Original Article

Oral vitamin B_{12} supplementation reduces plasma total homocysteine concentration in women in India

Chittaranjan S Yajnik MD FRCP[1], Himangi G Lubree MSc[1], Nileema V Thuse BHMS[1], Lalita V Ramdas MCom[1], Swapna S Deshpande MSc[1], Vaishali U Deshpande MSc[1], Jyoti A Deshpande MSc[1], Bhagyashree S Uradey MSc[1], Anjali A Ganpule MSc[1], Sadanand S Naik PhD[1], Niranjan P Joshi MD DNB[1], Hannah Farrant MSc[2] and Helga Refsum MD PhD[3]

[1]*King Edward Memorial Hospital, Pune, India*
[2]*Medical Research Council, Environmental Epidemiology Resource Centre, Southampton, UK*
[3]*Institute of Basic Medical Sciences, Department of Nutrition, University of Oslo, Norway; Oxford Centre for Gene Function, Department of Physiology, Anatomy & Genetics, University of Oxford, UK*

People in India have a high prevalence of low vitamin B_{12} status and high plasma total homocysteine (tHcy) concentrations. In a proof of principle trial, we studied the effect of oral vitamin B_{12} (500 μg) and/or 100 g cooked green leafy vegetables (GLV) every alternate day in a 2x2 factorial design over a 6-week period. Forty-two non-pregnant vegetarian women (age 20-50 years) were randomly allocated to four study groups. Clinical measurements were made at the beginning and at the end of the study, and blood samples were collected before, and 2 and 6 weeks after commencement of intervention. Forty women completed the trial. Twenty-six women had low vitamin B_{12} status (<150 pmol/L) and 24 had hyperhomocysteinemia (>15 μmol/L). GLV supplementation did not alter plasma folate or tHcy. Vitamin B_{12} supplementation increased plasma vitamin B_{12} concentration (125 to 215pmol/L, $p<0.05$) and reduced tHcy concentration (18.0 to 13.0 μmol/L, $p<0.05$) within first 2 weeks, both of which remained stable for the next 4 weeks. Plasma vitamin B_{12} and tHcy concentrations did not change in those who did not receive vitamin B_{12}, and there was no change in plasma folate concentration in any of the groups. Blood haemoglobin concentration increased marginally within first two weeks in those women who received vitamin B_{12} (by 3 g/L, $p<0.05$) and the number of women with macrocytosis decreased from 2 to zero. There was no change in vibration sensory threshold during the period of the study. High-dose per oral vitamin B_{12} supplementation significantly reduced plasma tHcy within 2 weeks but did not achieve normal plasma tHcy concentration even after 6 weeks.

Key words: vitamin B_{12}, total homocysteine, folate, supplementation, India

Introduction

Recent studies in Pune, India suggest that hyperhomocysteinemia and low vitamin B_{12} status are common in men and women while low folate status is relatively rare.[1] Hyperhomocysteinemia is a risk factor for cardiovascular disease (CVD), adverse pregnancy outcomes, birth defects, and psychiatric disorders, including dementia and Alzheimer's disease.[2-5]

We are particularly intrigued by the observation that low maternal vitamin B_{12} status and associated high levels of plasma total homocysteine (tHcy) concentration predict low birth weight in the baby.[3, 4] Low birth weight and associated changes in body composition are risk factors for future type 2 diabetes and CVD. Thus, improvement in vitamin B_{12} status and reduction in circulating levels of tHcy in the mothers might improve fetal growth, and reduce the risk of a number of conditions, including CVD and type 2 diabetes in South Asian Indians. There is little information on the effect of vitamin B_{12} supplementation in Asian Indians. In this preliminary study we investigated the effect of oral vitamin B_{12} supplementation on plasma tHcy concentration in non-pregnant Indian women.

Methods

Subjects

We invited 52 healthy, non-pregnant, lacto-vegetarian women not taking any vitamin supplements (staff members of the KEM Hospital) to participate in the trial. We measured their blood haemoglobin and plasma vitamin B_{12} concentrations. Two women with blood haemoglobin <90 g/L were excluded. Eight declined to participate in the trial. Forty-two women were randomly allocated to four study.

Corresponding Author: Dr Chittaranjan S Yajnik Director, Diabetes Unit, King Edward Memorial Hospital, Moodliar Road, Pune, India.
Tel/ Fax: 0091 20 26111958
Email: diabetes@vsnl.com
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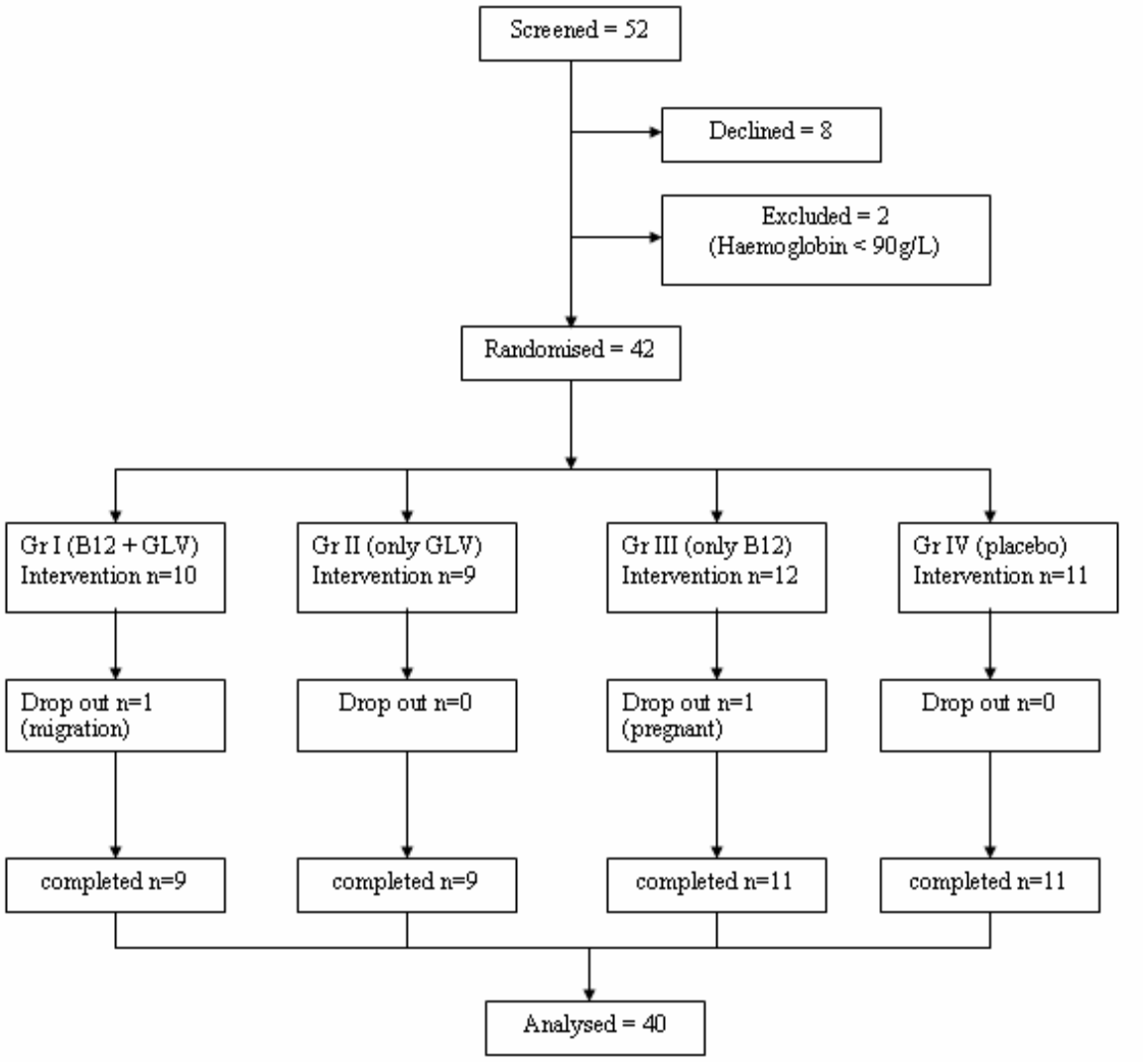

Figure 1. Study design and subject allocation

groups. However, two women dropped out (one conceived, one migrated). We present results on 40 women who completed the trial (Fig 1). Ethical committee of the KEM Hospital Research Center approved the study. All participants signed an informed consent.

Intervention

The intervention was by 2x2 factorial design; the two arms supplemented vitamin B_{12} or placebo and Green Leafy Vegetables (GLV) or control meal. Subjects were randomly allocated to four groups after minimization for age and plasma vitamin B12 concentration (above and below the median) to ensure comparability. Every alternate day, they were fed under supervision either vitamin B_{12} (500 µg methylcobalamin) or placebo, and 100 g of cooked seasonal GLV (fenugreek, spinach, amaranthus, chavali or coriander) or seasonal non-GLV (cauliflower, white pumpkin, brinjal (aubergine) or capsicum). The vegetables were carefully washed and cooked (avoiding overcooking), and served as common recipes.

Measurements

Standardised anthropometric parameters (weight and height), blood pressure (UA 767PC, A & D Instruments Ltd, Abingdon, Oxford, UK) and vibratory sensory threshold in the feet (Sensitometer, Dhansai Laboratory, Mumbai, India) were measured at the beginning and at the end of the study. A fasting blood sample was obtained before, two and six weeks after intervention in the supine position from a free flowing cannula placed in antecubital vein. The sample was collected into tubes containing EDTA, stored on ice and spun within half an hour at 2500 x g for 30 mins to obtain plasma. Aliquots of plasma were stored at –80^{o}c till further analysis. Haematological measurements were made within one hour of blood collection on Beckman Coulter Analyser (A^{C}.T diff ^{TM}, Miami, Florida). Plasma glucose, total and HDL cholesterol and triglycerides were measured on Alcyon 300 automated analyser (Abbott Laboratories, Abbott Park, IL, USA) using standard methods. Intra- and inter-batch coefficients of variation for all these assays were <5%. Pre- and post-intervention plasma vitamin B_{12}, tHcy and folate measurements were done in the same batch. Plasma vitamin B_{12} and folate were measured using a radioimmunoassay kit (Diagnostic Products Corporation, USA). Plasma tHcy was measured by immunofluores-

cence polarisation assay on an AxSYM system (Abbott, IL, USA, Intra and inter-batch CV was <10%).

Statistical methods

Data are presented as median and inter-quartile range. Differences between groups and between pre and post intervention measurements were tested by non-parametric tests for paired and unpaired samples as appropriate. Contribution of regression to mean (RTM) was assessed by the Oldham approach (6). Associations were tested by the Spearman correlation coefficient. A p value of <0.05 (two sided) was considered significant. SPSS version 11.0 was used for statistical analysis.

Results

Baseline results

Table 1 shows the baseline characteristics of women in vitamin B_{12} supplemented and non-supplemented groups. Of the 40 women who completed the study, twenty-six women (62%) had low vitamin B_{12} status (<150 pmol/L) and ten (25%) had low folate status (plasma folate <3.0 ng/mL). Twenty-four women (57%) had hyperhomocysteinemia (≥15 μmol/L). Seventeen (40%) women were anaemic (haemoglobin <115 g/L), 12 had microcytosis (mean corpuscular volume, MCV <80 fL) and three had macrocytosis (MCV >100 fL). All women had normal vibration sensory threshold.

Plasma vitamin B_{12} and folate concentrations were strongly correlated (r=0.73, p <0.001), and both of these variables were inversely related to plasma tHcy concentration (r ~ -0.50, P <0.001 both). MCV was significantly inversely related to plasma vitamin B_{12} and folate (r ~ -0.46, P <0.01 both) and tHcy concentrations (r=0.69, P <0.001). Vibration sensory threshold was not related to plasma vitamin B_{12}, folate and tHcy concentrations.

Effect of intervention

Dietary assessment of these women showed that the habitual intake of GLV or other vegetables did not change significantly during the study period. GLV supplementation did not significantly affect plasma folate, vitamin B_{12} or tHcy concentrations. We have therefore, analysed further results by pooling groups to compare the vitamin B_{12} supplemented (group I + III) and vitamin B_{12} not supplemented (groups II + IV) groups (Fig 1).

Plasma vitamin B_{12} and total homocysteine

Vitamin B_{12} supplementation led to an increase in plasma vitamin B_{12} concentration (from 125 to 215 pmol/L, p<0.001) in the first 2 weeks. After that, there was no further increase in the following four weeks. The number with low vitamin B_{12} status decreased from 14 to four in two weeks and then to three by end of six weeks. Vitamin B_{12} supplementation reduced plasma tHcy concentration by an average of 28% (18.4 to 13.4 μmol/L, p<0.01) in the first two weeks, and there was a further small (1%) but significant fall between two and six weeks. The number of women with hyperhomocysteinemia fell from 11 to seven after two weeks and remained the same after six weeks. Of these seven persistently hyperhomocystenemic women, five had achieved normal vitamin B_{12} status and all had normal folate status. Those with higher initial plasma tHcy concentration had a larger fall (r= -0.9, p<0.001) (Fig 2). Figure 3 shows the relationship between plasma vitamin B_{12} and tHcy concentrations before (3a) and six weeks (3b) after supplementation.

In vitamin B_{12} non-supplemented group, the fall in plasma tHcy concentration between 2nd and 6th week was explained by RTM (p<0.01, for RTM effect) but not the fall in the vitamin B_{12} concentration (p =0.81). The fall in plasma tHcy concentration in vitamin B_{12} supplemented group was significant after allowing effect of RTM (p=0.18).

Haematological and neurological parameters

In women who did not receive vitamin B_{12} supplementation, there was no significant change in any of the haematological parameters. Vitamin B_{12} supplementation led to a small but significant increase in blood haemoglobin concentration within two weeks (119 to 122 g/L, p<0.05),

Table 1. Baseline Characteristics of women in vitamin B12 supplemented and non-supplemented groups

	Group I (B12 + GLV, only B12) n = 20	Group II (Only GLV and Placebo) n=20
Age (years)	31 (23, 37)	29 (20, 35)
Height (cm)	155 (152, 160)	152 (149, 158)
Weight (kg)	57.1 (53.5, 62.8)	51.7 (44.7, 61.5)
BMI (kg/m^2)	23.9 (21.2, 25.5)	22.2 (19.6, 27.6)
Plasma vitamin B12 (pmol/L)	125 (101, 215)	136 (99, 198)
Low vitamin B12 status (<150 pmol/L)	14 (70%)	12 (60%)
Plasma total homocysteine (μmol/L)	18.4 (12.8, 39.1)	21.3 (13.6, 34.9)
Hyperhomocysteinemia (> 15 μmol/L)	11 (55%)	13 (65%)
Plasma folate (ng/mL)	4.4 (2.4, 5.8)	4.2 (3.2, 4.7)
Low folate status (<3 ng/mL)	6 (30%)	4 (20%)
Blood haemoglobin (g/L)	119 (111, 125)	113 (104, 123)
Anemia (<115 g/L) %	6 (30%)	11 (55%)
MCV (fL)	87 (76, 91)	85 (78, 92)
Macrocytosis (>=100fL)	2 (10%)	1 (5%)
Microcytosis (<80 fL)	6 (30%)	6 (30%)

Values are median (25th, 75th centile) or %

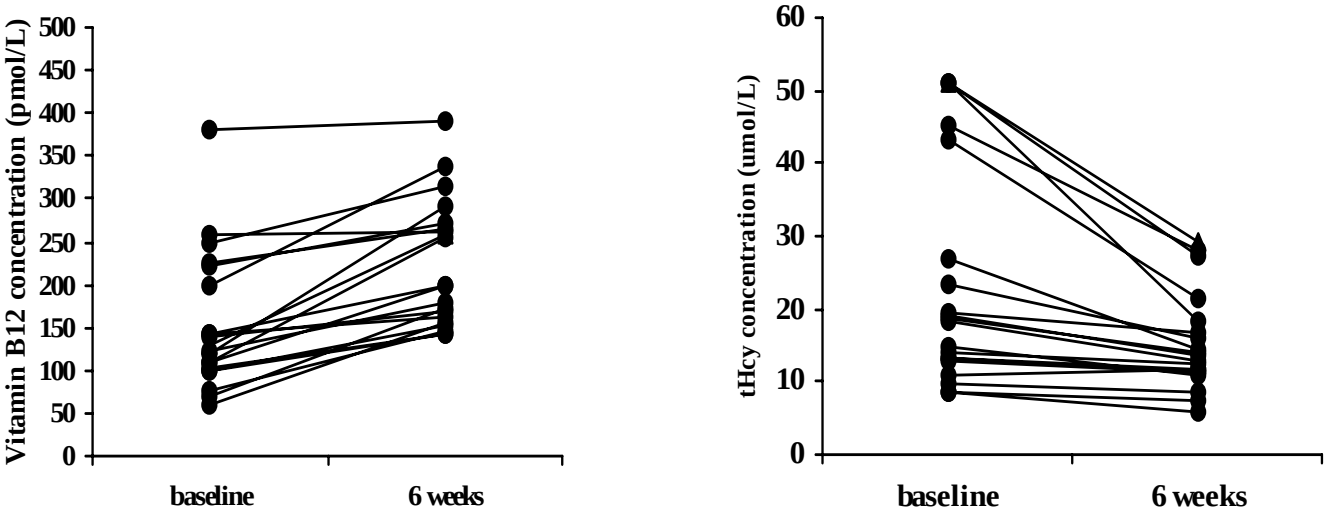

Figure 2. Plasma vitamin B12 and tHcy concentration at baseline and after 6 weeks of B12 supplementation

Table 2. Plasma concentrations of vitamin B12, tHcy and folate at baseline and after 2 and 6 weeks of supplementation

	B12 supplementation	Baseline	2 weeks	6 weeks
Vitamin B12 (pmol/L)	Yes	125 (101, 215)	215 (162, 291) ***	198 (158, 271) **
Vitamin B12 (pmol/L)	No	136 (99, 198)	131 (115, 175)	110 (73, 165) $
tHcy (µmol/L)	Yes	18.4 (12.9, 39.1)	13.4 (11.8, 28.4) **	13.2 (11.4, 18.0) ***, $$
tHcy (µmol/L)	No	21.3 (13.7, 34.9)	20.5 (15.4, 27.1)	18.8 (13.7, 25.7) $
Plasma folate (ng/mL)	Yes	4.4 (2.4, 5.8)	4.6 (3.6, 6.5)	4.9 (3.5, 6.6)
Plasma folate (ng/mL)	No	4.2 (3.3, 4.7)	4.2 (3.5, 5.4)	3.8 (3.0, 5.0) $
Haemoglobin (g/L)	Yes	119 (111, 125)	122 (116, 131) *	119 (112, 131)
Haemoglobin (g/L)	No	113 (104, 123)	117 (104, 127)	115 (103, 124)
MCV (fL)	Yes	87 (76, 91)	87 (77, 92)	85 (75, 90)
MCV (fL)	No	85 (78, 92)	86 (78, 93)	84 (78, 92)

Values are median (25^{th}, 75^{th} centile); *p<0.05, ** p<0.01, *** p<0.001, different than baseline values; $ p<0.05, $$$p$<0.01, $$$ p<0.001, different than 2 week value

there was no further change at six weeks. As a group, there was no significant change in MCV for those who received vitamin B_{12} supplementation, but two women who were macrocytic became normocytic after six weeks of vitamin B_{12} supplementation. Number of microcytic women (n=6) did not change after vitamin B_{12} supplementation.

There was no significant change in vibration sensory threshold in vitamin B_{12} supplemented and the control group, nor was there any change in anthropometric parameters and in the plasma glucose, total and HDL cholesterol, and triglycerides concentrations.

Discussion

Our study demonstrates that oral vitamin B_{12} supplementation reduces plasma tHcy concentration in otherwise healthy lacto-vegetarian Indian women. Vitamin B_{12} treatment was associated with a marginal increase in blood haemoglobin concentration and a fall in the red cell size (MCV) of two women with macrocytosis, but there was no change in vibration sensory threshold of peripheral nerves. The biochemical effect was visible within two weeks of starting the supplementation. This was a proof of principle trial and therefore we used large doses of oral vitamin B_{12} to ensure an effect in a short period of time. A moderate increase in the intake of local GLV recipes did not change circulating folate and tHcy concentrations. Our findings support a causal role for low vitamin B_{12} status in the aetiology of hyperhomocysteinemia in Indians. To the best of our knowledge this has not been demonstrated in Asian Indians in a randomised trial setting.

There are a number of interesting findings in our study: 1) plasma vitamin B_{12} concentration plateaued within two weeks of supplementation, and there was no further increase in the following four weeks. This could be due to diminished intestinal absorption of vitamin B12 due to 'saturation' of the absorptive mechanisms, or due to equilibrium between absorption and tissue clearance. 2) Normalization of tHcy was obtained in only 4/11 women,

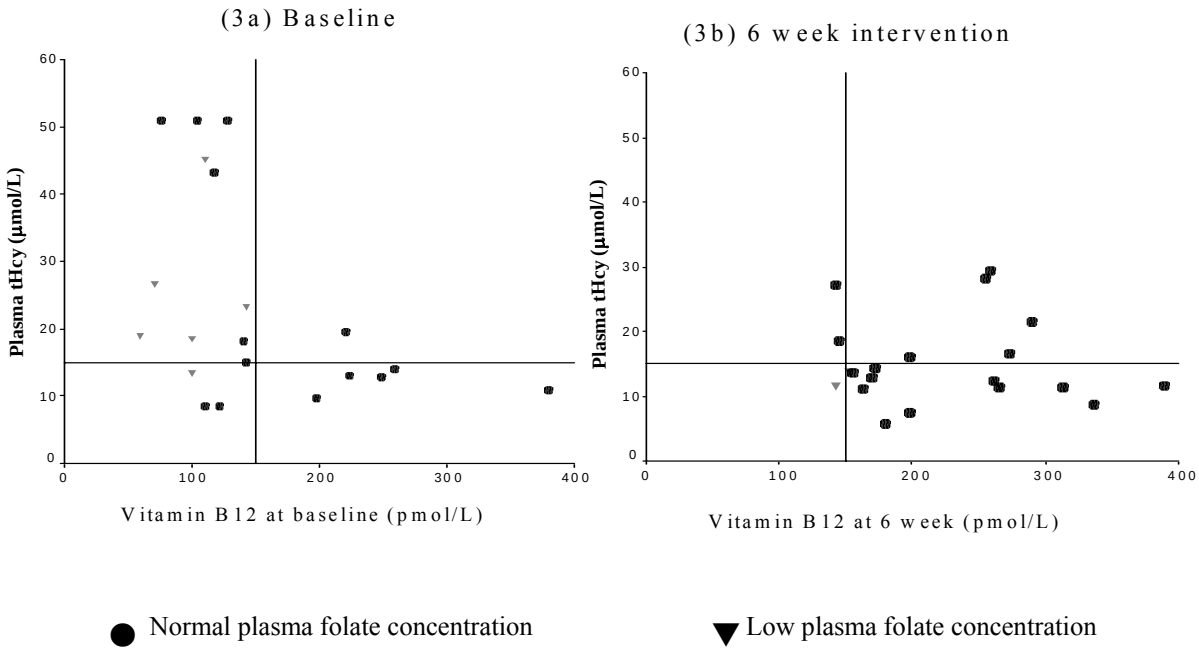

Figure 3. Plasma vitamin B12 and tHcy concentration at baseline and 6 weeks after vitamin B12 supplementation.

while seven women continued to be hyperhomocysteinemic despite improvement of vitamin B_{12} status to within 'normal' range and despite a normal folate status. The duration of our study may be too short to achieve the full effect of vitamin B_{12} supplementation. Alternatively, low vitamin B_{12} status may be only partially responsible for hyperhomocysteinemia, and factors other than low vitamin B_{12} and folate status may also contribute to hyperhomocysteinemia in these women. Low pyridoxine status, a common finding in vegetarians, could be one such factor.[7] Yet another possibility is that there might be some limiting factor preventing metabolic effects of vitamin B_{12}, such as transport into the cells or the presence of cobalamin analogues[8] 3) Fall in plasma tHcy concentration occurred predominantly in those with high baseline levels. Thus, vitamin B_{12} supplementation appears to be effective in reducing plasma tHcy concentrations in the hyperhomocysteinemic individuals. 4) Plasma folate concentration did not increase after GLV supplementation. This could be due to destruction of folate by cooking[9] if our precautions to avoid overcooking were not adequate. Another possibility is that the overall folate status in this population is good, despite low plasma values in 25% women (plasma folate is influenced by short term intake) [10] and our intervention, therefore, had insignificant effects. The association between vitamin B_{12} and folate concentrations was unexpected.

Our study has some limitations. The number of subjects was relatively small, we studied institutional volunteers, dose of vitamin B_{12} was considerably larger than recommended dietary allowance[11], and the duration of trial was short. This was mainly because we had planned only a 'proof of principle' trial. These limitations should not affect biological interpretation. The results of this study stress the need for a larger community-based intervention using low-dose vitamin B_{12} to assess the effect on plasma tHcy concentration in Asian Indians.

In conclusion, we have demonstrated that large dose oral vitamin B_{12} supplementation in lacto-vegetarian Indian women improved plasma vitamin B_{12} status and reduced circulating tHcy concentrations within two weeks of starting the supplementation. Our results suggest that oral vitamin B_{12} may be used in Asian Indians to test the putative effects of reduction in circulating tHcy concentrations on different outcomes.

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[1]*King Edward Memorial Hospital, Pune, India*
[2]*Medical Research Council, Environmental Epidemiology Resource Centre, Southampton, UK*
[3]*Institute of Basic Medical Sciences, Department of Nutrition, University of Oslo, Norway; Oxford Centre for Gene Function, Department of Physiology, Anatomy & Genetics, University of Oxford, UK*

口服維生素B_{12}補充劑可降低印度婦女血漿總同半胱胺酸濃度

印度的人民低維生素B_{12}狀況及高血漿總同半胱胺酸(tHcy)盛行率極高。在一個以證據原則的試驗，採用 2x2 因子設計，我們研究為期六週隔日口服維生素B_{12} (500 μg) 或 100g熟的綠色蔬菜(GLV)的效應。42 名未懷孕素食婦女(年齡 20-50 歲)被隨機分配到四個研究組別。在研究的最初及結束時作臨床檢測，在介入之前、介入開始後兩週及六週收集研究對象的血樣。共有 40 名婦女完成此試驗。26 名女性為低維生素B_{12}(<150 pmol/L)、24 名為高同半胱胺酸血症(>15 μmol/L)。GLV的補充沒有改變血漿葉酸或是tHcy。維生素B_{12}補充劑使血漿維生素B_{12}濃度增加(125 到 215pmol/L，p<0.05)，並在最開始的二週內降低tHcy濃度(18.0-13.0μmol/L， p<0.05)，上述兩者在接下來的四週均維持穩定。血漿維生素B_{12}及tHcy濃度在沒有接受維生素B_{12}的組別中則是沒有變化，而血漿葉酸在任何組別中均沒有改變。接受維生素B_{12}補充劑的婦女，在頭二週的血中血紅素濃度有微量的上升(約 3 g/L, p<0.05)，巨紅血球症的婦女性人數從 2 降為 0。在研究期間沒有震動知覺閾值的改變。補充口服高劑量維生素B_{12}可以在兩週內顯著降低血漿tHcy，但是即使在六週後仍無法達到正常血漿tHcy的濃度。

關鍵字：維生素B_{12}，總同半胱胺酸、葉酸、補充劑、印度。