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Original Article

# *In Vitro* protective effect of vitamin $B_{12}$ derivatives against thimerosal-Induced oxidative stress in human cultured neuronal cells, SH-SY5Y

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ABSTRACT

This study is directed towards investigating the ability of various forms of vitamin B12 to augment intracellular gluthathione (GSH) level as well as studying their possible protective effect against the thimerosal-inuced oxidative stress. SH-SY5Y human neuroblastoma cell culture system was used as a model for this study. Cells were acutely (60 minutes) treated with 3 different vitamin $B_{12}$ derivatives (methyl-, hydroxy- and cyanocobalamin) in the presence and absence of thimerosal, vaccine antimicrobial agent. The results revealed that only methylcobalamin augmented the GSH level, as compared to other forms (hydrorxy- and cyanocobalamin). Also methylcobalamin was the only derivative that showed a protective effect against the thimerosal-induced depletion of GSH. Our findings provide evidence that thimerosal induces oxidative stress in human neuronal cells by depleting GSH and only methylcobalamin protects the cells against such insult and therfore improving the antioxidant intracellular capacity against oxidative stress, a common cause for the pathogenesis of many diseases including, autism.



## 1. Introduction

Methylation reactions involve the transfer of a methyl group to another molecule and this process regulates cell function throughout the whole biological systems of our body. During methylation S-adenosylmethionine (SAM), the major intracellular methyl donor, donated its methyl group to a variety of acceptors, and then is converted to S-adenosylhomocysteine (SAH) by a variety of cellular methyltransferases present in all cells. SAH is subsequently hydrolyzed to homocysteine (HCY) and adenosine by SAH hydrolase (Figure 1).

HCY can either be: (a) recycled to methionine by accepting a methyl group provided by 5-methyl tetrahydrofolate (5-CH3-THF) and vitamin B12 in a reaction that is catalyzed by methionine synthase (MS); (b) methylated to methionine via the addition of a methyl group supplied by trimethylglycine (betaine), in a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT); or (c) utilized for glutathione (GSH) biosynthesis through transsulfuration pathway (Figure 1).

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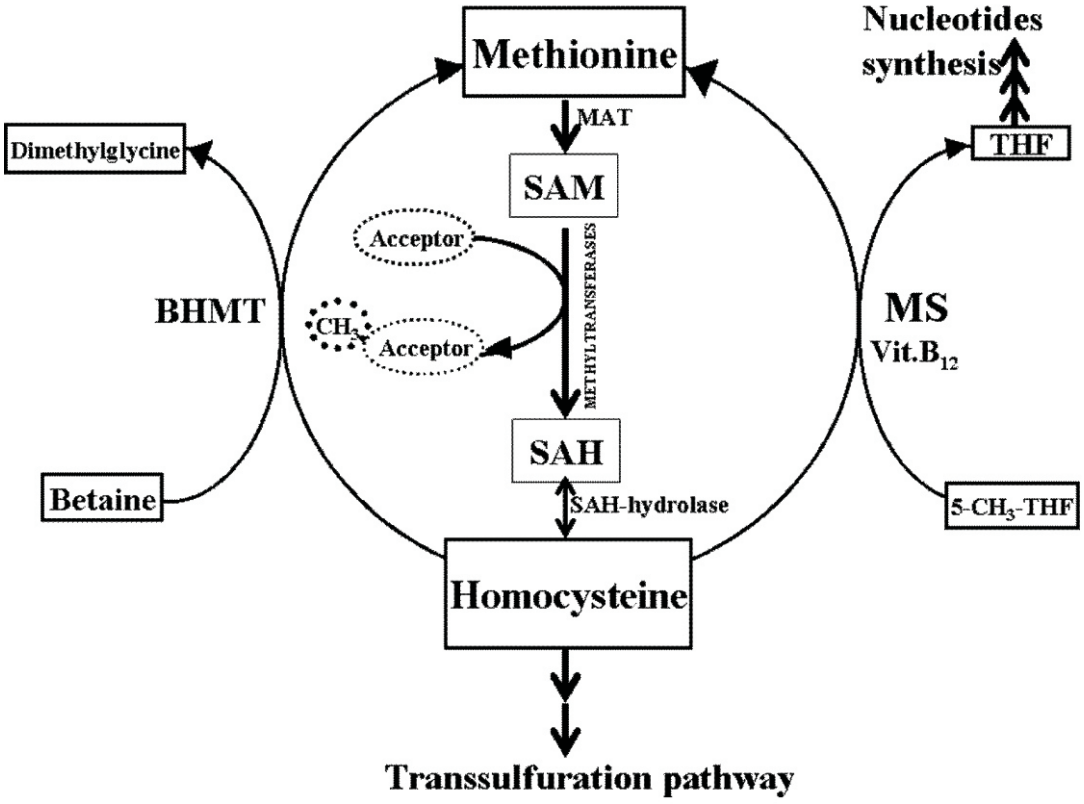
Recent studies show that inadequate folate and/or vitamin $B_{12}$ status or MS inhibition, could results in lower rates of SAM synthesis and hence impaired SAM-dependent methylation reactions with a consequent elevation in the plasma level of HCY [1-6].

It is of particular interest that in the brain, folate and vitamin $B_{12}$-dependent HCY remethylation cycle is the only available pathway for the generation of SAM and methylation of HCY that is because BHMT is not expressed in brain tissue [7]. Therefore in brain, folic acid and vitamin $B_{12}$ are major determinant of both homocysteine and SAM intracellular levels and their sequential metabolic fate.

Figure 1. Simplified scheme of the methylation cycles. Homocysteine (HCY) is converted into methionine by two enzymes: (1) methionine synthase (MS), which utilizes vitamin $B_{12}$ as a cofactor and acquires a methyl group from 5-methyltetrahydrofolate (5-CH3-THF). (2) betaine homocysteine methyltransferase (BHMT), which uses betaine as a methyl donor. BHMT-dependent HCY methylation is only active in peripheral tissues, such as liver, but not the brain. Methionine is further converted to S-adenosylmethionine (SAM) through the activity of methionine asenosyl transferase (MAT). SAM is the major methyl donor for all methyltransferases,which adds methyl groups to various acceptor molecules such as DNA, RNA, phospholipids and proteins. SAM is then converted to S-adenosylhomocysteine

(SAH), which is then reversibly converted to HCY in a reaction catalyzed by SAH-hydrolase. HCY is either remethylated back to methionine (as mentioned above) or flushed into transsulfuration pathway for the biosynthesis of GSH.




SAM-dependent methylation reactions are extensive and are required for the biosynthesis of a variety of cellular components including creatine, epinephrine, carnitine and membrane phospholipids and for regulatory modifications, methylation, of nucleic acids (DNA) [8,9]. DNA methylation is an epigenetic mechanism that is required for normal gene expression and transcription [10]. Generally in normal cell, the lack of methylation of certain nucleotides is a prerequisite for active transcription, meanwhile methylation of these nucleotides cause a transcriptional repression, inhibition of gene expression [11-12].

GSH is the major intracellular antioxidant and it undergoes oxidation to the disulfide form (GSSG), oxidized form, when scavenging reactive oxygen species (ROS) that are generated endogenously (from normal cellular metabolism) or from exogenous sources (such as ozone, smoking, ultraviolet light, trans fatty acids and other environmental insults). ROS are highly unstable molecules and they react readily with biological molecules, such as lipids, proteins, DNA and cell membrane phospholipids causing irreversible damage, which may lead to the development of many diseases.

Cellular Oxidative stress is rises, when the amount of endogenous antioxidants is not counterbalancing the amount of ROS. An Example of oxidative stress condition is low intracellular GSH level And high cellular level of GSSG, as represented by low ratio of GSH/GSSG.

The efficiency of HCY-dependent transsulfuration reactions, including GSH biosynthesis, varied upon the variation in the vitamin $B_{12}$ cofactor derivatives. Hyperhomocysteinemia may be considered as a functional indicator of folate and vitamin $B_{12}$ status [13]. Recent epidemiological evidences suggested that hyperhomocysteinemia, low serum levels of folate and vitamin B12 were associated with increased risk of occlusive vascular disease, stroke, thrombosis and cognitive dysfunction [14-16].

Several studies have found that certain compounds have an inhibitory effect on MS activity, these include methyl mercury [17] , nitrous oxide [17,18] , ethanol [19] and a series of cobalamin analogues [20,21], as well as the vaccine preservative, thimerosal [22]. Such inhibition might decrease the methionine-cycle turn over and sequentially low amount of HCY for biosynthesis of GSH.

The mercury-containing compound thimerosal has been suggested as a possible cause of autism [23-25]. Autism is a neurodevelopmental syndrome that most commonly shows itself during the second and third year of life. Autistic children have impaired social interaction and communication, failure to respond to stimuli and can exhibit repetitive actions.

The current study was undertaken to elucidate the effects of different vitamin $B_{12}$ derivatives (cyano, hydroxy, and methylcobalamin) on modulating the GSH intracellular content in human cultured neuronal cells and their possible protective action against the thimerosal-induced GSH depletion in human cultured cells.

## 2. Materials and Methods

The study design was to acutely incubate, 60 minutes, the human neuroblastoma cells in cell culture media supplemented with methylcobalamin, hydroxycobalamin or cyanocobalamin in the presence or absence of thimerosal. Thereafter GSH measurements will be conducted.

### *2.1. Materials*

Vitamin $B_{12}$ derivatives (hydroxy, cyano and methylcobalamin), alpha-modified Minimal Essential Medium (α-MEM) cell medium and thimerosal drug were purchased from Sigma Chemical Co., St. Louis, MI, USA

### *2.2. Cell Culture*

SH-SY5Y human neuroblastoma cells were purchased from European Collection of Cell Cultures (ECACC, Cat.No.94030304) and were frozen in liquid nitrogen before culturing. Cells were grown as monolayer in 10 cm Petri dishes that contain 10 ml of α-MEM supplemented with 1% penicillin-streptomycin-fungizone (PSF) and 10% fetal bovine serum (FBS). Cultured cells were placed in an incubator chamber containing humidified 95% air and 5% $CO_2$ at 37°C. For passage, confluent cells were detached with 1.5 ml trypsin-EDTA solution. They were then re-suspended in 12 ml of fresh medium and seeded at 100,000 cells per well in six well culture plates and incubated for 24 hrs before conducting the desired experiment.

### *2.3. GSH Assay*

At a predetermined day for the experiment, cells were acutely incubated for 60 minutes with 1 mM of one of the different vitamin $B_{12}$ derivatives in the presence/absence of 1nM thimerosal drug.

After 60 minutes treatments, cells were scraped, pelleted and approximately 108 cells were re-suspended in 1 ml of 100 mM phosphate buffer, pH 7.4. Cell membranes were disrupted by sonication on ice and the cell homogenate centrifuged at 4°C, 10,000 g for 10 minutes, supernatant (cell lysates) was separated from cell debris and a 100 µL aliquot of supernatant was transferred to a fresh tube and 2 µL of monochlorobimane (25 mmol/L) and 2 µL of glutathione-S-transferase reagents were added, as provided by a commercial kit (ApoGSH detection kit, BioVision, Inc, Cat.No.K251-100). After a 30 min incubation at 37°C, fluorescence was read at 380/460 nm. GSH content was determined by comparison with values from a standard curve using freshly prepared GSH. Data was normalized to the protein content of cell lysates, using the Lowry protein Assay [26].

### *2.4. Statistical Analysis*

Results are expressed as mean + standard error of the mean (S.E.M.) of six independent observations. Statistical analyses were conducted using GraphPad Prism software, version 4, Student's t-test was used to compare between means and $p < 0.05$ was considered to be significant.

## 3. Results and Discussion

Previous biochemical studies reported that 1 mM was the effective dose (ED50) of vitamin $B_{12}$ for the MS-methionine mediated cycle [27-30]. Therefore, in the current study, neuronal cell cultures were acutely (60 minutes) treated with 1 mM of different vitamin $B_{12}$ derivatives. Table (1) reveals that methylcobalamin caused two folds significant increase in GSH intracellular level at a concentration of 1mM, as compared to control, untreated cells ($t = 5.49$, $p<0.001$). Neither hydroxo- nor cyanocobalamin at concentrations of 1 mM induced any significant changes in GSH level as compared to control ($t = 0.01$, $p>0.05$).

**Table 1. Intracellular GSH measurement in SH-SY5Y human neuroblastoma cells**

| Treatment | GSH level nmol/mg protein |
|---|---|
| Control (nontreated) | 10.42 + 1.2 |
| Cyanocobalamin (1 mM; 60 min) | 10.39 + 1.7 |
| Hydroxycobalamin (1 mM; 60 min) | 10.43+ 1.4 |
| Methylcobalamin (1 mM; 60 min) | 20.98 + 1.5* |
| Thimerosal (10 nM; 60 min) | ND |
| Thimerosal + Cyanocobalamin | ND |
| Thimerosal + Hydroxycobalamin | ND |
| Thimerosal + Methylcobalamin | 10.11+ 0.9 |

Results are the mean S.E.M. of six independent observations.
*Statistically significant as compared to control group, $p<0.001$
ND = no detectable GSH level.

Waly et al. [24], showed that neurodevelopmental toxins such as lead, mercury and thimerosal, in a concentration as low as 1 nM, reduced MS-mediated methionine cycle enzyme activity to zero. Thus, the same concentration was used in this study to observe the effect of various derivatives of vitamin $B_{12}$ against the inhibitory effect of thimerosal on HCY-dependent MS-transulfuration cycle.

As demonstrated in table (1), 60 minutes incubation of the cells with 1 nM thimerosal depleted the intracellular GSH contents of the neuronal cells to a non-detectable level and this finding is consistent with the above mentioned study [24]. Also, when the cells were incubated, for 60 minutes, in concomitant with 1 nM thimerosal and 1mM of different vitamin $B_{12}$ derivatives, only methylcobalamin provided a protective effect against the thimerosal-depleting effect, and methylcobalamin- restoring effect was at a comparable level as that of the control group (10.11 + 0.9 nmol/mg protein). Meanwhile hydroxycobalamin and cyanocobalamin failed to show any protective effect.

Collectively, the preceding results suggest that methylcobalamin was the only vitamin $B_{12}$ derivatives that augments intracellular GSH even in the presence of the neurodevelopmental toxin, thimerosal.

Our study show that methylcobalamin modulates GSH in a mechanism that might provides methyl groups to the MS enzyme and hence the increase of rate of methionine-cycle and subsequent generation of more SAM and HCY, precursor of GSH.

We suggest that methylocbalamin supplementation improve the redox satus of the neuronal cells and decreasing the oxidative stress under various insults. In support for our suggestion, a recent study reported that supplementation of methylcobalamin improve the cognition and intellectual thinking of autistic children [31]

The potent depletion of GSH by thimerosal might contribute to oxidative stress-neurodevelopmental toxicity by limiting HCY transulfuration into GSH. Therefore, at least a portion of the thimerosal neurological toxicity might be mediated by depleting GSH intracellular content of neuronal cells and its consequences on cellular oxidative stress and cell death.

## 4. Conclusion

In conclusion, in the studied human neuronal cells population, our findings provide new insights into a direct modulating role of methylcobalamin over GSH biosynthesis eventually by being a significant source of methyl groups for HCY remethylation and transulfuration. Thimerosal potently interferes with HCY-dependent methylation and transulfuration pathways and depletes the intracellular GSH.

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