The Role of Some B Vitamins in Methionine-Induced Hyperhomocysteinemia in Male Rabbits

Abdul-Razzak Abdul-Latif [*], Haydar H. Al-Shalah[**], Ali A. Al-Beer [***]

[*] *Assistant Professor in Department of Pharmacology and Toxicology at College of Medicine/University of Babylon.*
[**] *Assistant Professor in Department of Biochemistry at College of Medicine/ University of Babylon.*
[***] *Clinical Pharmacist in Karbala Teaching Hospital for Children; Corresponding Author.*

Abstract

Background: Hyperhomocysteinemia has been associated with an increased risk of atherosclerosis and other cardiovascular diseases. Vitamin B_6, vitamin B_{12}, and folic acid are essential components in the metabolism of homocysteine.
Objective: This study aimed to assess the effects of vitamin B_6, vitamin B_{12}, folic acid, and the combination of them in the prevention of hyperhomocysteinemia.
Methods: Thirty six male local rabbits were used in the study. Methionine was given to rabbits to induce a model of hyperhomocysteinemia. Rabbits were divided randomly into 6 groups (6 rabbits in each group) as the following: **control group**: they were maintained on standard chow only; **methionine only group**: they were maintained on methionine only; **B_6 group**: they were maintained on methionine and vitamin B_6; **B_{12} group**: they were maintained on methionine and vitamin B_{12}; **folic acid group**: they were maintained on methionine and folic acid; **multivitamins group**: they were maintained on methionine, vitamin B_6, vitamin B_{12}, and folic acid.
Results: Multivitamins group showed no significant differences ($P>0.05$) in homo-cysteine levels at day 30 in comparison with day 0 and there are no significant differences ($P>0.05$) in homocysteine levels at day 30 between multivitamins group and control group. At day 30 and day 60, serum homocysteine levels in multivitamins group were significantly ($P<0.05$) lower than serum homocysteine levels in folic acid group**.**
Conclusion: Multivitamin combination composed of vitamin B_6, vitamin B_{12}, and folic acid reduces the hyperhomocysteinemic effect of methionine and it is better than folic acid alone, while folic acid is better than vitamin B_6 or vitamin B_{12} when they used alone.

Key words: Homocysteine, methionine, vitamin B_6, vitamin B_{12}, folic acid.

الخلاصة

مقدمة: ارتبطت حالة ارتفاع مستوى مادة الهوموسيستين في الدم مع زيادة خطر الاصابة بمرض تصلب الشرايين وأمراض القلب والأوعية الدموية الأخرى . ان فيتامين B_6 ، فيتامين B_{12} ، و حامض الفوليك هي عناصر أساسية في عملية الايض لمادة الهوموسيستين .
الهدف من الدراسة: هدفت هذه الدراسة لتقييم تأثير فيتامين B_6 ، فيتامين B_{12} ، حامض الفوليك ، و الخليط المؤلف منهم في الوقاية من حالة ارتفاع مستوى مادة الهوموسيستين في الدم .
طرق العمل: ستة و ثلاثون من ذكور الارانب المحلية تم استخدامها في الدراسة . تم اعطاء مادة الميثيونين للأرانب لاستحثاث حالة ارتفاع مستوى مادة الهوموسيستين في الدم . تم تقسيم الأرانب عشوائيا الى ستة مجاميع (ستة ارانب في كل مجموعة) : **مجموعة السيطرة** : تم اعطاء ارانب هذه المجموعة طعام الارانب القياسي فقط ؛ **مجموعة المثيونين فقط** : تم اعطاء ارانب هذه المجموعة مادة الميثيونين فقط ؛ **مجموعة B_6** : تم اعطاء ارانب هذه المجموعة مادة المثيونين و فيتامين B_6 ؛ **مجموعة B_{12}** : تم اعطاء ارانب هذه المجموعة مادة المثيونين و فيتامين B_{12} ؛ **مجموعة حامض الفوليك** :

تم اعطاء ارانب هذه المجموعة مادة المثيونين و حامض الفوليك ؛ **مجموعة الفيتامينات المتعددة** : تم اعطاء ارانب هذه المجموعة مادة المثيونين ، فيتامين B_6 ، فيتامين B_{12} ، و حامض الفوليك .

النتائج : لم تظهر مجموعة الفيتامينات المتعددة وجود فروق ذات دلالة إحصائية في مستويات مادة الهوموسيستين ($P> 0.05$) في يوم 30 بالمقارنة مع بدء التجربة و ليس هناك فروق ذات دلالة احصائية ($P> 0.05$) في يوم 30 بين مجموعة الفيتامينات المتعددة و مجموعة السيطرة . في يوم 30 و يوم 60 اظهرت مجموعة الفيتامينات المتعددة مستويات هوموسيستين أقل ($P <0.05$) من مستويات الهوموسيستين في مجموعة حامض الفوليك .

الاستنتاج : ان الخليط المؤلف من فيتامين B_6 ، فيتامين B_{12} ، و حامض الفوليك يقلل تأثير مادة المثيونين الرافع للهوموسيستين و هو افضل من حامض الفوليك لوحده بينما حامض الفوليك افضل من فيتامين B_6 او فيتامين B_{12} عندما يستخدموا لوحدهم .

Introduction

Homocysteine is a sulfur containing amino acid produced by the metabolism of methionine, which is one of the essential amino acids obtained from dietary proteins [(1)]. Homocysteine (Hcy) is a homologue of the amino acid cysteine, differing by an additional methylene **(-CH_2-)** group [(2)].

In 1969, McCully made the first clinical observation that linking elevated plasma homocysteine concen-trations with the vascular diseases. McCully reported autopsy evidence of extensive arterial thrombosis and atherosclerosis in two children with elevated plasma homocysteine concen-trations and homocystinuria. On the basis of this observation, he proposed that elevated plasma homocysteine level can cause atherosclerotic vascular disease [(3)]. Subsequent investigations have confirmed McCully's hypothesis, and it has recently become clear that hyperhomocysteinemia is independent risk factor for atherosclerosis and atherothrombosis [(4)].

Elevated homocysteine levels have been associated with an increased risk of atherosclerotic sequelae, including death from cardiovascular causes, coronary heart disease, clinical stroke, and carotid atherosclerosis.

Homocysteine is considered as a modifiable risk factor since plasma homocysteine levels can be lowered by supplementation with folic acid [(5)]. It is suggested that chronic elevations of plasma homocysteine concentrations has been shown to be associated with peripheral vascular disease and myocardial infarction [(6)].

High plasma homocysteine concen-trations accompanying low concen-trations of folate and vitamin B_6 (through their role in homocysteine metabolism), are associated with an increased risk of extracranial carotid artery stenosis [(7)]. Patients with classic homocystinuria (due to rare cystath-ionine β-synthase deficiency) suffer from premature vascular disease, with about 25% of them dying from throm-botic complications before 30 years of age [(8)].

Aim of the study: This study aimed to assess the effects of vitamin B_6, vitamin B_{12}, folic acid, and the combination of them in the prevention of hyperhomo-cysteinemia.

Materials and Methods

Choice of animals: Thirty six male local rabbits were used in the study. Their weight was 2 to 2.5 kg. The rabbits were housed in the animal house of Collage of Medicine- University of Babylon in individual cages and kept at room temperature of 25 $\pm$ 2 °C. After adaptation period for 6 weeks, the study started in **1 March 2012** and ended in **30 April 2012**. During the first 2 weeks of adaptation period alfalfa, carrots, and standard chow were given to rabbits; after that, alfalfa and carrots were withdrawn completely and the only diet given during the experiment period was standard chow. No mortality has been occurred during the study.

Design of the study: Rabbits were divided randomly into 6 groups (6 rabbits in each group) as the following: **Control group**: they were maintained on standard chow only; **Methionine only group**: they were

maintained on methionine only; **B_6 group**: they were maintained on methionine and vitamin B_6; **B_{12} group**: they were maintained on methionine and vitamin B_{12}; **Folic acid group**: they were maintained on methionine and folic acid; **Multi-vitamins group**: they were maintained on methionine, vitamin B_6, vitamin B_{12}, and folic acid.

Methionine was given to induce model of hyperhomocysteinemia in the rabbits. Dose of methionine used to induce hyperhomocysteinemia was 100 mg/kg/day [12]. Used dose of vitamin B_6 in the study was 25 mg/kg/day; used dose of vitamin B_{12} was 100 μg/kg/day; and used dose of folic acid was 20 mg/kg/day [12] (**Table 1**).

Preparation of methionine and drugs: Methionine powder was dissolved in water and the rabbits administered it orally via special graduated drinking bottle. The rabbits were adapted to take water via these graduated drinking bottles during the adaptation period. Vitamin B_6, vitamin B_{12}, and folic acid tablets were converted to small particles by grinding them by using electric grinder (**Table 2**), and after that the small particles of drugs were dissolved in water and the rabbits administered it orally via the same graduated drinking bottle.

Table 1. List of drugs and chemicals used in the study

Drug or Chemical	Company	Country
Pyridoxine 25 mg tablets	Jamieson	Canada
Cyanocobalamin 100 μg tablets	Jamieson	Canada
Folic acid 5 mg tablets	Actavis	England
Methionine	Sigma	USA
Ether	BDH	England

Table 2. List of instruments and tools used in the study

Instrument or Tool	Company	Country
ELISA reader	Biokit	USA
Freezer	Arçelik	Turkey
Centrifuge	Hettich	Germany
Incubator	Memmert	Germany
Microoscillator	Triup	India
Electric grinder	B & D	China
Electronic scale	Camry	China
Drinking bottle	Deluxe	China
Micropipette	Dragon	China
Gel tubes	Sun	Jordan

Preparation of sample: After anesthetization with ether, 5 ml of blood was drawn directly from hearts of the rabbits after overnight fasting. Blood samples were taken from the rabbits on day 0 (start of the study), day 30 (middle of the study), and day 60 (end of the study) and serum was used to measure serum homocysteine levels. After withdrawal, fresh blood was placed in test tubes and serum was isolated by centrifugation for 10 minutes at 3000 rpm. Sera were frozen at -20 °C. Serum homocysteine levels were determined by using diagnostic **ELISA kit** from **DRG International Company**, **Germany;** LOT number of Microtiter Strips: 802884012; LOT number of ELISA Standard Kit: 802882532.

Statistical analysis: The statistical method one way ANOVA (analysis of variance) and a post-hoc LSD approach was used for the comparison between the means of different groups of the study at the same time period, while repeated measures ANOVA was used for the comparison between the means of the same group at different time periods. Differences between P values of 0.05 or less were considered to be significant. Statistical analysis was done by using the **SPSS** software **Version 19** for Windows system from **IBM Corporation**, **USA**.

Results

Serum homocysteine concentrations were changed in the study groups as shown in **(Table 3)** and **(Figure 1)**. Methionine only, B_6, B_{12}, and folic acid groups showed significant increases ($P<0.05$) in homocysteine levels at day 30 and day 60 in comparison with day 0 which is the baseline levels.

Multivitamins group showed no significant differences ($P>0.05$) in homocysteine levels at day 30 in comparison with day 0 which is the baseline levels. Methionine only, B_6, and B_{12} groups showed significant increases ($P<0.05$) in homocysteine levels at day 60 in comparison with day 30.

Table 3. Means and standard deviations of serum levels of homocysteine in (micromole/liter)

Group	Mean	Standard deviation
Control group at day 0	11.57	1.29
Methionine only group at day 0	11.79	1.31
B_6 group at day 0	11.52	2.09
B_{12} group at day 0	11.77	2.22
Folic acid group at day 0	11.30	1.70
Multivitamins group at day 0	11.93	1.48
Control group at day 30	11.21	0.93
Methionine only group at day 30	41.44	3.46
B_6 group at day 30	29.92	1.45
B_{12} group at day 30	26.34	2.10
Folic acid group at day 30	20.61	3.36
Multivitamins group at day 30	13.71	1.50
Control group at day 60	12.24	0.90
Methionine only group at day 60	101.10	4.45
B_6 group at day 60	39.59	4.87
B_{12} group at day 60	37.13	3.94
Folic acid group at day 60	24.32	2.60
Multivitamins group at day 60	15.72	2.83

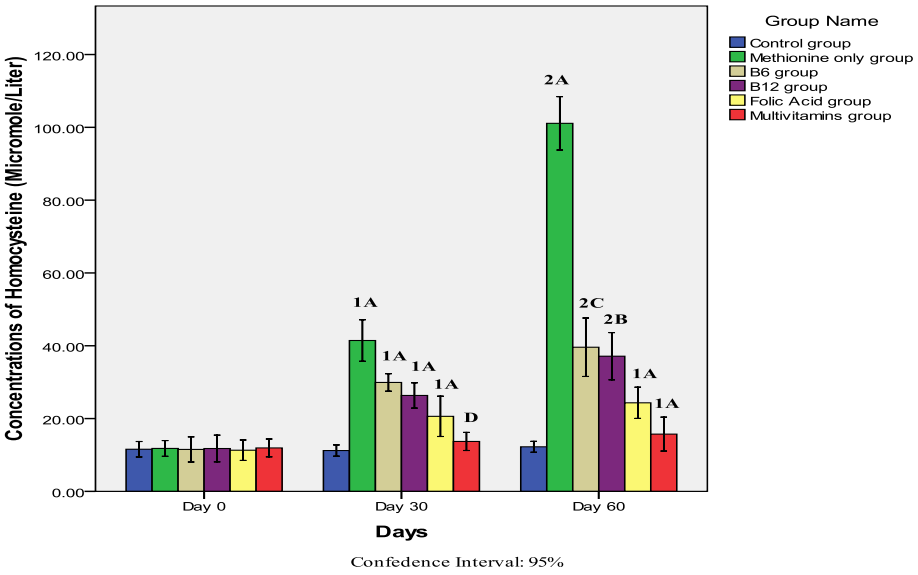

Figure 1. Changes in serum homocysteine concentrations.

1: Significant difference ($P<0.05$) from day 0.

2: Significant difference (P<0.05) from day 0 and day 30.
A: Significant difference (P<0.05) from other groups of the study.
B: Significant difference (P<0.05) from other groups of the study except B_6 group.
C: Significant difference (P<0.05) from other groups of the study except B_{12} group.
D: Significant difference (P<0.05) from other groups of the study except control group.

Folic acid group showed no significant differences (P>0.05) in homocysteine levels at day 60 in comparison with day 30. At day 30 and day 60, methionine only group showed significantly (P<0.05) higher serum homocysteine levels in comparison with other groups of the study. At day 30 and day 60 B_6, B_{12}, and folic acid groups showed significantly (P<0.05) higher serum homocysteine concentrations in comparison with control group.

At day 30, there were no significant differences (P>0.05) in serum homocysteine levels between multivitamins and control groups. Multivitamins group showed no significant differences (P>0.05) in homocysteine levels at day 60 in comparison with day 30.

At day 30 and day 60, folic acid group showed significantly (P<0.05) lower serum homocysteine levels in comparison with B_6 and B_{12} groups. At day 30 and day 60, multivitamins group showed significantly (P<0.05) lower serum homocysteine concentrations in comparison with B_6 and B_{12} groups. At day 30 and day 60, multivitamins group showed significantly (P<0.05) lower serum homocysteine concentrations in comparison with folic acid group.

Discussion

Evidences are accumulating that high levels of homocysteine in the plasma are a risk factor for various cardiovascular diseases. Hyperhomo-cysteinemia can result from genetic deficiencies of enzymes required for homocysteine metabolism or from nutritional deficits of the vitamins that serve as cosubstrates or cofactors for these enzymes. Methionine is an essential amino acid that is released during protein digestion. As methionine is metabolized inside the body, homocysteine is then formed [9].

In our study, plasma homocysteine levels were increased significantly (P<0.05) in methionine only group in comparison with control group. Many studies on rabbits showed similar results with this result [12, 13, 14, 15, 16].

At day 30 and day 60 of our study, serum homocysteine levels in folic acid group were significantly (P<0.05) lower than serum homocysteine levels in vitamin B_6 and vitamin B_{12} groups; this means that folic acid administration is better than vitamin B_6 or vitamin B_{12} when they used alone in the prevention of hyperhomocysteinemia.

Many studies agreed with our result [12, 19, 20]. In one of these studies [19], findings showed that folic acid supplementation to human patients reduced plasma homocysteine concentrations by 41.7% whereas vitamin B_{12} supplementation lowered homocysteine concentrations by 14.8%, and vitamin B_6 did not reduce significantly plasma homocysteine concentrations. In another study but on rabbits [12], results showed that folic acid alone is better than vitamin B_6 or vitamin B_{12} when they used alone to prevent methionine-induced hyperhomocysteinemia. In addition, a study on rats [20] showed that folic acid supplementation led to a significant antihyperhomocysteinemic activities in hyperhomocysteinemia model induced by a high methionine diet.

The results of our study showed that there are no significant changes (P>0.05) in serum homocysteine levels in multivitamins group at day 30 in comparison with day 0 and there are no significant changes (P>0.05) in serum homocysteine levels in multivitamins group at day 60 in comparison with day

30. In addition, there are no significant changes (P>0.05) in homocysteine levels between multivitamins group and control group at day 30. This result indicates that multivitamin combination composed of vitamin B_6, vitamin B_{12}, and folic acid can reduce the hyperhomocysteinemic effect of meth-ionine. Many studies agreed with this result [11, 17, 18]. In these studies, a multivitamin combination composed of vitamin B_6, vitamin B_{12}, and folic acid was given to human subjects and the combination was effectively reduced plasma homocysteine levels.

At day 30 and day 60 of our study, serum homocysteine levels in multivitamins group were significantly (P<0.05) lower than serum homo-cysteine levels in folic acid group; this result indicates that combination of multivitamin supplements consists of vitamin B_6, vitamin B_{12}, and folic acid is better than folic acid alone in the prevention of hyperhomocysteinemia; no data available to compare this result with it. Folate, vitamins B_6, and vitamin B_{12} are essential components in the metabolism of homocysteine inside the body, which occurs through 2 pathways either remethylation to methionine or transsulfuration to cysteine [10]. The breakdown of homocysteine to cysteine requires vitamin B_6-dependent enzyme; while the remethylation of homo-cysteine to methionine requires vitamin B_{12}-dependent enzyme, with folate as a substrate [11].

Conclusion

Multivitamin combination composed of vitamin B_6, vitamin B_{12}, and folic acid reduces the hyperhomocysteinemic effect of methionine and it is better than folic acid alone, while folic acid is better than vitamin B_6 or vitamin B_{12} when they used alone.

References

1. Narin F, Coşkun A, Narin N, Baykan A, Onan SH, Coşkun A, Poyrazoğlu MH, Erez R. An investigation of changes in blood flow in rabbits with long-term hyperhomocysteinemia using color doppler ultrasonography. *Turkish Journal of Medical Sciences*. 2011;41(6): 1007–1012.
2. Sharma VK. Homocysteine induced dementia: collecting evidences for Alzheimer's disease. *International Journal of Pharma and Bio Sciences*. 2010;1(1):1–14.
3. McCully KS. Chemical pathology of homo-cysteine: excitotoxicity, oxidative stress, endothelial dysfunction, and inflammation. *Annals of Clinical & Laboratory Science*. 2009;39(3):219–232.
4. Badawy AA. Moderate alcohol consumption as a cardiovascular risk factor: the role of homocysteine and the need to re-explain the French paradox. *Alcohol and Alcoholism*. 2010;36(3):185–188.
5. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, Dagostino RB, Wilson PW, Wolf PA. Homocysteine as a risk factor for dementia and Alzheimer's disease. *The New England Journal of Medicine*. 2002;346(7): 476–483.
6. Gurib FB and Subratty AH. Homocysteine potentiates esterase- induced contraction on rat aorta in vitro: a risk factor for athero-sclerosis. *Journal of Cell and Molecular Biology*. 2003;2:79–83.
7. Selhub J, Jacques PF, Bostom AG, Dagostino RB, Wilson BW, Belanger AG, Oleary DH, Wolf PA, Schaefer EG, Rosenberg IH. Association between plasma homo-cysteine concentrations and extracranial carotid artery stenosis. *The New England Journal of Medicine*. 1995;332(5):286–291.
8. Harvey RA and Ferrier DR. *Lippincott's Illustrated Reviews: Biochemistry*. 5th Edition. Philadel-phia. Lippincott Williams & Wilkins. 2011;263–378.
9. Symons JD, Mullick AE, Ensunsa JL, Ma AA, Rutledge JC. Hyperhomo-cysteinemia evoked by folate depletion effects on coronary and carotid arterial function. *Arterio-sclerosis,Thrombosis, and Vascular Biology*. 2002;22: 772–780.
10. Siri PW, Verhoef P, Kok FJ. Vitamin B_6, vitamin B_{12}, and folate: association with plasma total homocysteine and risk of coronary atherosclerosis. *Journal of the American College of Nutrition*. 1998;17(5):435–441.
11. Tucker KL, Olson B, Bakun P, Dallal GE, Selhub J, Rosenberg IH. Breakfast cereal fortified with folic acid, vitamin B_6, and vitamin B12 increases vitamin concentrations and reduces homocys-teine concentrations: a

randomized trial. *American Journal of Clinical Nutrition*. 2004;79:805–811.
12. Narin F, Narin N, Akcakus M, Ustdal M, Karaküçük I, Halici C. The effect of folic acid, vitamin B_6, and vitamin B_{12} on the homocysteine levels in rabbits fed by methionine enriched-diets. *Tohoku Journal of Experimental Medicine*. 2002;198:99–105.
13. Zulli A, Widdop RE, Hare DL, Buxton BF, Black MJ. High methionine and cholesterol diet abolishes the endothelial relaxation. *Arterioscle-rosis, Thrombosis, and Vascular Biology*. 2003;23:1358–1363.
14. Júnior HJS, Gil França LH, Stahlke PH, Stalhke BS. Hyperhomo-cysteinemia causing atherogenesis in rabbit aorta: an experimental model. *Journal Vascular Brasileiro*. 2004; 3(1):20–30.
15. Liu FY, Zhang JD, Yu SD, Wang R, Wang B, Lai LP, Yin HQ, Liu G. Effect of folate on neointima formation and matrix metalloproteinase-9 expression after the balloon injury in hyperhomocysteinemic rabbits. *Inter-national Journal of Cardiology*. 2008;131:59–65.
16. Yalçınkaya-Demirsöz S, Depboylu B, Doğru S, Ünlüçerçi Y, Uysal M. Effects of high methionine diet on oxidative stress in serum, apo-B containing lipoprotein, heart, and aorta in the rabbits. *Annals of Clinical and Laboratory Science*. 2009;39(4):386–391.
17. Heijer M, Brouwer IA, Bos GM, Blom HJ, Put NM, Spaans AP, Rosendaal FR, Thomas CM, Haak HL, Wijermans PW, Gerrits WB. Vitamin supplementation reduces homocysteine levels: a controlled trial in patients with venous thrombosis and healthy volunteers. *Arterio-sclerosis, Thrombosis, and Vascular Biology*. 1998;18:356–361.
18. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J. Homocysteine lowering with folic acid and B vitamins in vascular disease. *The New England Journal of Medicine*. 2006; 354:1567–1577.
19. Ubbink JB, Vermaak WJ, Merwe AV, Becker BJ, Delport R, Potgieter HC. Vitamin requirements for the treatment of hyperhomo-cysteinemia in humans. *Journal of Nutrition*. 1994;124:1927–1933.
20. Ansari MN, Nigam GK, Bhandari U. Effect of folic acid on hematological changes in methionine-induced hyperhomocysteinemia. *Indian Journal of Pharmaceutical Sciences*. 2009;71(3):270–275.