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Full Length Research Paper

Betaine reduction of hyperhomocysteinemia and enhancement of 5-hydroxyindoleacetic acid in ethanol-induced hyperhomocysteinemia in rabbits

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Hyperhomocysteinemia is a hypothesis for the association of homocysteine with cerebrovascular diseases, neurodegenerative diseases and depression of mood. Thus, we examined whether oral betaine can act as a preventive agent in ethanol-induced hyperhomocysteinemia on the monoaminergic system. A total of 32 New Zealand White rabbits were divided into four groups (n=8) among which is the control group (C) . The ethanol group (E) was administered ethanol at a dosage of 4 g/kg daily. The betaine group (B) received betaine at a dosage 1.5% (w/w) of the diet daily, and the betaine and ethanol group (B and E) was administered with the betaine group diet; after one hour the rabbits received ethanol at a dosage of 4 g/kg daily. Blood samples were taken in the morning of the day before beginning treatment (0.0 day) and on the 30th, 60th and 90th day of the treatment. Serum folate and vitamin B12 levels were determined using a radioimmunoassay, total plasma homocysteine (tHcy) level was determined by homocysteine EIA kit, and 5- hydroxyindoleacetic acid (5-HIAA) of plasma was measured with HPLC-ECD. There was a significant negative correlation between 5-HIAA and tHcy in the E group (r=-0.473, P=0.02), and compared to the E group the concentrations of 5-HIAA in the B and E group increased considerably (p<0.05). In contrast to the E group, significantly high concentrations of 5-HIAA were observed in the B and C groups. While the serum concentrations of vitamin B12 showed no significant difference in the B and E group on the 90th day compared to the control group, the serum concentrations of folate on the 90th day differed significantly (p<0.05). However, no significant difference was observed between tHcy and gender. Overall, oral pretreatment with betaine significantly prevented ethanol-induced hyperhomocysteinemia, subsequently increasing 5-HIAA in the plasma as well as vitamin B12 and folate in the serum. Thus, betaine may be recommended as a pretreatment method for depressive patients with alcoholism.

Key words: Betaine, hyperhomocysteinemia, 5-HIAA, ethanol, vitamin B12, folate.

INTRODUCTION

Chronic alcoholism leads to elevated plasma homocysteine levels, as shown by clinical investigations and animal experiments (Bleich et al., 2004). Homocysteine, a metabolite of the essential amino acid methionine, can be either remethylated to methionine by enzymes that require folate or cobalamin or catabolized by cystathionine -synthase, a pyridoxine-dependent enzyme, to form cysteine (Figure 1) (Kruman et al., 2000). The formation of methionine from homocysteine can occur either via betaine or via 5-methyl-tetrahydrofolate (MTHF). Animal studies have shown that both pathways are equally important and betaine is a vital methylating agent (Craig, 2004). Liver betaine homocysteine methyl transferase (BHMT) concentrations increase when rats are fed diets supplemented with betaine or choline, showing an adaptive change in the

catabolism of betaine (Finkelstein et al., 1983). Elevated total homocysteine (tHcy) level has been observed as a result of chronic alcohol consumption in rats and ethanol has altered sulfur amino acid metabolism, including decreased conversion of methionine to S-adenosylmethionine (SAMe) and homocysteine to methionine (Cravo et al., 1996).

Folate and homocysteine are related through the one-carbon cycle, which involves the production of S-adenosyl methionine from adenosine triphosphate and methionine. SAMe, which is uniformly distributed in the brain, serves as the major donor of methyl groups required in the synthesis of neuronal messengers and membranes (Papakostas et al., 2005). Folate and vitamin B12 deficiency, hyperhomocysteinemia and the T677 allele of the methylenetetrahydrofolate reductase (MTHFR) gene, which cause impaired methylation reactions in the central nervous system, have been associated with depressive disorders (Kim et al., 2008). In addition, methyl folate has been proved to have an antidepressant effect and correlates with cerebrospinal fluid 5-hydroxyindoleacetic acid (5-HIAA) (Atmaca et al., 2005). Patients with severe hyperhomocysteinemia exhibit a wide range of clinical manifestations including neurological abnormalities such as mental retardation, cerebral atrophy, and depression (Kruman et al., 2000). Monoaminergic abnormalities have been implicated in the pathophysiology of depression and alcoholism. For example, lower cerebrospinal fluid (CSF) 5- HIAA levels with alcoholism are associated with higher lethality of suicide attempts in major depression (Sher et al., 2007).

It is well known that alcoholism is associated with altered CSF monoamine metabolite levels. The reduction of serotonin metabolite, 5-HIAA, has been observed in the serum samples of depressive patients (Bose et al., 2004). Taking the above into consideration, we hypothesized that the oral administration of betaine prior to ethanol can act as a methylating agent to increase the level of 5-HIAA in ethanol-induced hyperhomocysteinemia in rabbits. We also investigated how plasma tHcy varied with concentrations of vitamin B12 and folate.

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Abbreviations: 5-HIAA, 5- hydroxyindoleacetic acid; **tHcy**, total homocysteine; **HPLC-ECD**, high performance liquid chromatography-electrochemical detector; **MTHF**, 5-methyl tetrahydrofolate; **BH4**, tetrahydrobiopterin; **BHMT**, betaine-homocysteine methyltransferase; **DHFR**, dihydrofolate reductase; **HVA**, homovanillic acid; **MTHFR**, methyltetrahydrofolate reductase; **NMDA**, N-methyl-D-aspartate; **SAMe**, S-adenosyl methionine; **SAH**, S-adenosyl homocysteine; **SAHH**, S-adenosyl homocysteine hydrolase; **CSF**, cerebrospinal fluid; **5HT**, serotonin; **CBS**, cystathionine beta-synthase; **GSH**, glutathione; **DMG**, dimethyl glycine; **MS**, methionine synthase; **MAT**, methionine adenosyl transferase.

MATERIALS AND METHODS

Alcohol (Ethanol 95%) and 1-octanesulfonic acid sodium salt were from Merck Chemical Company (Merck, Darmstadt, Germany). Betaine (Betafin® 96%) was obtained from Biochem Company (Lohne, Germany). 5-Hydroxyindoleacetic acid was purchased from Sigma (St Louis, MO, USA). SimulTRAC-SNB Radioassay kit vitamin B12 [57Co]/Folate [125I] was prepared by MP, Biomedical, LLC (CNI Pharmaceutical, USA) and the homocysteine kit was prepared by Axis® Homocysteine EIA (Axis-Shield AS, Germany). All other chemicals used were of analytical grade.

Animals and experimental design

All animal experimentation procedures were approved by the Institutional Animal Care and Use Committee of Shiraz University of Medical Science. A total of 32 adult New Zealand White rabbits (2.0 - 2.5 kg) obtained from the animal house of Shiraz University of Medical Science were housed under standard conditions of temperature (23±2°C) and illumination (12-h light–dark cycle). They were provided with standard chow diet (average 50 g/kg), and water *ad libitum* in 2-week of acclimation period. Then, animals were divided into four groups; the first group (Control) received standard chow diet. Second group (Ethanol) were administered with ethanol with a dosage of 4 g/kg per 500 ml water daily plus standard chow diet. Third group (Betaine), received the standard chow diet, plus betaine with a dosage 1.5% (w/w) of the diet soluble in water daily by using gavage and fourth group (Betaine and Ethanol) administered with the Betaine group diet, after one hour rabbits received ethanol with a dosage of 4 g/kg per 500 ml water daily (pretreatment method) (Ji and Kaplowitz, 2003; Song et al., 2003). Each group consisted of 8 (male and female) animals. The total period of study was 90 days. Weight gains and food consumption was determined at weekly intervals.

Blood samples and biochemical analyses

Blood samples were collected from the rabbits in a fasting state and were taken from the marginal ear vein in the morning of day before beginning treatment (0.0 day) and the 30th, 60th and 90th days of the treatment. 1.0 ml of whole blood were drawn into tubes of ethylenediamine tetra-acetic acid (EDTA), centrifuged, separated into plasma aliquots and the remaining whole blood was placed in another tubes and collection of serum assessed in micro tubes. Serum and plasma aliquots stored at -70°C until analysis. Serum folate and vitamin B12 levels were determined using a radioimmunoassay; (SimulTRAC-SNB Radioassay kit vitamin B12/Folate). In short, the sample volume used was 200 µl diluted serum (1/8). All experiments were performed in duplicate and the linearity of dilution that was used was 100 and 110% for vitamin B12 and folate analyses respectively. The intra- and inter-assay coefficients of variations for the determination of folate were less than 5.8 and 8.9%; and for vitamin B12 they were less than 6.2 and 7.5%, respectively (Ferrucci et al., 2007; Golbahar et al., 2005). Total plasma homocysteine level was determined by Axis® Homocysteine EIA kit. In brief, the sample volume used was 25 µl. Absorbance was measured at a wavelength of 450 nm using ELISA reader (STAT FAX 2100, USA). All estimations were performed in duplicate and the intra-assay coefficient of variation was <10% and the detection limit of the tHcy assay was 2.0 µM/l (Golbahar et al., 2005; Karthikeyan et al., 2007).

Determination of 5-HIAA

Plasma 5-hydroxyindoleacetic acid concentrations were measured

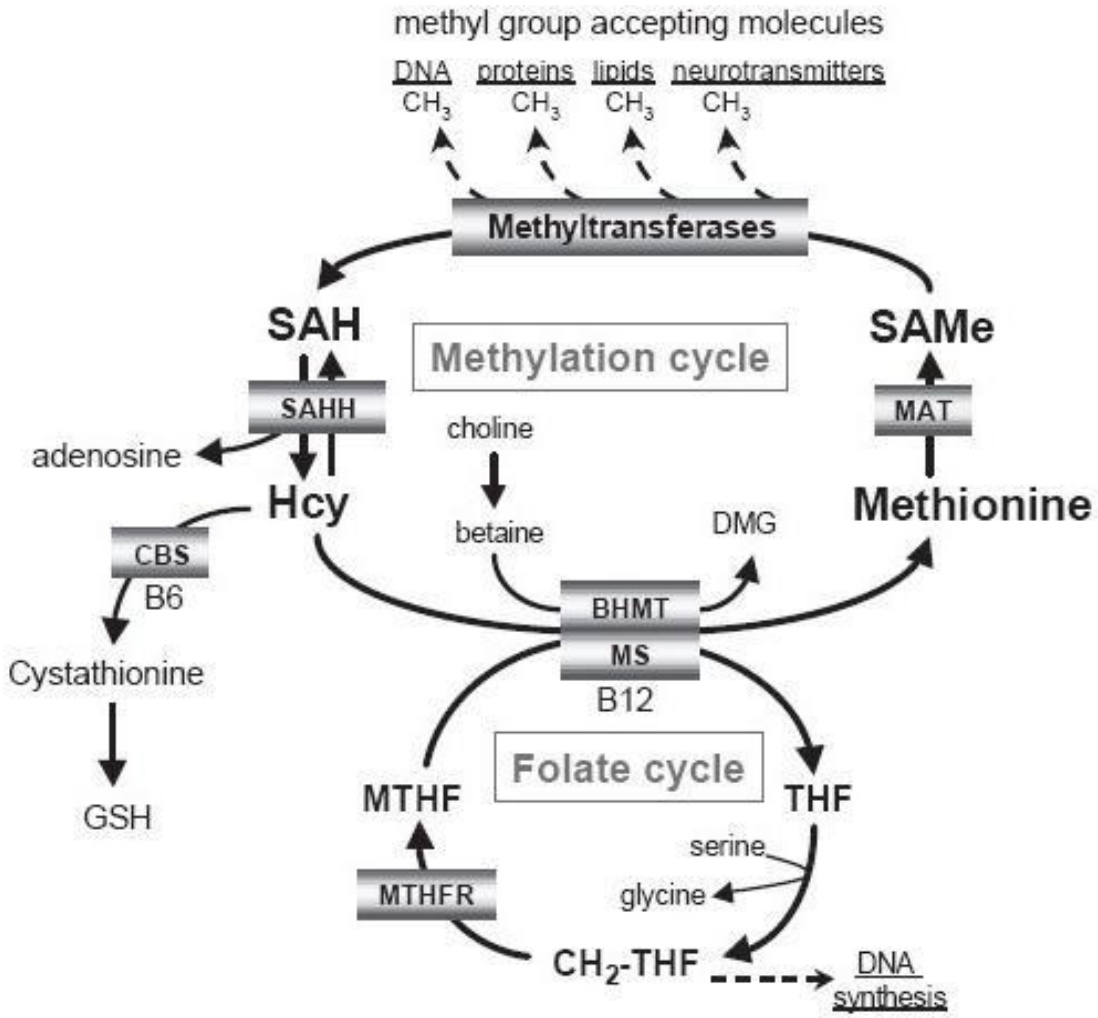

Figure 1. Homocysteine metabolism- Homocysteine has three main metabolic fates: to be remethylated to methionine, to enter the cysteine biosynthetic pathway, and to be released into the extracellular medium. Hcy, homocysteine; CBS, cystathionine beta-synthase; GSH, glutathione; DMG, dimethyl glycine; MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; MTHF,5-methyl tetrahydrofolate; MTHFR, methyltetrahydrofolate reductase; THF, tetrahydrofolate; MAT, methionine adenosyl transferase; SAMe, S- adenosyl methionine; SAH, S-adenosyl homocysteine; SAHH, S- adenosyl homocysteine hydrolase. (Bottiglieri, 2005). Bottiglieri T. Homocysteine and folate metabolism in depression. Progress in Neuropsychopharmacology and Biological Psychiatry (2005); 7:1103-1112. Bottiglieri T. Homocysteine and folate metabolism in depression. Progress in Neuropsychopharmacology and Biological Psychiatry 2005; 7:1103-1112

by high-performance liquid chromatography with electrochemical detection (Chi et al., 1999). In short, The HPLC system consisted of a Constametric1000 pump (Knauer, Germany), a manual Rheodyne7725 injection valve equipped with a 20- l loop, a 3 mm particle size (250 × 4.6 mm, I.D.) with C18 analytical column (Knauer, Germany). End-point detection was achieved with an Introamperometric detector (EC3000, GmbH, Germany) . The operating potential was 0.75 V. The mobile phase consisted of 0.1 M KH_2PO_4 acetonitrile (84:16, v/v) and 1-octane sulphonic acid (100 mg/l) adjusted to pH 4.75 (with 0.5 M K2HPO4). The flow-rate was 1.0 ml/min. Peak height rather than area in the chromatography was normally measured. Concentration of 5-HIAA was calculated by interpolation of its standard curve. Working standards for the assay were prepared using the mobile phase as the diluent and consisted of six concentration points over the range 2 - 32 ng/ml.

The plasma extraction procedure used was combination of a protein precipitation step via acetonitrile and centrifugation at 14500 g for 5.0 min at 4°C. Normally 20 µl of the supernatant was injected into the HPLC system.

Statistical analysis

Statistical analysis was performed using a computer statistical package SPSS 11.0 for windows (SPSS, Inc., Chicago, I L., U S A). The significance of the differences between the groups was assessed with One-Way ANOVA. Tukey's test was used after One-Way ANOVA to determine statistical differences among all of the groups. The significant differences within the groups at monthly intervals were assessed with repeated measures ANOVA. The

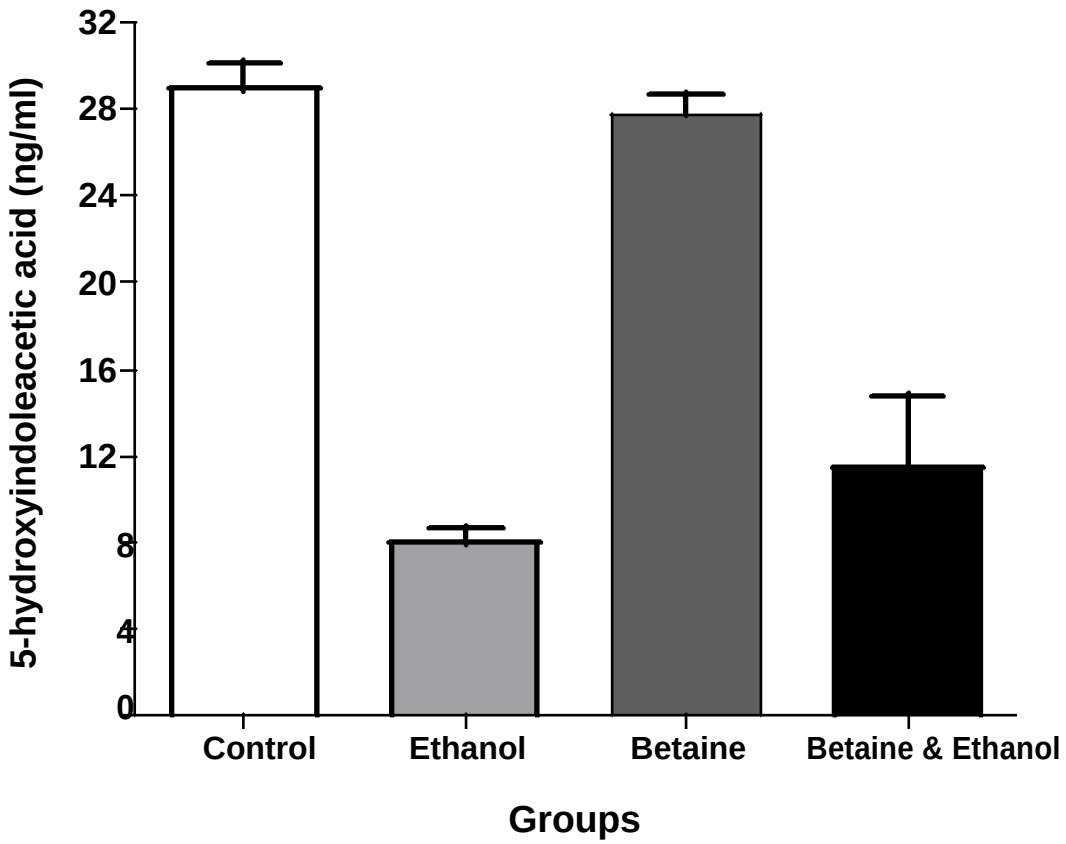

Figure 2. 5- HIAA levels of plasma between the control and treatment groups; on the 90th day of the treatment. Values represent mean ± SD of 5-HIAA; (*, **, ***) indicate statistical difference ($p < 0.05$) between the groups. Tukey's test was used after One-Way ANOVA to determine statistical differences among all of the groups.

relationship between tHcy and 5-HIAA in the plasma of the E group on the 90th day was calculated by Pearson's correlation test. Independent sample t-Test was used for tHcy in both male and female rabbits from the Ethanol group. Data were expressed as mean ± SD and p-values of <0.05 were regarded as statistically significant.

RESULTS

5-HIAA and tHcy were compared in the treatment groups and control having significant differences between the groups only on the 90th day. Therefore, the differences between the groups on the 90th day of the treatment for 5- hydroxyindoleacetic acid and tHcy have been illustrated in Figures 2 and 3 respectively. There was a significant negative correlation between 5-HIAA and tHcy in the E group ($r = -0.473$, $P = 0.021$), and a significant increase in the concentration of 5-HIAA in the B AND E group compared to the E group ($p<0.05$) . Significantly high concentrations of 5-HIAA were also observed in the B and C groups, in contrast to the E group ($p<0.05$). Figures 4 and 5 show the folate and vitamin B12 concentrations of the control and the treatment groups in one-month intervals. While vitamin B12 showed no significant difference in the B AND E group on the 90th day compared to the control group, the serum concentrations of folate on the 90th day differed significantly.

Significant differences were observed for folate on the 30th, 60th and 90th days of the treatment with regard to the 0.0 day for the all treatment groups (Figure 4). Also, significant differences for vitamin B12 on the 30th, 60th and 90th day of the treatment in the B and E groups and on the 30th and 60th day in the B AND E group were observed with respect to the 0.0 day (Figure 5). The plasma total homocysteine levels in the E group are not significantly higher in male (16.32 µM/l) versus female rabbits (14.75 µM/l). Therefore, no significant difference was observed between tHcy and gender.

DISCUSSION

Our results support the hypothesis that betaine reduces hyperhomocysteinemia (Finkelstein, 2007) and enhances 5-hydroxyindoleacetic in ethanol- induced hyperhomocysteinemia in rabbits. To the best of our

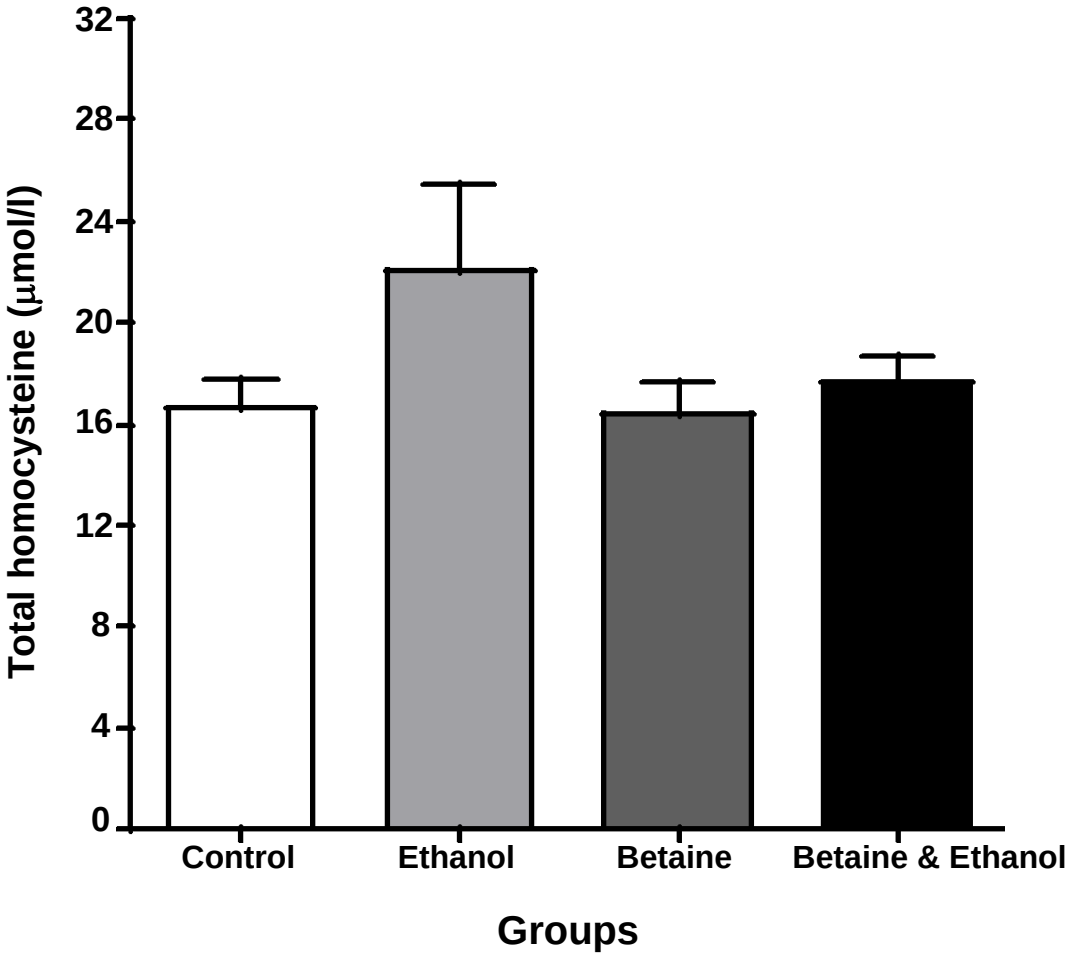

Figure 3. Total homocysteine levels of plasma between the control and treatment groups; on the 90th day of the treatment. Values represent mean ± SD of tHcy; (*, **) indicate statistical difference ($p < 0.05$) between the groups. Tukey's test was used after One-Way ANOVA to determine statistical differences among all of the groups.

knowledge this study is the first ethanol-induced hyperhomocysteinemia investigation of associations between folate, vitamin B12, total homocysteine and serotonergic system to determine therapeutic effects of betaine. The protective effects of betaine were confirmed by higher folate and vitamin B12 in serum and lower total homocysteine in plasma. The data we found provides new evidence that the tHcy of plasma decline, while 5-HIAA elevate with oral betaine. Also, there is a negative association between the 5-HIAA (metabolite of serotonergic system) and ethanol treatment. While vitamin B12 showed no decrease in E group on the 90th day with regard to the 30th day of the treatment, the concentration of folate differed and folate seems to be associated with increased risk for hyperhomocysteinemia. Furthermore, vitamin B12 showed no significant difference in Betaine and Ethanol group on the 90th day compared to the control group while, the concentration of folate differed significantly. Thus, the important role of folate rather than vitamin B12 is established and confirms by previous studies (Bottiglieri, 2005; Kim et al., 2008; Papakostas et al., 2005). Although, plasma total homocysteine levels were higher in the male versus the female rabbits, it was not significant. It is clear that this difference is related to sex steroids (Giltay et al., 1998).

Homocysteine is a non-essential, thiol containing and potentially cytotoxic 4-carbon -amino acid formed during methionine metabolism through the demethylation of methionine (Nasir et al., 2007). In recent years increasingly more evidences support the hypothesis that elevated total homocysteine is an independent risk factor for coronary vascular and neurodegenerative diseases (Bidulescu et al., 2009; Chandra et al., 2006; Folstein et al., 2007; Kim et al., 2008; Zieminska and Lazarewicz, 2006). High levels of homocysteine are associated with cerebrovascular disease, abnormality of monoamine neurotransmitters, and depression of mod. A plausible hypothesis for these associations is that of high homocysteine levels (Folstein et al., 2007). Traditional

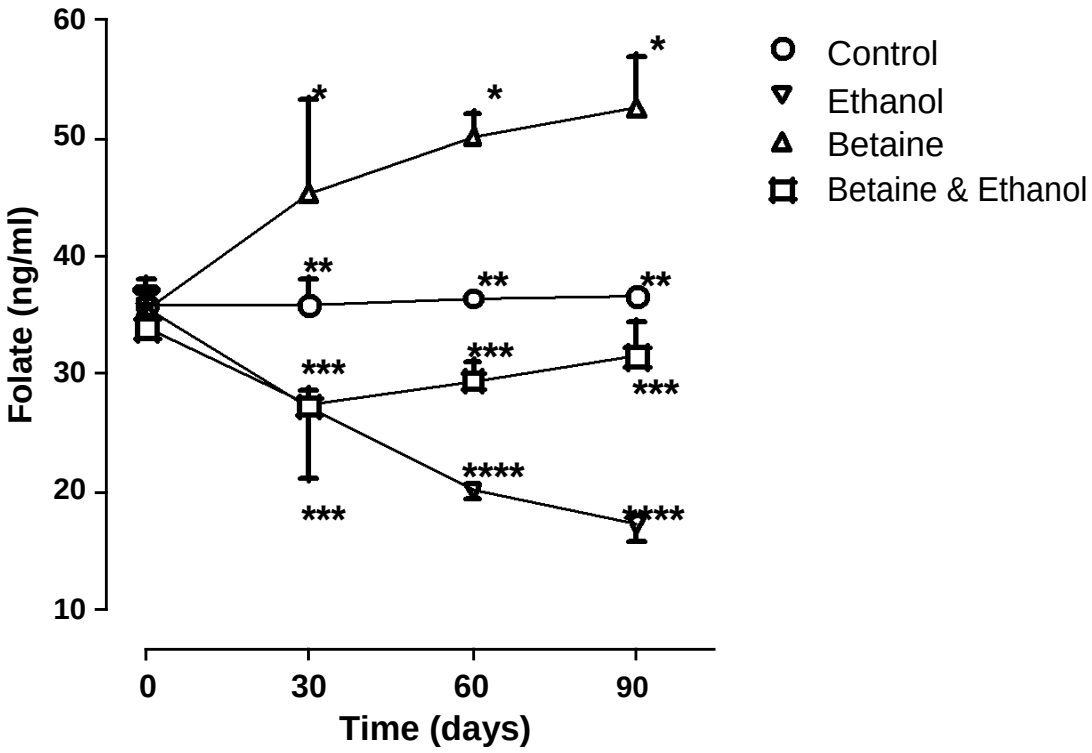

Figure 4. Effect of administration of betaine and ethanol on the serum folate. Values represent mean ± SD of Folate; (*, **, ***, ****) indicate statistical difference ($p < 0.05$) between the groups on the same days. Tukey's test was used after One-Way ANOVA to determine statistical differences among all of the groups.

explanations of the mechanism of Hcy neurotoxicity point to key role of disturbance in methylation and remethylation process. SAMe accumulated in cells in hyperhomocysteinemia is a very strong competitive antagonist of many transferase (Bottiglieri et al., 2000; Zieminska and Lazarewicz, 2006). Hcy toxicity and impaired methylation may be potential mechanisms involved in the clinical spectrum of MTHFR and cerebral folate deficiency. However, folate may also be directly involved in the regulation of neurotransmitter metabolism. Low concentrations of CSF 5-hydroxyindole acetic acid, a metabolite of serotonin (5- HT) that reflects global CNS tissue levels, have been reported in folate-deficient patients with various neuropsychiatric illnesses and severe depression (Bottiglieri, 2005). Our observation for vitamins, tHcy and 5-HIAA in the treatment groups of the present study supports the idea that vitamin B12 and folate are associated with 5-HIAA through their involvement in homocysteine remethylation.

A possible mechanism linking folate deficiency and perturbed monoamine neurotransmitter function may involve tetrahydrobiopterin (BH4) metabolism, a cofactor required in the synthesis of monoamine neurotransmitters. Due to the structural similarities between folate and BH4, the folate enzymes MTHFR and dihydrofolate reductase (DHFR) have been postulated to be involved in BH4 metabolism. However, in depressed patients, significant correlations between red cell folate and CSF BH4, and also between CSF monoamine metabolites have been reported (Bottiglieri, 2005; Bottiglieri et al., 2000). With regard to the fact that folate appears to influence the rate of synthesis of tetrahydrobiopterin, a cofactor in the hydroxylation of phenylalanine and tryptophan, rate-limiting steps in the biosynthesis of dopamine, norepinephrine, and serotonin, neurotransmitters postulated to play a role in the monoamine hypothesis of affective disorders. In addition when folate has been administered in parenteral and certain oral forms, both SAMe and methyl folate have been shown to have antidepressant efficacy. (Atmaca et al., 2005; Hofmann et al., 1996). In contrast, in some cases causative treatment of hyperhomocysteinemia and depression to reduce the levels of Hcy in human body fluid, include; supplementation of the diet with folic acid and//or Vitamin B12 and B6 it was unsuccessful (Zieminska and Lazarewicz, 2006). Therefore, we designed the present investigation to determine the preventive effect of betaine on ethanol-induced hyperhomocysteinemia. The results of the present study demonstrated that betaine supplementation to alcohol-fed rabbits promotes the generation of hepatic S-adenosyl methionine due to stimulation of methionine synthesis by the alternate BHMT pathway (Bottiglieri, 2005; Finkelstein, 2007). Betaine, a methyl-donor that

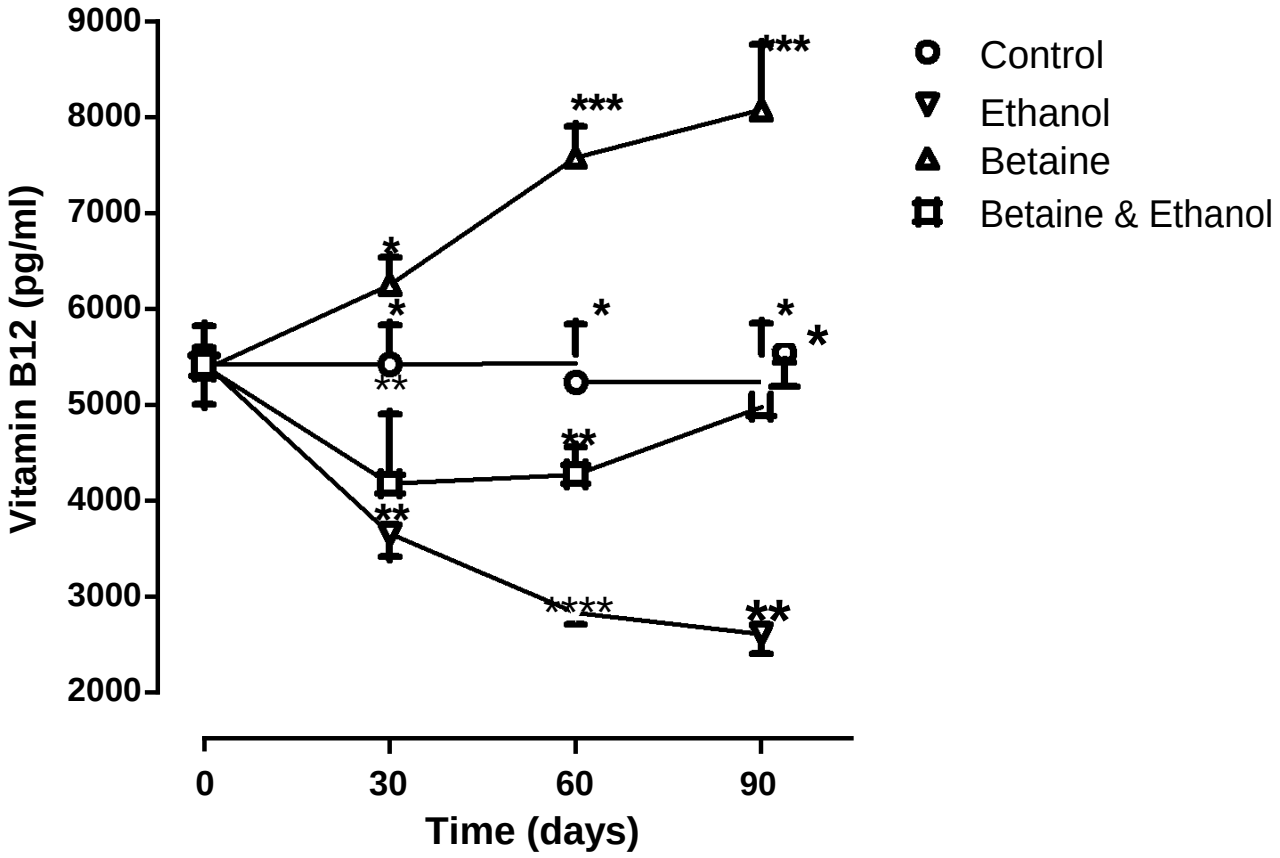

Figure 5. Effect of administration of Betaine and Ethanol on the serum vitamin B12.Values represents mean ± SD of vitamin B12; (*, **, ***, ****) indicate statistical difference ($p < 0.05$) between the groups on the same days. Tukey's test was used after One-Way ANOVA to determine statistical differences among all of the groups.

continuously generates S-denosyl methionine, is shown to lead to long term lowering of plasma homocysteine during supplementation in the dietary intake range of 1.5% (w/w) (Ji and Kaplowitz, 2003).

It seems the intracellular content of S-adenosyl methionine (SAMe) is the likely gauge for the availability of methionine. Changes in the concentrations of SAMe and S-adenosyl homocysteine (SAH) affect the methionine conserving enzymes of both of the methionine cycles. SAMe inhibits the activity of methionine adenosyl transferase (MAT) and also down-regulates the expression of the MAT gene in hepatocytes. Conversely, low concentrations of SAMe allow the expression of this gene in liver cells (Finkelstein, 2007). Thus, SAMe which issuees from BHMT pathway in our study via betaine supplementation can probably inhibit MTHFR, subsequently MS and ultimately increase vitamins (B12, folate) by saving its consumption through the classical pathway involving MS (Figure 1). With regard to some limitations, measurement of depression and the molecular mechanism of homocysteine neurotoxicity are not evaluated from the present investigation. One limitation of the present study is that there was no instrument or program to define depression of rabbits. Another limitation is the insufficiently sensitive index to measure the changes in depression and the timing of clinical improvement. In contrast, previous studies have reported that homocysteine, being an excitatory neurotoxic by excessive accumulation of cytosolic calcium, N-methyl D-aspartate (NMDA) receptor overstimulation, generating oxidative stress and activation of apoptotic pathway (Chandra et al., 2006).

According to the monoamine hypothesis of affective disorders, depression is due to a deficiency of 5-HT, or norepinephrine, or both these monoamines (Atmaca et al., 2005; Folstein et al., 2007). In the present study, the results of the ethanol group of rabbits showed an abnormality of tHcy and 5-HIAA that is related to deficiency of folate and vitamin B12. In animal studies, low dietary intake of choline and betaine results in aberrant DNA methylation and possible increased atherogenesis and independently of folate; the dietary intake of choline and betaine are inversely associated with plasma homocysteine (Bidulescu et al., 2009). In the present study, it was concluded that betaine is a preventable metabolic agent and that the ingestion of lipotropic agents is part of a preventative strategy.

Betaine prevented a decrease in the content of vitamin B12 and folate and decreased the tHcy in the betaine as well as betaine and ethanol groups of rabbits. It is well known that, depression may be the consequence of a developmental pathology affecting serotonergic and/or dopaminergic neuronal (Atmaca et al., 2005). The present study provides evidences for the serotonergic neurotoxicity of homocysteine (Bleich et al., 2004); it also indicates that the elevated levels of this excitatory amino acid in the ethanol group have been declined in the betaine and ethanol group of rabbits with long term betaine therapy. Therefore, these relationships probably have resulted in elevation of serotonin that plays a major role in the configuration of mood and depression (Bleich et al., 2004; Bottiglieri, 2005; Kim et al., 2008).

Conclusions

The results of the present study may have important implications in understanding the possible role of betaine on the treatment of human depression and betaine may be recommended in the pretreatment method of depressive patients with alcoholism. It appears that a limited betaine-dependent remethylation of homocysteine to methionine (BHMT pathway) also exists within mammalian brains (Finkelstein, 2007). Interestingly, the existence of brain BHMT pathway would enable explains details of our results. Nevertheless, future investigations with controlled human studies are required to confirm whether the betaine exactly increases 5-HT output or not.

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