Decreased serum homocysteine levels after micronutrient supplementation in older people

Pusparini*

ABSTRACT

Aging is associated with a gradual impairment in cognitive function. The elderly also show a high prevalence of undernutrition, whereas nutrition plays an important role in the metabolism of neuronal cells and enzymes. Homocysteine is an amino acid resulting from methionine metabolism and is dependent on intake of vitamin B₁₂, vitamin B₆ and folic acid. Homocysteine is said to play a role in cognitive function. The objective of this study was to evaluate the effect of micronutrient supplementation for 6 months on serum homocysteine levels and cognitive function in older people. This study was an experimental study of pre-post test design, carried out in Mampang subdistrict, South Jakarta. A total of 94 elderly people was recruited for this study, consisting of 44 females and 50 males. Serum homocysteine level was assessed by fluorescent polarization immunoassay and cognitive function by means of the mini mental state examination (MMSE) before and after micronutrient supplementation. Mean serum homocysteine concentration after supplementation decreased significantly to 14.8 ± 5.8 µmol/L, compared with mean serum homocysteine level of 15.9 ± 5.9 µmol/L before supplementation (p=0.000). Multiple regression analysis indicated that the factors influencing post-supplementation MMSE scores were gender (â=−0.350; p=0.000), education (â=0.510; p=0.000) and post-supplementation homocysteine levels (â=−0.201; p=0.000), while age, pre-supplementation homocysteine levels and BMI did not affect MMSE scores. Homocysteine concentration decreased significantly after 6 months of supplementation. The factors affecting post-supplementation MMSE scores were gender, level of education, and post-supplementation homocysteine level.

Keywords: Micronutrient, MMSE, serum homocysteine, cognitive function, elderly

INTRODUCTION

No other organ system in the human body is as dependent on nutritional intake as the central nervous system, while conversely this system also affects nutritional intake. Several theories have elucidated the functions of brain receptors for cholecystokinin, opioid-like endorphins and serotonin that play a role in determining human dietary habits.(1,2) Studies on experimental animals indicate that with advancing age there is a decline in
the functioning of the brain and in the number of brain receptors. In the elderly there is also a diminished sense of taste and a decline in olfactory nerve functions that affect appetite.\(^2,3\)

The nervous system requires a constant intake of glucose for adequate functioning of the brain, which is particularly dependent on intake of essential nutrients. Deficiencies of vitamins and minerals, such as vitamin B\(_{12}\), folate, vitamin B\(_6\), and trace minerals, may result in disturbances of brain functions such as cognitive functions.\(^4,5\) In the elderly, vitamin deficiencies are common, especially of the vitamin B group, caused by various factors, such as gastric atrophy with achlorhydria or hypochlorhydria, which in the elderly may account for up to 20-50\% of cases. Studies have yielded evidence for an association in the elderly between decreased levels of vitamin B\(_{12}\), folate, and vitamin B\(_6\) on the one hand and impaired cognitive function on the other.\(^1,5\)

One of the manifestations of impaired cognitive function is dementia, which is characterized by a progressive decline in cognitive function, resulting in a decreased ability for conducting activities of daily living. In Canada dementia affects 8\% of the elderly population over 65 years of age, and gives rise to more than 60,000 new cases annually. Another disease, Alzheimer’s, is the cause of 50\% of all cases of dementia,\(^6\) whereas other studies indicate that dementia due to Alzheimer’s disease may account for up to 70\% of all dementia cases.\(^7\) It is estimated that in the following 50 years the number of dementia cases may undergo a threefold rise.\(^8\) The prevalence of this disorder is extremely high and as the disorder may lower the quality of life of the elderly, prevention and early detection are important.\(^9\) Factors that have been associated with the risk of dementia are age and education, both of which are of the highest relevance for dementia. Recently the role of homocysteine in dementia has been elucidated, where high homocysteine levels have been associated with an increased risk of dementia.\(^6,7,9\)

Homocysteine is an amino acid resulting from the metabolism of methionine that is dependent on B\(_{12}\), vitamin B\(_6\), and folic acid.\(^8,10\) Plasma homocysteine level is an indicator for vitamin B status, including that of folic acid, where a high homocysteine concentration indicates an inadequate vitamin B status. Data from several laboratories have shown that high homocysteine level is extremely common in old age and does not depend on vitamin status.\(^11-13\) Total plasma homocysteine level is a major vascular risk factor, and raised plasma homocysteine level is associated with an increased risk of atherosclerotic sequelae, including death due to cardiovascular disease, coronary heart disease, carotid atherosclerosis and stroke.\(^7,14,15\)

Research results on the influence of homocysteine on cognitive function in the elderly are still subject to controversy. A number of studies have demonstrated a significant association,\(^17,15-17\) whereas other studies have shown contradictory results.\(^8,18,19\) Preliminary studies with small samples in a population of healthy elderly in Italy showed no significant correlation between MMSE scores and homocysteine levels. However, the same investigator found in larger samples that older persons with low MMSE scores had a higher risk of suffering from abnormal cognitive function with increasing plasma homocysteine levels. The study also found that homocysteine level was not directly associated with cognitive disorders.\(^9\)

The present study had as objectives to find a decrease in homocysteine concentrations after six months of supplementation with a combination of micronutrients and vitamins, and to determine whether decreased post-supplementation homocysteine levels can improve cognitive function in older persons.
METHODS

Design of the study
This study used an experimental pre-post test design to investigate the occurrence of a decrease in homocysteine concentrations after administration of a supplement comprising a combination of micronutrients and vitamins for a period of six months. The study was carried out from November 2008 to April 2009.

Subjects
The study subjects were residents aged 60+ years from the catchment area of the Mampang Prapatan Subdistrict Health Center in South Jakarta who met the following inclusion criteria: healthy males and females, mobile, independent, able to verbally communicate and willing to join the study by giving written informed consent. Subjects with acute infections, intake of vitamins, cod liver oil or other supplements within the previous month, and abnormal renal function, were excluded from the study. Selection of the subjects was by cluster and simple random sampling.

Supplementation
The supplements for the subjects were in the form of oral tablets, each containing 280 mg zinc gluconate (equivalent to 40 mg zinc), 120 mg ascorbic acid, 6 mg β-carotene, 15 mg α-tocopherol, and 400 µg folic acid. The tablets were administered to the study subjects by field workers who assisted in this study. To each study subject was allocated one bottle containing 30 tablets to be taken once daily. Compliance was monitored by the field workers and the investigator by counting the remaining tablets returned with the bottles each month.

Data collection
The field workers conducted interviews using a questionnaire that had been tested in a preliminary trial on elderly who were willing to participate in this study. The filling in of questionnaires was performed daily from Monday to Friday, and those subjects who had been interviewed were invited to come to the Mampang Prapatan Subdistrict Health Center on Saturday. The weekly number of elderly who completed their interviews was around 25. The subjects were asked to fast overnight on Friday for 10–12 hours prior to the collection of a blood sample for laboratory examination. The following Saturday morning the participants visited the health center for measurement of blood pressure, pulse rate, weight, height, waist circumference, and hip circumference. The measurements were taken by two previously trained nurses. Data were collected within six months, before and after the supplementation period. In addition to anthropometric assessment and laboratory examination, diet recall was performed 2x24 hours on work days and 1x24 hours on holidays. Assessment of dietary recall was done twice, namely at the start and at the end of the study.

Measurements
Height was measured to the nearest 0.1 cm using a portable microtoise and weight to the nearest 0.1 kg using Sage portable scales. Body mass index (BMI) was calculated as the weight (kg) divided by the square of the height (m²). Waist circumference (cm) and hip circumference (cm) of the subjects were measured using a standard tape measure accurate to 1 mm. Blood pressure measurements were performed with the subject sitting, after a rest period of 15 minutes, by means of a standard sphygmomanometer accurate to 5 mmHg. Hypertension was defined as a systolic blood pressure of ≥140 mmHg and/or a diastolic blood pressure of ≥90 mmHg.

Assessment of renal function
From each subject meeting the inclusion and exclusion criteria a 5 mL blood sample was collected using a vacutainer without
anticoagulant. The blood samples were centrifuged at 300 RPM for 10 minutes in order to obtain serum for assessment of renal function and homocysteine level. Since renal function affects the metabolism of homocysteine, subjects with a creatinine concentration above reference level were considered to have abnormal renal function. The reference level for females is 0.5-1.3 mg/dL and for males 0.5-1.4 mg/dL. Determination of homocysteine level was performed only on the serum of subjects with a renal function consistent with reference values. Homocysteine concentrations of all study subjects were divided into 3 categories, i.e. <11.7 µmol/L, 11.7-14.6 µmol/L, and >14.6 µmol/L. Creatinine concentration was determined by the Jaffe method on a TRX, whereas homocysteine was assessed by fluorescent polarization immunoassay on an Abbott IMX. The coefficient of variation (CV) of creatinine was 2.1% for normal concentrations and 1.6% for high concentrations, whilst the corresponding CV values for homocysteine were 3.4% and 2.3%, respectively. Serum for homocysteine assessment was stored at −70°C and examined after collection of the last blood sample.

**Assessment of cognitive function**

Cognitive function was assessed by means of the MMSE instrument. This comprises five categories of criteria, viz. criteria on orientation with a maximum score of 10, registration criteria with maximum score of 3, attention and calculation with maximum score of 5, recall criteria with maximum score of 3, and language criteria with maximum score of 9. In addition to these five criteria the subjects are also asked to write a sentence and copy a drawing. MMSE scores of 26-30 are designated questionably significant/no cognitive impairment, while scores of 21-25, 10-20 and 0-9 respectively indicate mild, moderate and severe cognitive impairment.

**Data analysis**

Data processing was performed by means of the Statistical Program for Social Sciences (SPSS) software version 15. Normality of data distribution was determined by the Kolmogorov–Smirnov (KS) test. For normally distributed data the mean and standard deviation (SD) was calculated and for non-normally distributed data the median. To determine the presence of a statistically significant difference between groups for

![Figure 1. Flow chart describing the progress of the subjects during the trial](image_url)
normally distributed data the analysis of variance (anova) was used, whilst for non-normally distributed data the Kruskal – Wallis test was applied. Multiple linear regression was used to identify the factors affecting MMSE levels. A value of p < 0.05 was taken to indicate a significant difference.

RESULTS

At the start of the study 137 respondents were recruited, among whom 107 were found to meet the inclusion and exclusion criteria. During the study period of six months 13 respondents dropped out, due to various reasons, viz. 3 persons moved to another area, 3 persons died, 5 persons withdrew from the study, and 2 persons were taken ill and thus unable to take the supplements for more than 7 days. At the end of the study the remaining subjects totaled 94 persons, comprising 44 females and 50 males (Figure 1).

Supplementation to respondents who participated up to the end of the study proceeded satisfactorily, since 98% of the respondents received the full supplementation for six months and 2% skipped or missed 1-2 days of supplementation. Monitoring of subject compliance was done by counting the remaining tablets returned with the bottles. No subject was dropped for non-compliance.

At the completion of the study there were 94 elderly, consisting of 44 (47 %) females and 50 (53%) males. The characteristics of the study subjects are listed in Table 1. The age of the subjects ranged from 60 to 79 years, with a mean age of 65.9 ± 4.7 years.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>44 (47%)</td>
</tr>
<tr>
<td>Male</td>
<td>50 (53%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.9 ± 4.7</td>
</tr>
<tr>
<td>Body Mass Index (BMI) (kg/m²)</td>
<td>22.4 ± 3.4</td>
</tr>
<tr>
<td>Level of education (years)</td>
<td>5.1 ± 4.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>59.9 ± 10.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>155.7 ± 4.6</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>83.3 ± 11.2</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>91.1 ± 7.8</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>143 ± 18</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.84 ± 0.2</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>15.9 ± 5.9</td>
</tr>
<tr>
<td>MMSE**</td>
<td>24.1 ± 5.9</td>
</tr>
</tbody>
</table>

*Paired t-test
** MMSE: mini mental state examination

There were 46 (48.9%) study subjects with homocysteine concentrations above 14.6 µmol/L, having at the start of the study homocysteine levels of 20.1± 5.9 µmol/L, which decreased to 18.9 ± 5.9 µmol/L at the completion of the study. Homocysteine concentrations before and after supplementation showed a significant difference (p=0.000), with decreased post-supplementation levels in all groups (Table 2).

The factors that were thought to be of influence on cognitive function were among others age, BMI, education, gender and blood homocysteine levels. From Table 3 it may been seen that MMSE scores were influenced by gender, post-supplementation homocysteine levels, and education. Age, BMI, and pre-
supplementation homocysteine levels did not affect MMSE scores.

DISCUSSION

The aging process is related to a reduction in various physiological functions. It is well known that physiological changes are the result of inadequate intake of vitamins, trace elements, minerals and other nutrients. Even nutritional intakes that are considered to be of high quality for health cannot comprise all nutritional elements required for promotion of health and prevention of acute as well as chronic diseases. The results of the present study showed that the pre-supplementation homocysteine concentration of 15.9 ± 5.9 µmol/L decreased significantly to 14.8 ± 5.8 µmol/L after micronutrient supplementation for six months. In a Chinese study on 381 subjects with mean age of 46.9 ± 8.3 years, who received supplementation of vitamin C 250 mg, vitamin E 100 mg and selenium 37.5 µg twice daily for 88 months, the plasma homocysteine concentration tended to decrease 6.4%, the decrease however being not statistically significant (95% CI: -13.5 to 1.3%) (p=0.10). In contrast, in a study on Guatemalan elderly, who were given a supplement containing zinc and folic acid, results similar to the present study were obtained, which may be due to the fact that in the Chinese study the supplement did not include these two micronutrients, which are capable of reducing serum homocysteine levels.

Homocysteine is a sulfur-containing amino acid closely associated with the metabolism of methionine and cysteine. There is no DNA that codes for homocysteine synthesis. Homocysteine is not synthesized in nature but results from the metabolism of the amino acid methionine through a methylation cycle as a unique source of homocysteine. Methionine is obtained through intake of protein in the daily diet.

Homocysteine occurs in several forms in plasma. The sulfhydryl or reduced form is designated homocysteine and the disulfide or oxidized form is designated homocystine. The disulfide form may occur together with cysteine and with proteins having reactive cysteine residues (protein-bound homocysteine), and this form is called mixed disulfide. The oxidized forms account for most of the homocysteine in plasma (98-99%), whilst the reduced forms make up only 1% of total homocysteine. Total homocysteine (tHcy) is the sum total of all homocysteine forms present in plasma.

Homocysteine is converted by the enzyme cystathionine β synthase (CBS) into cysteine with vitamin B₆ as cofactor, in a process called demethylation. In the reverse process of remethylation, homocysteine is reconverted into methionine by the enzymes methylene tetrahydrofolate (MTHFR) and methionine synthase (MS), with folic acid and vitamin B₁₂ as substrates and cofactors. Folic acid, vitamin B₁₂ and vitamin B₆ are substrates of the cofactors in the metabolism of methionine and homocysteine, thus a deficiency of one of these vitamins leads to hyperhomo-cysteinemia.
Under normal conditions blood homocysteine levels are relatively low, ranging from 5-15 µmol/L. The homocysteine level in the extracellular compartment is determined by its intracellular synthesis, metabolism and excretion. If intracellular homocysteine synthesis exceeds the metabolic capacity, homocysteine is released into the extracellular compartment. In contrast, decreased synthesis leads to a fall in release of homocysteine from the cell. This mechanism maintains intracellular homocysteine at a low level. The homocysteine equilibrium may be upset by abnormal enzyme activity or as a result of a reduction in the number of cofactors that play a role in its metabolism.(25,27) In Table 2 homocysteine levels were subdivided into three categories, i.e. <11.7 µmol/L, 11.7-14.6 µmol/L and >14.6 µmol/L. This was based on the results of a study conducted by Quadri(15) with the objective of finding a reference value for hyperhomocysteinemia, as currently there are no standard criteria for defining hyperhomocysteinemia. A number of investigators have used variable values for hyperhomocysteinemia, e.g. Ravaglia(9) used a homocysteine level of >15 µmol/L, whilst Seshasdri(7) and Dufouil(2) used levels of >14 mol/L and >15 µmol/L, respectively. In the present study homocysteine levels of >14.6 ± 6.1 µmol/L were found in 46 subjects (48.9%) with mean level of 20.06 ± 5.9 µmol/L at the start of the study, decreasing to 44 subjects (46.8%) with mean level of 18.9 ± 5.9 µmol/L. In comparison, in the study by Quadri(15) homocysteine levels of >14.6 µmol/L were found in 51.85% of subjects with vascular dementia and in 56.36% of control subjects. In Quadri’s study, mean homocysteine level was 14.6 ± 6.1 µmol/L in control subjects with mean age of 75.6 ± 8.5 years, whereas subjects with vascular dementia with mean age of 80.5 ± 5.7 years had a mean homocysteine level of 18.9 ± 7.9 µmol/L and those with Alzheimer’s disease and mean age of 79.1 ± 7.7 years showed a homocysteine level of 16.8 ± 7.0 µmol/L. The different results may be due to the lower mean age of the subjects (65.9 ± 4.7 years) in the present study.

In the present study determination of homocysteine concentration was performed after the subjects had fasted at least 10 hours. Assessment of homocysteine levels without fasting results in a rise in homocysteine levels of around 20%, compared with those in fasting subjects.(7) Seshadri’s study(7) revealed a tendency for increased plasma homocysteine levels to precede the onset of dementia. Therefore it is probable that the increased homocysteine levels in the present study mark the early stages of a developing dementia, although the MMSE scores were categorized as mild cognitive impairment only. Seshadri’s results are also consistent with the results of the present study, where homocysteine levels showed a significant post-supplemental decrease, although the increases in MMSE scores did not reach statistical significance.

There are several factors that are considered to be of influence on cognitive function, namely age, gender, education, BMI and pre- and post-supplementation homocysteine levels. The results of the multiple regression analysis in this study revealed that gender, education, and post-supplementation homocysteine levels constituted the factors affecting post-supplementation MMSE scores, whereas age, BMI, and pre-supplementation homocysteine levels did not affect post-supplementation MMSE scores. These results are similar to the results of Quadri’s study,(15) indicating that education also differed significantly between the vascular dementia group and the control group.

With higher educational levels the MMSE scores are found to increase, indicating better cognitive functioning. Several investigators used subjects of similar educational backgrounds to determine an association between homocysteine levels and MMSE scores. The study by Ravaglia(9) indicated that
in subjects with a similar educational background hyperhomocysteinemia was an independent predictor for the occurrence of dementia or Alzheimer’s disease. The present study results showed that post-supplementation MMSE scores were not influenced by age, whereas in the cross-sectional study by Pusparini\(^2\) age was found to be an influencing factor in males (p=0.006) and in females (p=0.000). Both studies, the one of cross-sectional design as well as the study of pre-and-post design, yielded similar results, in that education affected MMSE scores. In the study by Pusparini\(^2\) initial homocysteine levels had no effect on MMSE scores, which was consistent with the results of the present study and those of the study by Joosten,\(^2\) where no significant difference was found between homocysteine levels and cognitive function. The study conducted by Bell\(^3\) found an association between plasma homocysteine levels and MMSE scores only in subjects with depression and not in normal subjects. In the cross-sectional studies conducted by Borroni\(^4\) and Miller\(^5\) it was found that increased plasma homocysteine concentration was not a causative factor in dementia and Alzheimer’s disease, but only an accompanying marker of vascular disorders and was independent of individual cognitive status. Intervention studies are necessary for providing evidence of an association between hyperhomocysteinemia and cognitive function. The study by Seshasdri\(^6\) revealed a correlation between raised plasma homocysteine levels and decreased MMSE scores, but the correlation was found only after 4 years of monitoring.

One limitation of this study is the lack of a control group for comparative purposes, thus it cannot prove that the decrease in post-supplementation homocysteine levels was the result of the supplementation factor itself rather than other causes. In addition, in this study there was no assessment of folic acid and vitamin B concentrations, which affect the metabolism of homocysteine, both initially and at the completion of the study, so that the investigator could not ascertain whether the administered supplementation was capable of increasing homocysteine levels through increases in folic acid and vitamin B levels or whether there were other causes.

**CONCLUSIONS**

From the results of this study it may be concluded that homocysteine levels decreased after six months of micronutrient supplementation. The main factors influencing post-supplementation MMSE scores were gender, post-supplementation homocysteine levels, and education.

**ACKNOWLEDGEMENTS**

The author wishes to express her gratitude to the Medical Faculty of Trisakti University, to the study subjects for their willingness to participate in this study, and to all parties who facilitated this study.

**REFERENCES**