

Effect of in vitro B-6 Vitameric Forms on Lymphocyte Proliferation in Healthy Young Women with Oral Vitamin B-6 Supplementation

Ho Kyung Kwak,^{1)2)†} James E. Leklem²⁾

Department of Home Economics,¹⁾ Korea National Open University, Seoul, Korea

Department of Nutrition and Food Management,²⁾ Oregon State University, Corvallis, OR, USA

ABSTRACT

A vitamin B-6 (B-6) intake higher than the current Recommended Dietary Allowance (RDA) has been found to provide an improvement in immune system. Seven premenopausal women consumed their usual diet with the exception of foods relatively high in vitamin B-6 for a total of 27 d. After 7 d, all subjects received a multivitamin supplement containing 2mg B-6 and 4 subjects were given an additional 50mg of B-6 supplement for 20 d. Lymphocyte response to phytohemagglutinin (PHA) was measured before and after the supplementation. To determine the effect of different forms of B-6 on lymphocyte proliferation, cell culture media supplemented with pyridoxal (PL) and PLP, as well as B-6 free media, were tested. A 50mg B-6 supplement significantly increased vitamin B-6 status. There was no further enhancement on lymphocyte proliferation when subjects were taking an additional 50mg of vitamin B-6 supplement. In general, lymphocyte proliferation in media with either PLP or PL did not show any prominent difference. These findings suggest that there may be no further benefits of a B-6 dose beyond twice that of the current RDA on lymphocyte proliferation. Further studies are necessary to examine the effect of the B-6 intake level on activities of enzymes involved in cellular B-6 metabolism in lymphocytes to provide substantial insight into the mechanisms underlying the role of B-6 in the lymphocyte proliferation. (*J Community Nutrition* 7(2) : 79~84, 2005)

KEY WORDS : Vitamin B-6 · supplementation · lymphocyte proliferation · women.

Introduction

It has been known for years that vitamin B-6 status influences immune function. Vitamin B-6 plays a critical role in nucleic acid and amino acid metabolism, and a B-6 deficiency can significantly alter immune responses (Axelrod, Trakattellis 1964). Even though vitamin B-6 deficiency is not common in humans, particular subgroups of the population may be at a greater risk, for example the elderly (Lowik et al. 1990), patients with kidney disease (Dobbelstein et al. 1974 ; Casciato et al. 1984) and in persons with human immunodeficiency virus (HIV) infection (Baum et al. 1991). In these subpopulations, a decreased B-6 status has shown to be associated with immunological decline. Vitamin B-6 supple-

mentation has resulted in a significant increase in indicators of immune functions in the elderly (Tabott et al. 1987) and uremic patients (Dobbelstein et al. 1974 ; Casciato et al. 1984). In addition to a single vitamin B-6 supplement, a multivitamin containing 3mg of vitamin B-6 (Bogden et al. 1994) and a supplement of coenzyme Q10 with pyridoxine (Folkers et al. 1993) have shown to improve the delayed hypersensitivity skin test and the blood levels of immunoglobulin G, respectively.

Lymphocyte proliferation has often been used to determine immune response of humans (Talbot et al. 1987 ; Wang 1990 ; Meydani et al. 1991). This immunological index has been shown to be significantly increased not only with a high B-6 supplementation (50mg) (Talbot et al. 1987), but also with a moderate B-6 supplementation (1.5mg) (Wang 1990). In addition, previous studies have shown that lymphocyte proliferation was significantly increased with a B-6 intake higher than the current recommendation in the elderly (Meydani et al. 1991) and the young (Kwak et al. 2002). With regard to the mechanism of B-6 involvement in lympho-

† Corresponding author : Ho Kyung Kwak, Department of Home Economics, Korea National Open University, 169 Donsung-dong, Chongno-gu, Seoul 110-791, Korea
Tel : (02) 3668-4649, Fax : (02) 3668-4188
E-mail : hkkwak@knou.ac.kr

cyte proliferation, PLP plays a role as a coenzyme in the reaction of serine hydroxymethyltransferase (SHMT). SHMT activity in lymphocytes is significantly increased by phytohemagglutinin (PHA) stimulation for 72 hours (Eichler et al. 1981 ; Trakatellis et al. 1994), and inhibited by 4-deoxy-pyridoxine, an antagonist of vitamin B-6 (Scountzou et al. 1989 ; Trakatellis et al. 1995). Nevertheless, it has not been clearly determined if the PLP concentration in lymphocytes is related to their proliferative response.

To participate in intracellular enzymatic reactions, circulating PLP has to cross the cell membrane, via the action of alkaline phosphatase (ALP) (Merill et al. 1984). In the regulation of the cellular content of PLP, hydrolysis of PLP by phosphatase was found to be an important controlling factor of cellular concentration of PLP in erythrocytes (Lumeng, Li 1974) and isolated hepatocytes (Li et al. 1974). However, it is still unclear if intracellular availability of PLP is altered by changes in activities of enzymes involved in B-6 metabolism following changes in B-6 status.

In the present study, two different levels of vitamin B-6 supplementation were tested for 20 d in order to determine if there is any further enhancement of lymphocyte proliferative response with a high dose of B-6. In addition, the effect of the in vitro supplementation of different forms of vitamin B-6 on lymphocyte mitogenic response was examined.

Subjects and Methods

1. Subjects

Eight premenopausal nonsmoking women participated in this study. Subject criteria were (1) age between 20 and 50 years old ; (2) not taking any supplement or medication that may affect vitamin B-6 metabolism and immune response or willing to stop taking supplement at least 2 weeks before study ; (3) not pregnant ; (4) a body weight no more than 35 % above the ideal body weight and not less than 110 pounds ; (5) exercise of less than 1 hour per day. The study group was made up of 2 Africans, 3 Asians, and 3 Caucasians. No subject reported any chronic disease or other health problem in the interview prior to the study. Subjects were asked to maintain their activity level throughout the study. The basic outline and demands of the study were thoroughly explained to all subjects. All participants provided informed consent. All study procedures with human subjects were approved by Oregon State University Committee for the

Protection of Human Subjects.

2. Experimental design

Subjects consumed their usual diet for 27 d with the exception of foods relatively high in vitamin B-6 and with some foods permitted in a given amount per day. Subjects completed 3-day diet records (2 weekdays and 1 weekend) during the first week and every 10 days of the study. The diet records were analyzed using Food Processor for Windows (version 7.5, ESHA Research, Salem, OR).

Subjects were randomly divided into two groups at the beginning of the study. During the 1st week, subjects were asked to consume a diet with exception of foods high in vitamin B-6 and no supplementation was provided. For the subsequent 20 days, all subjects remained on the same regime of the food selection but were given a daily multivitamin supplement containing 2.0mg of vitamin B-6 (One Daily Multivitamin Supplement with Iron, BI-Mart, Eugene, OR). Four of the eight subjects were given an additional B-6 supplement of 50mg (BI-Mart B-6 50mg, BI-Mart, Eugene, OR) during the 20-day supplement period. The actual content of vitamin B-6 in each supplement was analyzed by microbiological assay (Horwitz 1980).

3. Sample collection and analyses

Following the 7 days of adjustment period with a diet only, baseline blood sample was collected. During the 20 days of supplementation period, subsequent blood samples were collected at days 3, 10 and 20. All blood samples were drawn into heparinized vacutainer tubes after an overnight fast. Twenty four-hour urine samples were collected from each subject to determine subjects urinary B-6 excretion.

On the day of the blood draw, an aliquot of each blood sample was centrifuged at $1800 \times g$ at $4^{\circ}C$ for 15 minutes and the plasma stored at $-30^{\circ}C$ until analysis. The remainder was layered onto an equal amount Histopaque-1077 (Sigma Diagnosis, Sigma Chemical Co., St. Louis, MO) in sterilized conical tubes, and centrifuged at $400 \times g$ at room temperature for 30 minutes. Peripheral blood mononuclear cells (PBMC) were isolated from the interface between plasma and Histopaque using sterilized Pasteur pipettes.

The isolated PBMC were transferred to another tube and washed 3 times with 10mL of Dulbecco's phosphate buffered saline (Sigma Chemical Co., St. Louis, MO). The resulting cell pellets were dispersed into phosphate buffered saline. To determine viability of cells and concentration of

cells, trypan blue (Sigma Chemical Co., St. Louis, MO) was used. The number of cells per mL was counted using a hemocytometer. In order to adjust the concentration of cells to $5 \times 10^6/\text{mL}$, the cell suspension was diluted with phosphate-buffered saline.

Immunocompetence was assessed by measuring lymphocyte proliferation in response to PHA stimulation. To examine the effect of exogenous vitamin B-6 supplementation as different vitameric forms on lymphocyte mitogenic response, PL-HCl (Sigma Chemical Co., St. Louis, MO) and PLP (Sigma Chemical Co., St. Louis, MO), was added to pyridoxine-free customer formulated RPMI 1640 (Gibco BRL®, Life Technologies, Rockville, MD). The concentration of vitamin B-6 in each media containing either PL or PLP was $4.864 \mu\text{M}$, which was the same as the concentration of PN in the standard RPMI 1640. Vitamin B-6 free media and PN-containing standard RPMI 1640 were also tested. The diluted cell suspension was adjusted to $1 \times 10^6/\text{mL}$ using complete RPMI-10 prepared using each media containing L-glutamine plus 100U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 50 μM 2-mercaptoethanol, and 10% bovine calf serum (Hyclone, Logan, UT). Bovine calf serum was previously analyzed by HPLC method as described in Kwak et al. (2002) and contained 49.4nM PLP, 38.7nM 4-PA and 264.8nM PN. Three different concentrations of PHA, 6.25, 12.5 and 25 $\mu\text{g}/\text{mL}$, were prepared using 4 different complete RPMI-10 media. One hundred μL of the cell suspension (1×10^5 /well) and an equal amount of the respective PHA was dispersed into each well of a flat-bottomed 96-well plate. All conditions were performed in triplicate. Cells were incubated for 3 d in a humidified 37°C , 5% CO_2 incubator. Eighteen hours before terminating culture, 1.0 μCi of ^3H -thymidine (ICN, Costa Mesa, CA) was pulsed to each well. Cells were harvested using a semiautomated multiwell harvester, which aspirates and lyses cells, and transfers DNA onto filter paper. Completely dried filter dots for each well were transferred into scintillation vials and scintillation fluid added. Lymphocyte thy-

midine uptake was measured using a liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

4. Statistical analysis

Statistical analyses were done with using SAS (version 8, SAS Institute, Cary, NC) computer program. For each group at each time point, means and standard deviations were calculated for all measures. The Mauchly test of the Huynh-Feldt condition for repeated measures was performed. Tests of hypotheses for group, media and group \times media were performed using type III mean square for subject nested by group and media interaction as an error term. Significant differences in lymphocyte proliferation were assessed by ANOVA. When there was a significant ($P < 0.05$) difference, means of proliferation activity of each media at each time point were compared by Tukey-Kramer multiple comparison.

Results

Characteristics of the subjects are shown in Table 1. Mean hemoglobin (Hb) and hematocrit (Hct) of each group at the beginning and end of study are shown in Table 2. Throughout the study period, the mean vitamin B-6 intakes obtained from foods were not significantly different between groups. The mean (\pm SD) vitamin B-6 intake was $0.9 \pm 0.3\text{mg}$ and $0.9 \pm 0.2\text{mg}$ for groups with 2 and 50mg supplementation, respectively. From the beginning of the study through the end of the study, one subject in 2mg group showed 2 to 8 fold higher values of vitamin B-6 status indicators than the means of the other 3 subjects in the same group. This particular subject was considered to not have adhered to the protocol of

Table 2. Mean (\pm SD) hemoglobin concentration and hematocrit at the beginning and the end of the study ($n = 7$)¹

	Beginning		End	
	2mg	50mg	2mg	50mg
Hb(g/L)	139 \pm 13	140 \pm 10	138 \pm 19	137 \pm 14
Hct(%)	40.0 \pm 4.3	40.4 \pm 4.0	41.3 \pm 4.2	41.8 \pm 3.7

¹ 2mg group ($n = 4$), 50mg group ($n = 3$)

Table 1. Subject characteristics

Group	Age (y)		Weight (kg)		Height (cm)	
	2mg	50mg	2mg	50mg	2mg	50mg
	32	38	75	64	173	160
	20	33	54	73	166	163
	24	30	59	73	163	168
	20		64		160	
Mean \pm SD	25 \pm 6	30 \pm 8	62.7 \pm 11	68.5 \pm 5	167 \pm 5	163 \pm 4

the study. Therefore, the values of this subject were not included in the results. The actual B-6 content of multivitamin and 50 mg B-6 supplement (analyzed by microbiological assay) was 1.81mg and 52.95mg, respectively.

At 3 time points following the oral supplementation, vitamin status measured by plasma PLP and urinary 4-PA was about 3 and 10 times higher ($P < 0.005$), respectively, in the 50mg group compared to the 2mg group (Kwak, Leklem 2002).

There was no significant difference in lymphocyte proliferation between the two groups, and the trend of change over time points was similar in each group (Table 3). Thus values from two groups were not separately analyzed. For the three PHA concentrations used (6.25, 12.5 and 25 $\mu\text{g/mL}$), optimal lymphocyte response to PHA was observed at a concentration of 12.5 $\mu\text{g/mL}$, while the trend of response change was similar among three concentrations of PHA. Fig. 1 demonstrates the mean values of mitogenic response to PHA at 12.5 $\mu\text{g/mL}$ from all subjects.

Compared to the baseline values, lymphocyte proliferation was enhanced after 3 d and 10 d of B-6 supplementation when PBMC was incubated with PLP supplemented media. This enhanced lymphocyte mitogenic response returned to baseline values after 20 d of B-6 supplementation. When cells were incubated with PL supplemented media, no change was

observed in lymphocyte proliferative activity. Lymphocyte mitogenic response to PHA was gradually increased when incubated with PN containing media after 3 and 10 d of oral B-6 supplementation, but was decreased when supplementation was prolonged to 20 d. When no vitamin B-6 was added to the cell culture media, overall lymphocyte proliferative response to PHA was similar throughout the study, although there was a slight difference in response between day 3 and 10. After 10 d of B-6 supplementation, lymphocyte proliferative activity was higher when cells were incubated with media containing PN as a B-6 source than with media containing PLP, PL or no B-6.

In general, no significant difference was found in proliferative activities between B-6 free, PLP and PL supplemented media, however, small differences were notable.

After 10 d of supplementation, lymphocyte proliferation was 37% greater in PLP-containing media compared with B-6 free media. In addition there was a 30% higher proliferation with PL-containing media compared to PLP media at day 20 of oral B-6 supplementation.

PN containing culture media was prepared with regular RPMI (company formulated), and the other three culture media were prepared using B-6 free RPMI (customer formulated). Therefore, the molar concentrations of PLP and PL in the respective media was adjusted to the concentration

Table 3. Mean (\pm SD) lymphocyte proliferation in groups with 2 different levels of vitamin B-6 supplements (N = 7)¹

	BL		3 days		10 days		20 days	
	2mg	50mg	2mg	50mg	2mg	50mg	2mg	50mg
PLP	3.82 \pm 0.66	3.89 \pm 1.22	7.9 \pm 0.76	7.58 \pm 2.23	8.20 \pm 0.16	6.95 \pm 2.49	5.50 \pm 0.32	4.89 \pm 2.45
PL	7.42 \pm 0.80	6.72 \pm 1.77	8.12 \pm 0.67	7.22 \pm 2.08	8.08 \pm 0.81	7.13 \pm 2.41	7.64 \pm 1.31	6.34 \pm 2.82
PN	6.49 \pm 0.60	6.33 \pm 1.90	7.84 \pm 0.69	7.12 \pm 2.37	10.77 \pm 0.27	9.64 \pm 3.84	6.55 \pm 0.60	5.76 \pm 3.09
B6 Free	7.22 \pm 0.90	7.00 \pm 2.22	8.12 \pm 1.77	7.91 \pm 2.69	5.46 \pm 0.20	5.00 \pm 1.99	7.45 \pm 1.04	6.13 \pm 2.93

¹ cpm ($\times 10^4$); incubated with media containing PLP, PL, PN and no vitamin B-6; lymphocytes were stimulated by PHA at 12.5 $\mu\text{g/mL}$; 2mg group (n = 4), 50mg group (n = 3)

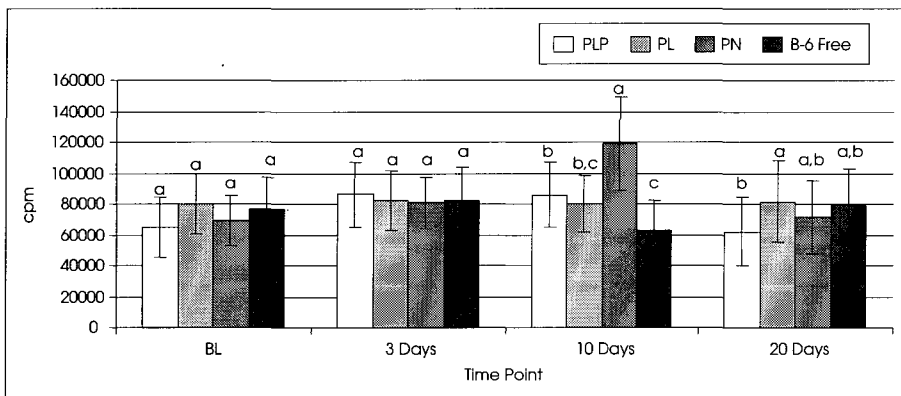


Fig. 1. Comparison of lymphocyte proliferation cultured with different forms of B-6 at each time point; lymphocytes were stimulated by PHA at 12.5 $\mu\text{g/mL}$; a-b Different letters for a given time point indicate a significant difference in lymphocyte proliferation between different media, $P < 0.05$; error bars represent standard deviation.

of PN in company formulated RPMI. The actual concentration of PN in the company formulated RPMI as analyzed by HPLC was close to the concentration used in preparation of the PLP and PL containing media.

Discussion

This study supports the findings of the previous study (Kwak et al. 2002) in which 2.1mg B-6 may be an adequate intake level to optimize lymphocyte proliferation for young women. Previously, we found no difference in lymphocyte response to PHA between 2.1mg and 2.7mg of vitamin B-6 intake (Kwak et al. 2002), once the response was significantly increased with 7 d of additional 0.6mg of B-6 to 1.5 mg daily B-6 intake. This finding led us to investigate if a higher B-6 intake than 2.7mg/d is associated with any further enhancement of lymphocyte response. Even though no attempt was made to determine the effect of B-6 levels between 2.7 and 50mg, the result of the current study clearly showed that an intake of 50mg B-6 did not provide a further benefit to the lymphocyte mitogenic response beyond that achieved from approximately 2.7mg B-6.

Lymphocyte mitogenic response has shown to be enhanced with an intake of vitamin B-6 higher than the current recommendation for the elderly (Meydani et al., 1991) and the young (Wang 1990 ; Kwak et al. 2002). In the previous study (Kwak et al. 2002), an additional 1.1 – 1.7mg of B-6 resulted in a significant increase in lymphocyte proliferation compared to 1mg intake of B-6. In the 1st week of the present study, subjects consumed approximately 0.9mg/d B-6. A subsequent B-6 supplementation regime (provided 1.8 or 53mg of B-6) for 10 days provided an enhancement of lymphocyte mitogenic response in PN containing media compared to the first week without supplementation. However, the reason for the decreased proliferative activity seen after 10 additional days of B-6 supplementation is not clear. No decreased activity was observed over the 4 weeks with the 2.1 and 2.7mg/d B-6 intake in the previous study, and over the 8 weeks with a 50mg/d B-6 supplement (Talbot et al. 1987). Because of these conflicting results, additional studies should be performed to evaluate how lymphocyte response to PHA changes with chronic B-6 supplementation over several months. Lymphocyte proliferation results, obtained other than from PN containing media, cannot be directly compared to findings from other studies because PN is the form

used in RPMI 1640, which is normally used for the lymphocyte proliferation assay in other studies.

In the present study, it was assumed that vitamin B-6 supplementation might be associated with alteration of enzyme activity which could affect the cellular utilization of B-6 vitamers. Therefore, an attempt was made to test how in vitro PLP and PL contribute differently to lymphocyte proliferation when cells are activated by a mitogen. Alkaline phosphatase (ALP) is involved in the hydrolysis of PLP to PL. ALP activity in leukocytes was found to increase slightly with 10mg/d PN supplementation for 7 days (Coburn, Seidenberg 1969). However, people who have previously taken multivitamin supplementation did not show increased ALP activity in leukocytes (Coburn, Seidenberg 1969), which might be the reason why lymphocyte proliferation in PLP containing media was enhanced with B-6 oral supplementation for 3 to 10 days, thereby returning back to the BL value with subsequent 10 days of supplementation. Increased ALP activity following B-6 supplementation for the first 10 days might facilitate cellular uptake of PLP from media by increasing hydrolysis of PLP, followed by utilization of PLP by PLP dependent enzymes in cells such as serine hydroxymethyltransferase (SHMT). Increased uptake of PLP from cell culture media might meet the demand of SHMT whose activity in lymphocytes was shown to be increased 5 – 20 times 72 hours after PHA stimulation (Eichler et al. 1981 ; Trakatellis et al. 1994). Mitogenic stimulation was found to be a factor affecting membrane ALP activity in murine lymphocytes, but only B-lymphocytes have been shown to be increased by mitogenic stimulation (Garcia-Rozas et al. 1982 ; Kasyapa, Ramanadham 1992). Thus, mitogenic stimulation might not be a factor affecting ALP activity in the present study since only a T-cell mitogen was tested. Nevertheless, the presence of an abundant amount of PLP or PL in cell culture media did not significantly affect lymphocyte proliferation in the current study since, in general, no significant difference of lymphocyte proliferative activity was found between PLP or PL containing media and B-6 free media. Even though PLP deficiency induced by the presence of in vitro 4-deoxyperidoxine (dB6) was found to decrease in lymphocyte proliferation, addition of PN into the culture did not show a further effect on improvement of proliferative function with an absence of dB6 (Trakatellis et al. 1992). In addition, lymphocyte proliferation was not shown to be significantly correlated with PBMC PLP in the previous study

(Kwak et al. 2002) and PBMC PLP concentration was not significantly changed with a range of intake levels at 1 to 2.7mg (Kwak et al. 2002). PLP plays an important role in lymphocyte proliferation, however, PLP concentration in PBMC may not be a primary factor affecting lymphocyte proliferative function once lymphocytes accumulate a certain amount of PLP. Therefore, future studies are suggested to determine the extent to which exogenous PLP and PL contribute to the lymphocyte proliferative function when PBMC PLP was lowered following B-6 deficiency. In addition, to better establish the intake level of vitamin B-6 that optimizes lymphocyte proliferation, future research efforts should focus on determining the mechanism underlying the effect of vitamin B-6 on the lymphocyte proliferation. Evaluating the effect of the B-6 intake level on enzyme activities (SHMT and enzymes involved in cellular B-6 metabolism) in PBMC may also provide substantial insight into the mechanisms underlying the role of B-6 in the lymphocyte proliferation and regulation of B-6 content in PBMC.

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