Military Hospital, Rawalpindi. After ruling out any illness and not taking any B-complex vitamins for the last 4 months through history, they were included in the study after informed consent. Female pregnant subjects were also excluded from the study. Forty six were smokers, while only 3 were vegetarians. The study was approved by the Ethics Review Committee of the Aga Khan University.

Venous blood was obtained after at least 8 hours of overnight fasting. Blood was drawn from the cubital vein of the subject in sitting position. Plasma and serum were separated within 2 hours after sampling. The blood samples were kept at 4°C during this period of time. Plasma/serum was stored frozen at -60°C in small aliquots until analysis. These were analyzed for folate, vitamin B_{12} and Pyridoxal Phosphate (PLP), a coenzymic form of vitamin B_6 using radioassay.[6,7] Samples were also analyzed for plasma homocysteine using kit method based on fluorescence polarization immunoassay.

Vitamin B_{12} deficiency was defined as a serum concentration less than 200 pg/ml (147 pmol/l). Folate deficiency was defined as a serum concentration less than 3.5 ng/ml. PLP deficiency was defined as a plasma level less than 20 nmol/l.[8]

The analyses were performed with the help of SPSS version 13 for Windows. Mean values for serum/plasma folate, vitamin B_{12}, PLP and homocysteine were expressed as mean ± SD. Mean values of serum B_{12} and plasma PLP were compared using independent sample t-test because the data pertaining to these variables were normally distributed. However, Mann-Whitney test was employed for serum folate and plasma homocysteine as their values were not following normal distribution. Percent folate, vitamin B_{12} and vitamin B_6 deficiencies among males and females were compared using Chi-square test. Multiple linear regression was performed to determine the nature of relationship between plasma homocysteine and serum folate, serum vitamin B_{12} and plasma PLP, adjusted for age, gender, smoking and Body Mass Index (BMI). Strength of association was determined using correlational analysis. Since the data were not normally distributed, Spearman's rho was applied. A p-value less than 0.05 was considered significant.

RESULTS

There were 219 males and 71 females with mean age of 46 ±10.5 years, ranging from 30 to 75 years; mean Body Mass Index was 23.5 ±3.8. Mean concentration values of plasma/serum homocysteine, folate, vitamin B_{12} and PLP in normal healthy adults were found to be 17.95±8.4 μmol/l, 5±3.9 ng/ml, 525±296 pg/ml and 21.6±14 nmol/l, respectively (Table I). Mean value of plasma homocysteine was found to be significantly higher in males compared to females (p=0.001). The value of homocysteine in the study subjects was found to be very high compared to fasting homocysteine levels in normal healthy adults in several populations in the world (Table II).

Serum/plasma levels of folate, vitamin B_{12} and vitamin B_6 were found to be negatively correlated with homocysteine (Spearman's rho coefficients=-0.367, p<0.001; -0.173, p=0.004, -0.185, p=0.002, respectively). The correlation of folate with homocysteine was stronger compared to either of vitamin B_{12} or PLP. Multiple linear regression analysis revealed that serum/plasma levels of folate and PLP were inversely related with homocysteine adjusted for age, gender, smoking and BMI (Table III; p<0.001 and p=0.003, respectively). In the adjusted regression model, vitamin B_{12} levels were not found to be significantly related with plasma homocysteine (p=0.419).

Table I: Concentration values of serum/plasma folate, B_{12}, PLP, and homocysteine in normal healthy subjects (mean ±SD).

Variables	Total (n=290)	Males (n=219)	Females (n=71)
*Folate (ng/ml)	5±3.9	4.74±3.8	6.0±4.1
Vitamin B_{12} (pg/ml)	525±296	519±292	538±304
PLP (nmol/l)	21.6±14	21.6±14.5	21.7±15.2
*Homocysteine (μmol/l)	17.95±8.4	19.43±8.7	13.2±4.13*

**Mann-Whitney U-test was used to compare mean values of folate and homocysteine between males and females. Independent samples t-test was employed for the remaining variables (vitamin B_{12} and PLP).*

Table II: Fasting homocysteine levels in normal healthy subjects in different populations including the population investigated in this study (mean ±SD).

Population	No. (n)	Homocysteine concentration (μmol/l)	Investigator(s) year of publication	Reference
Hong Kong Chinese	23	12.1±4.7	Lolin *et al.*, 1996	16
Bostonian (USA)	118	9.6±3.3	Verhoef *et al.*, 1996	17
Taiwanese	40	11.02±2.85	Chou *et al.*, 2000	18
French				
- Males	931	10.82±3.49	Mennen *et al.*, 2000	19
- Females	1139	8.74±2.7		
Indian Asian (UK)	381	10.8±3.86	Chambers *et al.*, 2000	2
South Indian	90	18.04±10.69	Sastry *et al.*, 2001	10
Thai	178	19.69±8.1	Leowattana *et al.*, 2000	11
Turkish	91	15.6±10	Tokgozoglu *et al.*, 1999	12
Syrian				
- Males	92	14±6.2	Herrmann *et al.*, 2003	
- Females	67	11.2±3.6		14
German				
- Males	31	9.2±3.6	Herrmann *et al.*, 2003	
- Females	44	9.5±2.3		14
Polish	120	10.99±4.49	Dzielinska *et al.*, 2000	20
Danish	235	9.23±2.6	Chambers *et al.*, 2000	2
Iranian				
- Males	486	19.02±1.46	Fakhrzadeh *et al.*, 2006	
- Females	786	14.05±1.45		13
Pakistani				
- Rawalpindi	100	10.8±4.1	Aamir *et al.*, 2004	22
- Karachi	126	16.42±4.94	Iqbal *et al.*, 2005	21
- Rawalpindi and Karachi	290	17.95±8.4	Present study	

Table IV shows percent folate, B_{12} and B_6 deficiencies in Pakistani normal healthy subjects. Deficiencies of folate, vitamin B_6 and vitamin B_{12} were found to be 39.7%, 52.8% and 6.8%, respectively, while percentage of hyperhomocysteinemia (homocysteine levels >15 µmol/l) was found to be 57.2%. The proportion of hyperhomocysteinemia in males and females were 89.8% and 10.2%, respectively indicating that males were significantly more hyperhomocysteinemic compared to females ($p<0.01$). Similarly, folate deficiency and vitamin B_6 deficiencies were more pronounced in males compared to females.

Table III: Relationship of serum/plasma folate, vitamin B_{12} and PLP with plasma homocysteine adjusted for gender, age, smoking and BMI*.

Variables	B-coefficient	Standard error	p-value
Folate (ng/ml)	-0.51	0.123	<0.001
Vitamin B_{12} (pg/ml)	-0.0013	0.002	0.42
PLP (nmol/ml)	-0.097	0.032	0.003

**Multiple linear regression was used to study this relationship.*

Table IV: Percent folate, vitamin B_{12} and vitamin B_6 deficiencies in Pakistani normal healthy adults.

Micronutrient	Deficiency* %(n)			p-value
	Total (n=290)	Males (n=219)	Females (n=71)	
Folate	39.7 (115)	80.8 (93)	19.2 (22)	0.0001
Vitamin B_{12}	6.6 (19)	73.7 (14)	26.3 (5)	0.14
Vitamin B_6	52.8 (153)	77.8 (119)	22.2 (34)	0.0001

**Deficiency of each micronutrient has been defined in Subjects and Methods section.*
***P-value is based on test of association using Chi-square. The proportions of males and females were compared.*

DISCUSSION

These preliminary results point towards the enormity of the problem that exists in this part of world. B_6 deficiency of 52.8% and folate deficiency of 39.7% suggest that nutritionally, these vitamins appear to have been most neglected.

The value of plasma PLP as standard for adequate B_6 status merits some discussion. According to the Standing Committee on Scientific Evaluation of Dietary Reference Intake, USA, a plasma PLP concentration of 20 nmol/l was not accompanied by observable health risk,[8] and was chosen as the minimum standard for adequate status. In the absence of any data on plasma levels of PLP in Pakistani healthy adults, this value was taken as the minimum value for adequate status of B_6. Since, nearly 53% of the normal healthy subjects had values lower than that, it is possible that for this population, the normal range might be different from the Western population. For example, Robinson *et al.* reported that only 2% of control subjects in Cleveland, USA, had levels of PLP below 20 nmol/l.[9]

Plasma homocysteine values obtained in the present study are quite high. Similar high values of homocysteine in healthy subjects have also been reported for a South Indian population, a Thai population, a Turkish population, an Iranian population and a Syrian population.[10-14] Such high levels of homocysteine, which fall into the category of "mild hyperhomocysteinemia" [15-30 µmol/l] have also been observed in our younger age group. Similar findings have been reported by Refsum *et al.* on a population from South India.[15] Compared to these values, plasma concentrations of homocysteine in Hong Kong Chinese, Bostonians, USA, Taiwanese, French males and females and Poles appear to be quite low.[16-20] The result of this study further substantiate the initial report of high levels of plasma homocysteine on a relatively small number of subjects (n=126) from the Karachi population.[21] However, the current values for homocysteine were found to be higher than that reported for a Rawalpindi population by Aamir *et al.*[22] The discrepancy could be due to the reason that we excluded all those subjects who had been taking folate, vitamin B_{12} and vitamin B_6 for the last 4 months, while this was not part of the exclusion criteria in the study by Aamir *et al.*[22] In a recent study from Canary Islands, the percent hyperhomocysteinemia in Canarian men was found to be 32.2%, considerably higher than the other Spanish population.[23] Canary Islands residents have been known to present highest age – adjusted mortality rate for CVD in all of Spain. Similarly, high prevalence of hyperhomocysteinemia due to low folate, vitamin B_{12} and vitamin B_6 status has been reported in Chinese men from Northern China.[24] In comparison with these reports, the percent hyperhomocysteinemia in Pakistani adult males (89.8%) in the present study is remarkably high. This may explain why rates of coronary artery disease are highest in our population and why the South Asian men are at greater risk of developing coronary artery disease at a younger age.[2,25]

WHO has proposed lower limit of 6 ng/ml for folate concentration.[26] It is important to note that 74% of our normal healthy subjects fall below this limit (data not shown). Since homocysteine concentration has been shown to increase when folate levels fall below 5.5 ng/ml,[27] it is conceivable that low folate is one of the major causes for the increase in homocysteine levels observed in our population.

A number of randomized-controlled trials have demonstrated that folic acid supplementation can significantly reduce plasma homocysteine levels, even when plasma levels of homocysteine and folate are already in the normal range.[28] In a recent meta-analysis by Bazzano *et al.* role of folate supplementation has been questioned in reducing the risk of CVD in persons with a history of vascular disease.[29] However, there has been hardly any report on folate supplementation not being beneficial in normal healthy subjects.

More recently, low folate and vitamin B_6 have been reported to be associated with retinal vein occlusion

independently of homocysteine levels.[30] This report points towards a wider role played by B-vitamins. This also suggests that mass supplementation of these micronutrients would be necessary to prevent deficiency associated health problems. A number of developing countries are turning towards fortification of staple food with B-vitamins to overcome the problems related to their deficiencies.[31] Pakistan is yet to take an initiative in that direction.

Despite the limitation of this study that its normal healthy subjects do not represent the general population, it definitely alludes to the problem of deficiency of these micronutrients in this country.

The data, however, are suggestive that there is a need for supplementation trials with folate and vitamin B_6 and possibly vitamin B_{12} in our population to lower the high levels of plasma homocysteine. Large scale "community-based' studies would be required to assess the prevalence of the deficiency of these three vitamins in our population and how these could be associated with hyperhomocysteinemia before embarking on intervention strategies. A special emphasis should be on women of fertile age and those who are pregnant to avoid adverse pregnancy outcome and possibly lasting endothelial dysfunction.

CONCLUSION

High prevalence of folate and vitamin B_6 deficiencies along with hyperhomocysteinemia in healthy Pakistani adults working in healthcare institutions is suggestive that these deficiencies could be very common in our community (general) population as well. Therefore, a serious consideration must be given towards mass micronutrient supplementation to prevent high incidence of CVD in this population.

Acknowledgement: The study was supported by a grant from the Pakistan Science Foundation, S-AKU/MED (210). We gratefully acknowledge the help of Dr. Majid Shafiq for procuring samples from personnel of the Armed Forces Institute of Cardiology and Military Hospital, Rawalpindi.

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