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Effects of multivitamin B containing vitamins B6, vitamin B12 and folic acid on age- associated changes of rat brain glutamate metabolism

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***Abstract*—Glutamate (Glu) acts as an excitatory neurotransmitter in brain. Excessive levels of Glu in the brain are cytotoxic and lead to several neurodegenerative processes. Rapid removal of the released Glu in synaptic cliff may prevent the excessive excitation of Glu receptors. Several enzymes such as glutamine synthetase (GS) glutamate decarboxylase (GAD) and aminotransferases (GOT and GPT) are important for maintaining Glu concentrations below excitatory levels in the synaptic cleft. Since multivitamin B supplementation has been recommended as adjunctive treatment in Alzheimer's disease, this study was undertaken to investigate, .the efficacy of vitamins B6 and vitamin B12 and folic acid on the activities of GS, GAD, GOT and GPT in aging rat brain. Male Wistar rats (3 and 30 months old) were used. The animals were injected with vitamins B6 and vitamin B12 and folic acid (10mg/Kg/day) for 30 days and the day after the last injection the animals were killed by decapitated. Forebrains were homogenized in phosphate buffer and the activities of the enzymes were measured in the supernatant. The enzyme activities in aged rat brain were considerably lower compared to young animals. Vitamin B6 induced activation of GAD, GOT and GPT in both ages, but, the differences were more pronounced in aged animals. Vitamin B12 and folic acid stimulate the activity of GS in both young and old animals, but had lintel effects on GAD, GOT and GPT of both ages. It is concluded that Glu metabolism might be considered as a therapeutic target for prevention of neurodegenerative disorders and age related symptoms.**

Keywords*— *Aging, Alzheimer's disease, Glutamate, Multivitamin B, Neurotransmitter

I. Introduction

The aging brain is characterized by increased risks for degenerative diseases exhibiting varying degrees of brain biochemical changes. Numerous metabolic dysfunctions have been reported to occur in aging brain. A number of evidences are in favor of significant changes in several major neurotransmitters [1, 2]. The amino acid glutamate (Glu) is known as an excitatory neurotransmitter which interacts with N-methyl-D-aspartate (NMDA) receptors for basal excitatory synaptic transmission. Gila also serves as the immediate precursor of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). As a result of this, Glu/GABA homeostasis is quite complex, involving several cell specific elements, including membrane transporters and enzymes in both neurons and astrocytes [3]. Neurophysiological studies indicated that Glu causes many forms of synaptic plasticity such as long-term ostentation and depression, which are thought to influence learning and memory [4, 5]. However, excessive levels of extracellular Glu in the nervous system are excitotoxic and lead to neuronal death and several neurodegenerative processes [6, 7]. Several lines of evidence suggested that increased extracellular Glu, can give rise to many potentially damaging mechanisms which may be pathologically important. Of particular interest are the beneficial therapeutic effects of Glu receptor antagonists in Alzheimer's disease [8]. While there is little doubt that the high level of Glu is neurotoxic, diminutive evidence points towards the enzymatic control of Glu metabolisms of the brain. Several enzymes are involved in the removal of Glu from synaptic regions; glutamine synthetase (GS), which brings nitrogen into metabolism by condensing ammonia and glutamate, with the aid of ATP, to yield glutamine [9]. Except for trace amounts, this enzyme is mainly located in astrocytes [10]. Glutamate decarboxylase (GAD) that catalyzes the conversion of Glu to GABA, which acts as the main inhibitory neurotransmitter in the brain [11], and aminotransferases which change Glu to alpha- ketoglutarate. This can then enter the citric acid cycle for further metabolism. Age related decreases in the expression and or activities of these enzymes in the brain have been very well established [12, 13]. The finding, which shines a spotlight on these enzymes only recently thought to play a role in the biochemistry of "anti-aging," has attracted the interest of several groups seeking to study vitamins that delay the aging process and age-related diseases. It is believed that vitamin deficiencies could influence memory function and might contribute to age-associated cognitive impairment and dementia. [14]. Folic acid and vitamin B12 are vitamins essential to the development of the central nervous system [15,

16]. Recently we have reported that the activity of GAD in aged rat brain was 54% lower than that of young animals, which could be reactivated to the levels equivalent to young animals by administration of vitamin B6 [17]. Vitamin B6 acts as co-enzyme for GAD and aminotransferases and vitamin B12 and folic acid are also involved in the biosynthesis of a wide variety of biological substances, including DNA, proteins, phospholipids, and neurotransmitters, thereby regulating their function [18]. Despite being widely consumed, the effects of multi-vitamin supplements on enzymatic activities on Glu metabolism and thus its regulation have received little research attention. This study was therefore conducted to examine the effects of vitamin B6, vitamin B12 and folic acid administration on the brain GAD, glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT)) in young and old rats.

II. Materials and Methods.

Animals; Male Wistar young rats (3 months old) with weight ranging from 200 to 250 g and old rats (30 months old) with weight ranging from 650 to 720 g were used. Animals were maintained with respect to the animal welfare regulation in animal house until the desired age was attained. Each group were assigned to 3 treatments subgroups: (I) vitamin B6 rats; (II) vitamin B12 rats; and (III) foliate rats. Vitamin B6, vitamin B12, and folic acid in saline were injected in doses of 10 mg/kg /kg body weight /day for a period of 30 days. Control groups of young and aged animals were injected with saline only. The control, and experimental groups were housed (6 rats in each subgroup) in a regulated environment (25 ± 1℃; 50 to 55 % relative humidity; 12 h light/dark cycle), with free access to food and water. The day after last injection the animals were killed by decapitation after anesthesia and the forebrains were carefully removed and placed on a Petri dish over crashed ice and chopped into the consistency of mince, which was rapidly transferred into 8- 14 ml phosphate buffer (pH 7.4) containing 25 mM potassium phosphate, 1mM EDTA, 0.1 mM PMST, 1% and 1% aprotonin, and 0.5% Triton X-100, and homogenized in crashed ice, to give a 10% (w/v) homogenate. This homogenate was centrifuged for 20 min at 70000 g and the enzyme activities were measured in the clear supernatant. Protein concentrations were determined by the method of Lowry et al. [19] with BSA as the standard.

Enzyme assays; Activity of GAD in the rat brain supernatant was measured by a fluorimetric method of Messripour [20]. Briefly, 100 µl aliquots of the supernatant with the protein concentration of 8-10 mg /ml were transferred in the fluorimetric cuvet (LSE specrrophotofluorimeter, Perkin-Elmer, Norwalk, CT) containing 1ml phosphate buffer (pH7.4) and pre-incubated in for 5 min at 37°C. The reaction was started by addition of 100µl Glu solution (10 mM) and increasing of fluorescence intensities (ΔF) was monitored at the excitation wavelength of 495 nm and emission wavelength of 540 nm, for 15 min against a blank containing all components except Glu. The results are expressed as ΔF /min/mg protein.

The specific activity of GS was measured in the brain supernatant by a colorimetric assay based on the catalysis of gamma glutamylhydroxamate from Glu and hydroxylamine (21). The basic reaction mixture contained 80mM-Tris-acetate pH7.4, 40mM-hydroxyalanine, 20 mM-MgSO4, 20mM-mercaptoethanol and 10 mM-ATP (Sigma chemical Co). Ouabain (0.5mM) and oligomycin (1.6ug/ml) were added as ATPase inhibitor. The assay started by addition of 100µl Glu solution (10 mM) to 1ml of assay mixture and incubated 15min at 37. The reaction was stopped by adding 1,5ml ferric chloride reagent and changes of optical density (OD) at 575nm was monitored chlorimetrically, against a blank containing all compounds except Glu. The results are expressed as ΔOD /min/mg protein. The activity of GPT and GOT were measured in the supernatant by diagnostic kits (Pars Azmun Co, Tehran, Iran).

Statistical analysis; The obtained data were subjected to statistical analysis using SPSS software (version 18). In all cases, the one-way analysis of variance (ANOVA) was used to compare the mean of each group with the control group. The LSD complementary test was conducted to elucidate the exact differences at p-value lower than 0.05. Data are presented as mean ± SD for all cases.

III. Results

The specific activities of GAD, GPT, GOT and SG in rat forebrains supernatant of 3 and 30 month old rats are summarized in *Table 1*. The activities of the enzymes in aged rat brain were significantly lower than corresponding enzyme in the brain of young animals ($P<0.05$). As has been indicated previously (17), administration of vitamin B6 in doses of 10 mg/Kg body weight stimulated the GAD activity in the samples in both young and old rats more than 2 and 3 fold respectively *(Fig. 1)*.

Table 1. Specific activities of GAD, GPT, GOT and GS in the supernatant of young and old rat brain

Enzymes	3mo. rats	30mo. rats
GAD	137.4 ±11.2	63.3±4.6*
GPT	22.4±1.6	11.2±0.7*
GOT	40.1±10.9	25.8±1.7*
GS	56.2 ± 5.0	24.5 ±2.3*

The specific activities of GAD, GPT, GOT and GS were assayed in the brain supernatant. Results are expressed as mean ±SD of 6 separate experiments. *The differences are statistically significant at $P<0.05$*.

Similarly, vitamin B6 increased activity of the GPT and GOT of young rats by about 27% and 30%, but activation in old animals were about, 60 and 80% respectively. The differences are significant at a level $P<0.05$, compared with respective control values. However, as indicated in *Fig. 1* vitamin B6 has little effect on SG activity of both young and old animal brains. Administration of vitamin B12 (10 mg/kg) increased the activities of GAD (18%), GOT (19%), GPT (27%) and GS (41%) of the brain of young animals, whereas the rate of activation was lower in aged rats. The results are shown in *Fig.2.* Higher activations were observed in the brain enzymes of young rats as compared to those of older animals, though statistically insignificant. *Fig. 3* shows the percent activation of the examined enzymes by folic acid. The percent of activation of GS in both young and old rat brain (approximately %50) was significantly higher when compared to other examined enzymes (about 20%). However, the rate of activations were not different when in young and old animals rats as compared to those of young animals. Lower activities of these enzymes indicated lower rate of Glu metabolism, that might be interpreted as being consistent with the accumulation of Glu in the bran synaptic regions of glutmitergic neurons [i.e. 13, 14].

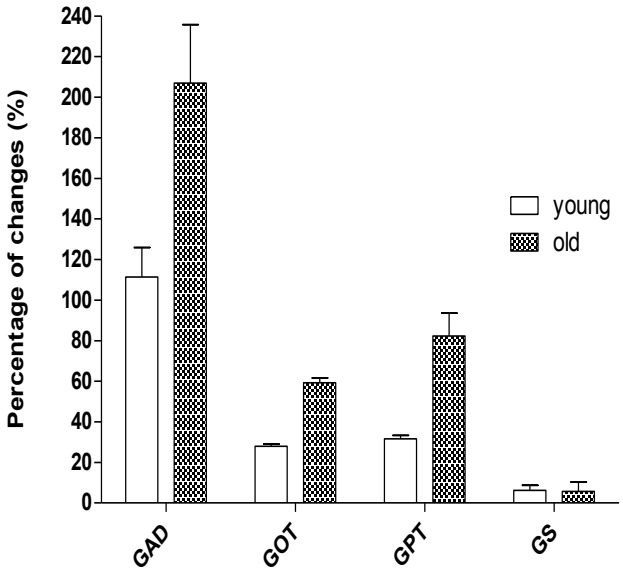

Fig.1: Percent change in the activities of GAD, GOT, GPT and GS of rat brain following administration of vitamin B6 in young and old rats. Results are expressed as mean ±SD of 6 separate experiments of the treated animal over the respected control values of the same age.

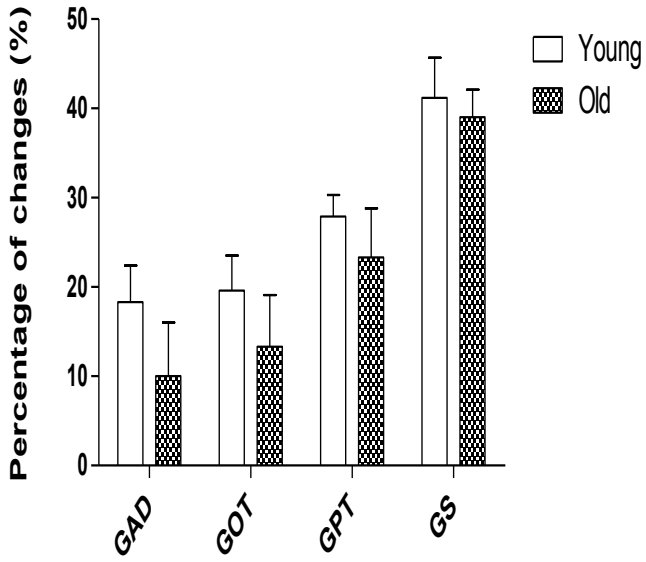

Fig 2: Percent change in the activities of GAD, GOT, GPT and GS of rat brain following administration of vitamin B12 in young and old rats. Results are expressed as mean **±SD** of 6 separate experiments of the treated animal over the respected control values of the same age.

IV. Discussion

The first part of this in vivo study sought to measure the specific activities of GAD, GPT, GOT and SG in the rat brain as a function of the age. The results indicate that the activities of these enzymes are significantly lower in the brain of old

In situations where there is high excitatory neurotransmitter activity, the brain typically responds with an increase in the inhibitory GABA activity as well. However, in aged brain, the slows down GABA neurotransmission may occur essentially by decreasing GAD activity. Under normal conditions, normal levels of GABA are sufficient to maintain control of the excitatory stimuli. But, if GABA function is impaired, then higher levels of Glu accumulation can cause excitotoxicity. Evidence from clinical studies indicated that vitamin B6 plays a role in cognitive development through the biosynthesis of certain neurotransmitters [15, 22]. It is very well known that the enzymes GAD, GPT and GOT but not SG use vitamin B6, in the form of Pyridoxal 5'-phosphate (PLP) as cofactor. Since, vitamin B6 increased the activities of GAD, GPT and GOT in both ages, it appears that in the active site of these enzymes PLP is at suboptimal levels. However, the rate of activation of the enzymes in the brain of aging rats was considerably greater than in young animals. Although provided data is not adequate to suggest about the interaction of PLP with the active sites of these enzymes at the molecular levels, it seems that the greater activation of GAD, GPT and GOT in the aged rat brain by administration of vitamin B6 might be resulted from either; lower availability of vitamin B6

in aged animals, or; the lower affinity of these enzymes for PLP. The latter is likely to be related to the posttranslational modifications of the proteins as a consequence of aging [23, 24)]. The result provided synergistic support for the suggestion that PLP is an important cofactor involved in the natural synthesis of GABA, to balance between inhibition and excitation, which are known to alter GABAergic or glutamatergic neurotransmission [24, 25]. In addition, extracellular Glu accumulation is reported to cause a deficiency in GS activity in astrocytes [26], thus the removal of Glu from extracellular regions may be taken as a possible indirect effect of vitamin B6 on GS activity as well.

The associations between administration of vitamin B12 and acid folic on the activities of the rat brain GAD, GPT, GOT and GS, observed in the present study is in accord with the biochemical studies that vitamin B12 and folic acid are encompassed in the biosynthesis of DNA, proteins and neurotransmitters [18]. The results are also consistent with epidemiological and clinical data that persons with low vitamin B12 and folic acid levels are at increased risk of Alzheimer's disease [27, 28, 29]. The data suggested that restoring the Glu enzymatic machinery in aging to the levels equivalent to young animals might prevent the Glu neurotoxicity. In conclusion, Glu metabolizing enzymes might be considered as a therapeutic target for prevention of neurodegenerative disorders and age related symptoms.

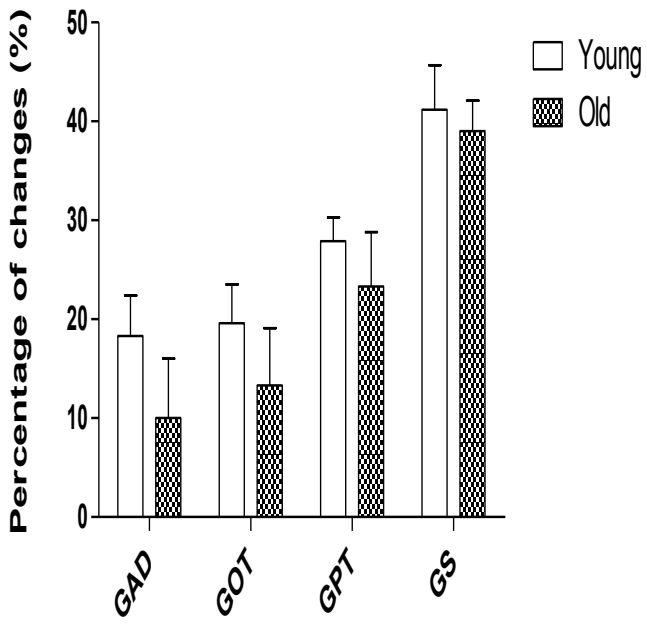

Fig 2: Percent change in the activities of GAD, GOT, GPT and GS of rat brain following administration of vitamin B12 in young and old rats. Results are expressed as mean **±SD** of 6 separate experiments of the treated animal over the respected control values of the same age.

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