

EFFECTS OF ISOLATED VITAMIN B₆ SUPPLEMENTATION ON OXIDATIVE STRESS AND HEART FUNCTION PARAMETERS IN EXPERIMENTAL HYPERHOMOCYSTEINEMIA

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ABSTRACT

Introduction: The purpose of this study was to investigate the effects of isolated vitamin B₆ (VB₆) supplementation on experimental hyperhomocysteinemia (Hhe) induced by homocysteine thiolactone (HcyT).

Methods: Fifteen male Wistar rats were divided into three groups according to their treatment. Animals received water and food *ad libitum* and an intragastric probe was used to administer water for 60 days (groups: CB₆, HcyT, and HB₆). On the 30th day of treatment, two groups were supplemented with VB₆ in the drinking water (groups: CB₆ and HB₆). After 60 days of treatment, homocysteine (Hcy), cysteine, and hydrogen peroxide concentration, nuclear factor (erythroid-derived 2)-like 2 (NRF2) and glutathione S-transferase (GST) immunocontent, and superoxide dismutase (SOD), catalase (CAT), and GST activities were measured.

Results: The HcyT group showed an increase in Hcy concentration (62%) in relation to the CB₆ group. Additionally, GST immunocontent was enhanced (51%) in the HB₆ group compared to the HcyT group. Also, SOD activity was lower (17%) in the HB₆ group compared to the CB₆ group, and CAT activity was higher in the HcyT group (53%) compared to the CB₆ group. Ejection fraction (EF) was improved in the HB₆ group compared to the HcyT group. E/A ratio was enhanced in the HB₆ group compared to the CB₆ group. Correlations were found between CAT activity with myocardial performance index (MPI) ($r = 0.71$; $P = 0.06$) and E/A ratio ($r = 0.6$; $P = 0.01$), and between EF and GST activity ($r = 0.62$; $P = 0.02$).

Conclusions: These findings indicate that isolated VB₆ supplementation may lead to the reduction of Hcy concentration and promotes additional benefits to oxidative stress and heart function parameters.

Keywords: Homocysteine; oxidative stress; vitamin B₆

Homocysteine (Hcy) is recognized as an independent risk factor for atherosclerosis, which is a leading cause of vascular disease worldwide. It may participate in the development of cardiovascular diseases through its effects on smooth muscle and vascular endothelium cells¹. In addition, ischemic heart disease remains a leading cause of premature adult mortality².

Hcy is a non-protein-forming, sulfur-containing amino acid which is metabolized through two pathways: transsulfuration to cysteine (Cys) and remethylation to Met³. Hyperhomocysteinemia (Hhe) can be defined as Hcy plasma concentration > 12 μmol/L, which is considered a risk factor for cardiovascular disease^{4,5}. Vitamin B₆ (VB₆) participates in the transsulfuration of Hcy as an enzymatic cofactor of CβS (cystationine β synthase) with cysteine as a final product⁶.

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Apart of its role in Hcy metabolism, VB₆ consumption decreases the risk for neuropsychiatric disorders, including seizures, migraine, chronic pain, and depression, and cardiovascular diseases, such as atherosclerosis and endothelial cell proliferation^{7,8}.

Nutritional factors play a fundamental role on Hcy metabolism. VB₆ is a water-soluble vitamin that can be found in different types of food, including fish, poultry, whole grains, potatoes, vegetables, and nuts. It exists in several forms such as pyridoxal, pyridoxine, pyridoxamine, and its active form, pyridoxal 5'-phosphate (PLP). Low VB₆ concentrations could reflect, thus, an increased consumption of PLP that is associated with an accelerated synthesis of inflammatory cytokines⁹. Additionally, VB₆ appears to control reactive oxygen species (ROS) production similarly to vitamins C and E. It is well established in the literature that increased oxidative stress is associated with VB₆ deficiency¹⁰⁻¹². Moreover, a recent case-control study found that a lower status of B vitamins (B₁₂, B₆, and folate) in the diet and in the serum concentration are involved in the etiology of hyperhomocysteinemia (Hhe), cardiovascular disease, and oxidative stress¹³.

Thus, the aim of this study was to evaluate oxidative stress and heart function parameters after isolated VB₆ supplementation in an experimental Hhe.

METHODS

Animals and Treatments

The experiment was in accordance with the *Guidance for the Description of Animal Research in Scientific Publications* from the National Research Council (US) Institute for Laboratory Animal Research¹⁴.

Fifteen male Wistar rats (30 days old) from the Animal House of the Universidade Federal de São Paulo, maintained in standard conditions (12h/12h light/dark cycle, room temperature at 21°C, food and water *ad libitum*), were divided into three groups: 1) CB₆: rats received water by intragastric probe for 60 days, and from the 30th to the 60th day they also received supplementation with pyridoxine hydrochloride (60 mg/kg) diluted in the water; 2) HcyT: rats received water containing homocysteine thiolactone (L-homocysteine thiolactone hydrochloride, Sigma; 100 mg/kg) by intragastric probe for 60 days; 3) HB₆: rats received water containing homocysteine thiolactone (100 mg/kg) by intragastric probe for 60 days, and from the 30th to the 60th day they also received supplementation with pyridoxine hydrochloride (60 mg/kg). Animals were weighed at the beginning and at the end of the experiment. After 60 days, animals were euthanized by decapitation, and blood and hearts were collected.

Blood was immediately processed, and hearts were stored at -80°C for posterior biochemical analysis.

Homocysteine and Cysteine Assay

Blood was centrifuged for 5 minutes at 3,000 g in tubes containing anticoagulant, and plasma was collected. Total homocysteine and cysteine levels were determined by high performance liquid chromatography (HPLC) with fluorescence detection, as performed by Oliveira et al.¹⁵.

ROS Evaluation

Hydrogen peroxide was measured through the horseradish peroxidase (HRPO)-mediated oxidation of phenol red. Slices of left ventricular (LV) tissue were incubated for 30 minutes at 37°C in 10 mmol/L phosphate buffer (140 mmol/L NaCl and 5 mmol/L dextrose). Supernatants were transferred to tubes with 0.28 mmol/L phenol red and 8.5 U/mL HRPO. After 25 minutes of incubation, 1 mol/L NaOH was added and the absorbance of the solution was measured at 610 nm. Results were expressed in nmoles H₂O₂ per g of tissue¹⁶.

Western Blot Analysis

Thirty micrograms of protein were submitted to a 12% one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)¹⁷. The separated proteins were transferred to nitrocellulose membranes using a buffer containing 20 mmol/L Tris, 150 mmol/L glycine, 20% (v/v) methanol, 0.1% (w/v) SDS, pH 8.2, in a cooled Bio-Rad TransBlot unit. Non-specific protein-binding sites were blocked with non-fat milk in Tris-buffer for 1 hour¹⁸. The membranes were processed for immunodetection using rabbit anti-NFR2 (52kDa) and mouse anti-GST (26kDa) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) incubated at 4°C. Bound primary antibodies were detected using rabbit anti-goat and rabbit anti-mouse, and membranes were developed using chemiluminescence. Autoradiographic images were scanned and analyzed using an image software (Image master VDS CI, Amersham Biosciences Europe, IT). Results of each membrane were normalized through Ponceau S staining¹⁹.

Determination of Protein Concentration

Protein was measured by the method of Lowry et al. (1951) using a standard solution of bovine serum albumin²⁰.

Preparation of Erythrocytes

Heparinized venous blood samples were washed in a solution of 9 g/L sodium chloride and centrifuged three times at 3000 g for 10 minutes at

room temperature. White cells were discarded by aspiration. Erythrocytes were diluted 1/10 in 1 mM acetic acid and 4 mM magnesium sulfate, placed on an ice bath for 10 minutes, and centrifuged at 4200 g for 20 minutes at 0°C. Supernatant was used for enzyme assays²¹.

Determination of Antioxidant Enzyme Activities in Erythrocytes

The quantification of superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of superoxide radical reaction with pyrogallol²². Catalase (CAT) activity was determined by following a decrease in hydrogen peroxide (H₂O₂) absorbance at 240 nm. It was expressed as nanomol of H₂O₂ reduced per minute per milligram of protein²³. Glutathione-S-transferase (GST) activity, expressed as nanomol per milligram of protein, was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm²⁴.

Echocardiographic Evaluation

A transthoracic echocardiography was performed in all experimental animals at the end of treatment using a SEQUOIA 512 equipment (ACUSON, Mountain View, CA, USA), with a 13-MHz linear transducer. Rats were anesthetized with a combination of ketamine (80 mg/kg, ip) and xylazine (10 mg/kg, ip). Images were obtained with the transducer placed on the animal's shaved chest in the supine position and were optimized using a transmission gel. All measurements were based on an average of three consecutive cardiac cycles. Echocardiographic indices were obtained according to the recommendations of the American Society of Echocardiography. Ejection fraction (EF) was calculated from M-mode recordings to assess the following parameters: (1) morphometric: LV mass, LV diastolic

diameter corrected by weight (LVDD/kg), and relative wall thickness (RWT); (2) systolic function: EF; (3) diastolic function: LV isovolumic relaxation time (IVRT) and ratio of peak velocity of early (E) and late (A) diastolic filling (E/A ratio); and (4) overall function: myocardial performance index (MPI), as previously described²⁵.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Data were tested for normal distribution using the Shapiro-Wilk test, and the one-way analysis of variance followed by the Tukey test was used to compare groups. Pearson's correlation was used to study the association between heart function parameters and CAT (pmol/mg protein) and GST (nmol/mg protein). Significance level was established at $P \leq 0.05$. Data were analyzed using SPSS, version 19.0.

RESULTS

Total Hcy plasma concentration (μmol/L) was higher (98%) in the HcyT group after treatment compared to the CB₆ group, demonstrating the efficiency of our experimental protocol. Cysteine concentration and body weight were not affected by the treatment with HcyT (table 1).

Regarding antioxidant enzyme activities, there was a decrease in SOD activity (U/mg protein) and GST activity (nmol/mg protein) in erythrocytes of the HB₆ group compared to the CB₆ group. Conversely, the HB₆ group showed an increase in GST activity compared to the HcyT group. Furthermore, CAT activity (pmol/mg protein) was lower in the HcyT group compared to the CB₆ group (table 2).

There was an increase (62%) in H₂O₂ concentration in the HcyT group compared to the CB₆ group.

Table 1: Metabolic evaluations in studied groups.

	CB ₆	HcyT	HB ₆
Hcy concentration (μmol/L)	8.6 ± 0.9	17.0 ± 1.4*	9.0 ± 1.0**
Cys concentration (μmol/L)	294.2 ± 32.5	327.9 ± 26	331.5 ± 36.1
Initial body weight (g)	249.0 ± 6.5	260.0 ± 18.0	244.5 ± 27.5
Final body weight (g)	444.0 ± 22.6	420.3 ± 35	456.0 ± 12.5

CB₆ = control + vitamin B₆; HcyT = homocysteine thiolactone; HB₆ = homocysteine + vitamin B₆. Data are reported as mean ± SD. * $P < 0.05$ vs CB₆; ** $P < 0.05$ vs HcyT.

Table 2: Antioxidant enzyme activities in erythrocytes.

Parameter	CB ₆	HcyT	HB ₆
SOD (U/mg protein)	13.88 ± 2.79	8.17 ± 1.14*	11.51 ± 2.14
CAT (pmol/mg protein)	89 ± 17	131 ± 19*	72 ± 15**
GST (nmol/mg protein)	98 ± 19	24 ± 4	61 ± 17*,**

CB₆ = control + vitamin B₆; HcyT = homocysteine thiolactone; HB₆ = homocysteine + vitamin B₆. Data are reported as mean ± SD. * $P < 0.05$ vs CB₆; ** $P < 0.05$ vs HcyT. SOD = superoxide dismutase; CAT = catalase; GST = glutathione S-transferase.

However, H₂O₂ concentration in the HB₆ group was similar to that in the CB₆ group (figure 1). The HcyT group showed a significant decrease (46%) in NRF2 immunocontent in relation to the CB₆ group (figure 2). Furthermore, the HB₆ group showed an increase in GST immunocontent (51%) compared to the HcyT group (figure 3).

In relation to heart function, the HB₆ group showed increased LVDD/Kg in relation to the CB₆ and HcyT groups. Moreover, RWT is decreased in HB₆ group in relation to the HcyT group. Considering LV systolic function variables, EF was significantly decreased (9.5%) in the HB₆ group compared to the CB₆ group, as well as in the HcyT group compared to the CB₆ (20%) and HB₆ (10%) groups. Global LV function assessment with MPI was increased in the HcyT group in relation to the CB₆ (29.5%) and HB₆ (30%) groups. E/A ratio was enhanced in the HcyT group compared to the CB₆ group. Conversely, the HB₆ group showed decreased IVRT in relation to the CB₆ and HcyT groups (table 3).

Correlations were found between increased CAT activity and increased MPI ($r = 0.71$; $P = 0.006$), as well as in increased E/A ratio ($r = 0.6$; $P = 0.01$).

Systolic cardiac function, measured by echocardiogram and represented by EF, was correlated with increased concentration of GST activity ($r = 0.62$; $P = 0.02$).

DISCUSSION

The main findings of the present study were that isolated VB₆ supplementation reduced plasma concentration of Hcy, restored antioxidant enzyme activities in erythrocytes, reduced H₂O₂ concentration,

and improved heart function parameters in this model of Hhe.

We observed that Hcy plasma concentration decreased significantly after the CB₆ and HB₆ groups received isolated VB₆ supplementation for 30 days compared to the HcyT group. Previous studies have demonstrated that VB₆ deficiency induced a significant Hhe. We also observed that folate supplementation improves Hhe induced by VB₆ deficiency²⁶. Conversely, epidemiological studies have demonstrated that VB₆ has an important role in the prevention of atherosclerosis regardless of the reduction of plasma homocysteine concentration, but the exact mechanism responsible for this remains unclear²⁷⁻²⁹. One plausible

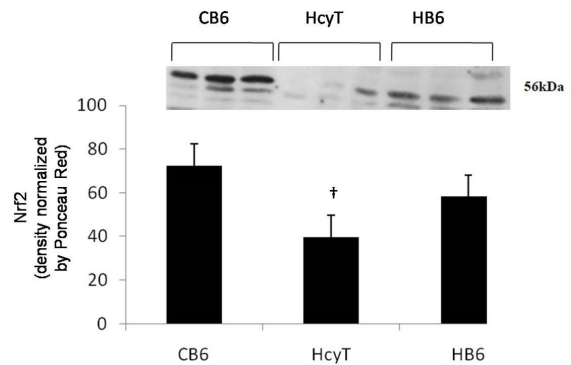


Figure 2: Nrf2 immunocontent in cardiac tissue: Protein expression of Nrf2 was measured by western blot technique in cardiac tissue of CB₆ (n=5), HT (n=5) and HTB₆ (n=5) animals. Image represents a blot containing Nrf2 immunocontent of three animals per group. Data are represented by mean \pm SD of mega pixels of each experimental. † $p < 0.05$ vs. CB₆ and ‡ $p < 0.05$ vs. HT ($p < 0.05$).

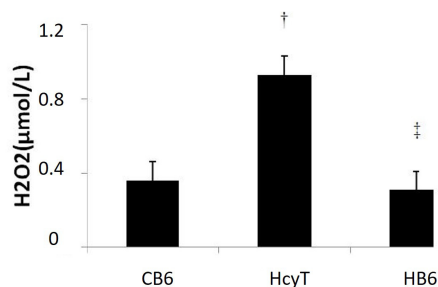


Figure 1: The effect of VB₆ supplementation on H₂O₂ levels in hyperhomocysteinemia (Hhe) model. H₂O₂ concentration was measured in cardiac tissue by spectrophotometry at the end of experimental protocol in CB₆ (0.36 \pm 0.06), HT (0.93 \pm 0.14) and HTB₆ (0.31 \pm 0.06) groups. Values are presented as mean \pm SD from 5 animals per group. † $p < 0.05$ vs. CB₆ and ‡ $p < 0.05$ vs. HT ($p < 0.05$).

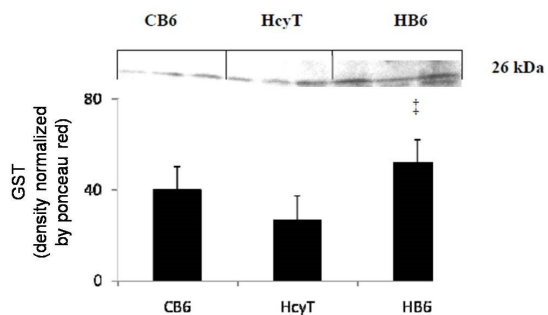


Figure 3: Glutathione S- Transferase immunocontent in cardiac tissue: Protein expression of GST was measured by western blot technique in cardiac tissue of CB₆ (n=5), HT (n=5) and HTB₆ (n=5) animals. Image represents a blot containing GST immunocontent of three animals per group. Data are represented by mean \pm SD of mega pixels of each experimental. † $p < 0.05$ vs. CB₆ and ‡ $p < 0.05$ vs. HT ($p < 0.05$).

Table 3: Parameters on echocardiography of experimental groups.

Parameter	CB ₆	HcyT	HB ₆
Heart rate (bpm)	234 ± 25	258 ± 17	243 ± 12
LVM (g/kg)	2.60 ± 0.13	2.70 ± 0.45	2.90 ± 0.33
RWT (cm)	0.40 ± 0.04	0.44 ± 0.02	0.36 ± 0.02**
LVDD (cm/kg)	1.53 ± 0.18	1.56 ± 0.13	1.70 ± 0.13*,**
E/A ratio	1.75 ± 0.25	2.16 ± 0.22*	1.99 ± 0.10
EF (%)	84.00 ± 0.07	69.00 ± 0.04*	76.00 ± 0.04*,**
MPI	0.31 ± 0.16	0.44 ± 0.11*	0.30 ± 0.11**
IVRT (ms)	25.00 ± 1.46	25.00 ± 1.40	22.00 ± 1.75*,**

CB₆ = control + vitamin B₆; HcyT = homocysteine; HB₆ = homocysteine + vitamin B₆. Data are reported as mean ± SD. **P* < 0.05 vs CB₆; ***P* < 0.05 vs HcyT. Heart rate (bpm) = beat per minute; LVM (g/kg) = left ventricular mass on echocardiography (adjusted per weight); RWT (cm) = relative wall thickness; LVDD (cm/kg) = left ventricular diastolic diameter (adjusted per body weight); E/A ratio: ratio of peak velocity of early (E) and late (A) diastolic filling; EF = ejection fraction; MPI: myocardial perfusion imaging; IVRT (ms) = isovolumic relaxation time.

mechanism is the endogenous production of H₂S by the transsulfuration pathway that mediates cysteine production from catabolism of Hcy. H₂S is a gaseous signaling molecule that modulates physiologic actions, such as relaxation of smooth muscle, and attenuates myocardial ischemia-reperfusion injury by protecting mitochondrial function³⁰. These findings, together with ours, suggest that VB₆ is important for cerebrovascular protection and that the mechanism involved in this process could be related to the transsulfuration pathway.

Furthermore, VB₆ supplementation did not change the body weight of the animals. This result demonstrates that VB₆ supplementation did not influence the metabolic control of animals (data not shown).

The groups that received VB₆ supplementation (CB₆ and HB₆) showed a significant reduction in H₂O₂, a signaling molecule, indicating that VB₆ might act as a ROS quencher. A recent study reported that a VB₆ deficient diet seemed to mediate the oxidative stress in connection with the redistribution of glutathione from liver to plasma, but did not further affect glutathione-related enzyme activities in mice with homocysteine-induced oxidative stress³¹. Another previous study with Japanese women demonstrated that VB₆ plays a role against oxidative DNA damage, but not folate and homocysteine³². One of the possible reasons for our results is the ability of VB₆ to inhibit xanthine oxidase activity, which is an enzyme responsible for the formation of uric acid and hydrogen peroxide³³.

The detoxification of xenobiotics seems to be related with the GST enzyme and is regulated by NRF2. In the present study, GST immunocontent increased significantly in the groups that received VB₆ supplementation, and the HB₆ group showed the highest level of GST immunocontent. One plausible explanation for this result is that VB₆ supplementation

preserved GST immunocontent. Conversely, the depletion of GST occurs during Hhe due to the role of this enzyme in cysteine metabolism.

In the present study, the HB₆ group showed an increase in NRF2 immunocontent compared to the HcyT group. The method used for this evaluation is suitable for the cytoplasmic portion; however, it is known that when activated NRF2 is in the nuclear portion. It is a possible explanation for an increased NRF2 immunocontent in the cytoplasmic portion. Other study using hepatoma cell line demonstrated that Hcy activates NRF2 transcription (from the cytoplasmic portion to the nuclear portion) inducing antioxidant-related genes³⁴.

Regarding antioxidant enzyme activities in erythrocytes, we observed that SOD and GST activities decreased in the HcyT group compared to the CB₆ group. We also found that CAT activity increased in the HcyT group when compared to the CB₆ and HB₆ groups. Additionally, GST activity increased in the HB₆ group compared to the HcyT group. The relevance of measuring blood oxidative stress parameters by standardized methods may be useful to define the role of oxidative stress in different diseases and also for clinical diagnosis^{21, 35}.

The exact mechanism by which VB₆ acts as an antioxidant is not clear yet. One possible direct mechanism might be that VB₆ has both hydroxyl and amine groups as substitutes on a pyridine ring, which may react with peroxy radicals and thereby scavenge radicals and lipid peroxides. Moreover, an indirect mechanism by which VB₆ compounds play the role of antioxidant may be through serving as a coenzyme in the glutathione enzyme system³⁶.

In the present study, LV mass and LVDD/kg was increased in the HB₆ group when compared to the other groups. A previous study with chronic uremic patients demonstrated a direct correlation between total plasma homocysteine level and LV mass index³⁷.

One possible reason for this result is the duration of isolated VB₆, which was not long enough to protect cardiac morphological against the Hhe effect.

Conversely, concerning systolic function, EF was decreased in the HB₆ group compared to the CB₆ group. However, the HB₆ group showed a significant improvement in relation to the HcyT group. A previous study investigated the effect of micronutrient supplementation (calcium, magnesium, zinc, copper, selenium, vitamin A, thiamine, folate, vitamin B₁₂, C, E, D, and coenzyme Q10, riboflavin, VB₆) for 9 months on elderly patients with chronic cardiac failure and observed a significant improvement in EF³⁸. This result, in agreement with ours, reinforces the hypothesis of association between redox balance and cardiac function.

In relation to LV diastolic function, IVRT was reduced in the HB₆ group in relation to the CB₆ and HcyT groups. This finding might be suggestive of diastolic dysfunction, probably by the HcyT effect of increased endothelial-myocyte uncoupling, resulting in hypertension³⁹. E/A ratio was increased in the Hcy group compared to the CB₆ group. In agreement with our findings, other study investigated the effects of plasma Hcy on LV diastolic function of Chinese patients with essential hypertension and found a lower E/A ratio and significant correlations between LV diastolic function indices and Hcy levels⁴⁰. Our results suggest that the period used for VB₆ supplementation was not enough to protect diastolic function from Hhe.

LV global function by pulsed-wave MPI was increased in the HcyT group in relation to the CB₆ group.

However, the HB₆ group showed a decrease in MPI, as expected. MPI has prognostic values in different diseases, including chronic obstructive pulmonary disease and pulmonary artery hypertension^{41,42}. We found positive correlations between increased CAT activity and increased MPI, which could be explained by the high production of H₂O₂ in the body that will need to be converted by CAT. H₂O₂ toxicity will probably act negatively on heart function. Considering the increase in E/A ratio, we suggest that exists an association between increased H₂O₂ production, which is converted by CAT, and the myocardial stiffness caused by increased E/A ratio. EF was also correlated with an increase in GST activity, indicating a protective role of this enzyme in systolic heart function. This correlation reinforces the association of oxidative and heart function parameters in this experimental model.

Collectively, these findings demonstrated that isolated supplementation of VB₆ seems to modulate antioxidant enzyme activities and some heart function parameters.

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Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

- Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. *Nutr J*. 2015;14(1):6. PMID:25577237. <http://dx.doi.org/10.1186/1475-2891-14-6>.
- Herrington W, Lacey B, Sherliker P, Armitage J, Lewington S. Epidemiology of Atherosclerosis and the Potential to Reduce the Global Burden of Atherothrombotic Disease. *Circ Res*. 2016;118(4):535-46. PMID:26892956. <http://dx.doi.org/10.1161/CIRCRESAHA.115.307611>.
- Chen NC, Yang F, Capecci LM, Gu Z, Schafer AI, Durante W, et al. Regulation of homocysteine metabolism and methylation in human and mouse tissues. *FASEB J*. 2010;24(8):2804-17. PMID:20305127. <http://dx.doi.org/10.1096/fj.09-143651>.
- Cohen E, Levi A, Vecht-Lifshitz SE, Goldberg E, Garty M, Krause I. Assessment of a possible link between hyperhomocysteinemia and hyperuricemia. *J Invest Med*. 2015;63(3):534-8. PMID:25646870. <http://dx.doi.org/10.1097/JIM.0000000000000152>.
- Wang B, Lin L, Zhao C. Related factors of serum uric acid in patients with primary hypertension and hyperhomocysteinemia. *Clin Exp Hypertens*. 2016;38(3):312-6. PMID:27028508. <http://dx.doi.org/10.3109/10641963.2015.1107088>.
- Selhub J, Byun A, Liu Z, Mason JB, Bronson RT, Crott JW. Dietary vitamin B6 intake modulates colonic inflammation in the IL10^{-/-} model of inflammatory bowel disease. *J Nutr Biochem*. 2013;24(12):2138-43. PMID:24183308. <http://dx.doi.org/10.1016/j.jnutbio.2013.08.005>.
- Dhalla NS, Takeda S, Elimban V. Mechanisms of the beneficial effects of vitamin B6 and pyridoxal 5-phosphate on cardiac performance in ischemic heart disease. *Clin Chem Lab Med*. 2013;51(3):535-43. PMID:23314545. <http://dx.doi.org/10.1515/cclm-2012-0553>.
- Krause D, Roupas P. Effect of Vitamin Intake on Cognitive Decline in Older Adults: Evaluation of the Evidence. *J Nutr Health Aging*. 2015;19(7):745-53. PMID:26193858. <http://dx.doi.org/10.1007/s12603-015-0539-3>.
- Lotto V, Choi SW, Friso S. Vitamin B6: a challenging link between nutrition and inflammation in CVD. *Br J Nutr*. 2011;106(2):183-95. PMID:21486513. <http://dx.doi.org/10.1017/S0007114511000407>.
- Cabrini L, Bergami R, Fiorentini D, Marchetti M, Landi L, Tolomelli B. Vitamin B6 deficiency affects antioxidant defences in rat liver and heart. *Biochem Mol Biol Int*. 1998;46(4):689-97. PMID:9844729.

11. Anand SS. Pyridoxine attenuates chromium-induced oxidative stress in rat kidney. *Basic Clin Pharmacol Toxicol.* 2005;97(1):58-60. PMID:15943760. http://dx.doi.org/10.1111/j.1742-7843.2005.pto_97109.x.
12. Matxain JM, Padro D, Ristilä M, Strid A, Eriksson LA. Evidence of high *OH radical quenching efficiency by vitamin B6. *J Phys Chem B.* 2009;113(29):9629-32. PMID:19558175. <http://dx.doi.org/10.1021/jp903023c>.
13. Waly MI, Ali A, Al-Nassri A, Al-Mukhaini M, Valliatte J, Al-Farsi Y. Low nourishment of B-vitamins is associated with hyperhomocysteinemia and oxidative stress in newly diagnosed cardiac patients. *Exp Biol Med (Maywood).* 2016;241(1):46-51. PMID:26246496. <http://dx.doi.org/10.1177/1535370215596860>.
14. National Research Council. Institute for Laboratory Animal Research. *Guidance for the description of animal research in scientific publications.* 8th ed. Washington, DC: The National Academies Press; 2011: ix, 31 p. [cited 2017 Jan 17]. Available from: <https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf>
15. Oliveira AC, D'Almeida V, Hipólido DC, Nobrega JN, Tufik S. Sleep deprivation reduces total plasma homocysteine levels in rats. *Can J Physiol Pharmacol.* 2002;80(3):193-7. PMID:11991229. <http://dx.doi.org/10.1139/y02-018>.
16. Pick E, Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J Immunol Methods.* 1980;38(1-2):161-70. PMID:6778929. [http://dx.doi.org/10.1016/0022-1759\(80\)90340-3](http://dx.doi.org/10.1016/0022-1759(80)90340-3).
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227(5259):680-5. PMID:5432063. <http://dx.doi.org/10.1038/227680a0>.
18. Araujo AS, Ribeiro MF, Enzweiler A, Schenkel P, Fernandes TR, Partata WA, et al. Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism. *Mol Cell Endocrinol.* 2006;249(1-2):133-9. PMID:16574313. <http://dx.doi.org/10.1016/j.mce.2006.02.005>.
19. Klein D, Kern RM, Sokol RZ. A method for quantification and correction of proteins after transfer to immobilization membranes. *Biochem Mol Biol Int.* 1995;36(1):59-66. PMID:7545052.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193(1):265-75. PMID:14907713.
21. Repetto MG, Reides CG, Evelson P, Kohan S, de Lustig ES, Llesuy SF. Peripheral markers of oxidative stress in probable Alzheimer patients. *Eur J Clin Invest.* 1999;29(7):643-9. PMID:10411672. <http://dx.doi.org/10.1046/j.1365-2362.1999.00506.x>.
22. Marklund SL, Adolfsson R, Gottfries CG, Winblad B. Superoxide dismutase isoenzymes in normal brains and in brains from patients with dementia of Alzheimer type. *J Neurol Sci.* 1985;67(3):319-25. PMID:3989575. [http://dx.doi.org/10.1016/0022-510X\(85\)90156-X](http://dx.doi.org/10.1016/0022-510X(85)90156-X).
23. Aebi H. Catalase in vitro. *Methods Enzymol.* 1984;105:121-6. PMID:6727660. [http://dx.doi.org/10.1016/S0076-6879\(84\)05016-3](http://dx.doi.org/10.1016/S0076-6879(84)05016-3).
24. Mannervik B, Guthenberg C. Glutathione transferase (human placenta). *Methods Enzymol.* 1981;77:231-5. PMID:7329301. [http://dx.doi.org/10.1016/S0076-6879\(81\)77030-7](http://dx.doi.org/10.1016/S0076-6879(81)77030-7).
25. Salemi VM, Bilate AM, Ramires FJ, Picard MH, Gregio DM, Kalil J, et al. Reference values from M-mode and Doppler echocardiography for normal Syrian hamsters. *Eur J Echocardiogr.* 2005;6(1):41-6. PMID:15664552. <http://dx.doi.org/10.1016/j.euje.2004.06.001>.
26. Yamamoto K, Isa Y, Nakagawa T, Hayakawa T. Involvement of 5-methyltetrahydrofolate in the amelioration of hyperhomocysteinemia caused by vitamin B(6) deficiency and L-methionine supplementation. *Biosci Biotechnol Biochem.* 2013;77(2):378-80. PMID:23391913. <http://dx.doi.org/10.1271/bbb.120661>.
27. Hron G, Lombardi R, Eichinger S, Lecchi A, Kyrle PA, Cattaneo M. Low vitamin B6 levels and the risk of recurrent venous thromboembolism. *Haematologica.* 2007;92(9):1250-3. PMID:17666362. <http://dx.doi.org/10.3324/haematol.11318>.
28. Sofi F, Marcucci R, Bolli P, Giambene B, Sodi A, Fedi S, et al. Low vitamin B6 and folic acid levels are associated with retinal vein occlusion independently of homocysteine levels. *Atherosclerosis.* 2008;198(1):223-7. PMID:17945240. <http://dx.doi.org/10.1016/j.atherosclerosis.2007.09.009>.
29. Vanuzzo D, Pilotto L, Lombardi R, Lazzarini G, Carluccio M, Diviacco S, et al. Both vitamin B6 and total homocysteine plasma levels predict long-term atherothrombotic events in healthy subjects. *Eur Heart J.* 2007;28(4):484-91. PMID:17267459. <http://dx.doi.org/10.1093/eurheartj/ehl470>.
30. DeRatt BN, Ralat MA, Kabil O, Chi YY, Banerjee R, Gregory JF 3RD. Vitamin B-6 restriction reduces the production of hydrogen sulfide and its biomarkers by the transsulfuration pathway in cultured human hepatoma cells. *J Nutr.* 2014;144(10):1501-8. PMID:25165392. <http://dx.doi.org/10.3945/jn.114.196808>.
31. Hsu CC, Cheng CH, Hsu CL, Lee WJ, Huang SC, Huang YC. Role of vitamin B6 status on antioxidant defenses, glutathione, and related enzyme activities in mice with homocysteine-induced oxidative stress. *Food Nutr Res.* 2015;59(1):25702. PMID:25933612. <http://dx.doi.org/10.3402/fnr.v59.25702>.
32. Kuwahara K, Nanri A, Pham NM, Kurotani K, Kume A, Sato M, et al. Serum vitamin B6, folate, and homocysteine concentrations and oxidative DNA damage in Japanese men and women. *Nutrition.* 2013;29(10):1219-23. PMID:23800563. <http://dx.doi.org/10.1016/j.nut.2013.03.014>.
33. Danielyan KE, Simonyan AA. Protective abilities of pyridoxine in experimental oxidative stress settings in vivo and in vitro. *Biomed Pharmacother.* 2016;86:537-40. PMID:28024289. <http://dx.doi.org/10.1016/j.biopha.2016.12.053>.
34. Mani M, Khaghani S, Gol Mohammadi T, Zamani Z, Azadmanesh K, Meshkani R, et al. Activation of Nrf2-Antioxidant Response Element Mediated Glutamate Cysteine Ligase Expression in Hepatoma Cell line by Homocysteine. *Hepat Mon.* 2013;13(5):e8394. PMID:23967023. <http://dx.doi.org/10.5812/hepatmon.8394>.
35. Gutierrez LL, Mazzotti NG, Araújo AS, Klipel RB, Fernandes TR, Llesuy SF, et al. Peripheral markers of oxidative stress in chronic mercuric chloride intoxication. *Braz J Med Biol Res.* 2006;39(6):767-72. PMID:16751982. <http://dx.doi.org/10.1590/S0100-879X2006000600009>.

36. Cheng CH, Huang SC, Chiang TY, Wong Y, Huang YC. Higher plasma pyridoxal phosphate is associated with increased antioxidant enzyme activities in critically ill surgical patients. *Biomed Res Int*. 2013;2013:572081.
37. Sagheb MM, Ostovan MA, Sohrabi Z, Atabati E, Raisjalai GA, Roozbeh J. Hyperhomocysteinemia and cardiovascular risks in hemodialysis patients. *Saudi J Kidney Dis Transpl*. 2010;21(5):863-6. PMID:20814121.
38. Witte KK, Nikitin NP, Parker AC, von Haehling S, Volk HD, Anker SD, et al. The effect of micronutrient supplementation on quality-of-life and left ventricular function in elderly patients with chronic heart failure. *Eur Heart J*. 2005;26(21):2238-44. PMID:16081469. <http://dx.doi.org/10.1093/eurheartj/ehi442>.
39. Sen U, Tyagi SC. Homocysteine and Hypertension in Diabetes: Does PPARgamma Have a Regulatory Role?. *PPAR Res*. 2010;2010:806538.
40. Ruhui L, Jinfa J, Jiahong X, Wenlin M. Influence of hyperhomocysteinemia on left ventricular diastolic function in Chinese patients with hypertension. *Herz*. 2015;40(4):679-84. PMID:24863078. <http://dx.doi.org/10.1007/s00059-014-4098-x>.
41. Sebbag I, Rudski LG, Therrien J, Hirsch A, Langleben D. Effect of chronic infusion of epoprostenol on echocardiographic right ventricular myocardial performance index and its relation to clinical outcome in patients with primary pulmonary hypertension. *Am J Cardiol*. 2001;88(9):1060-3. PMID:11704014. [http://dx.doi.org/10.1016/S0002-9149\(01\)01995-6](http://dx.doi.org/10.1016/S0002-9149(01)01995-6).
42. Das M, Tapadar SR, Mahapatra AB, Chowdhury SP, Basu S. Assessment of RV Function in Patients of (COPD). *J Clin Diagn Res*. 2014;8(3):11-3. PMID:24783066.

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