BETAINE REDUCES HYPERHOMOCYSTEINEMIA AND ENHANCES 5-HYDROXYINDOLEACETIC ACID IN ETHANOL-INDUCED HYPERHOMOCYSTEINEMIA IN RABBITS

Masoud Alirezaei¹, Mehdi Saeb¹, Katayoun Javidnia², Saeed Nazifi³ and Najmeh Khalighyan²

¹Department of Biochemistry, School of Veterinary Medicine-Shiraz University, Shiraz, 71345, Iran
²Medicinal and Natural Products Chemistry Research Center-Shiraz University of Medical Sciences, Shiraz, Iran
³Department of Clinical Pathology, School of Veterinary Medicine- Shiraz University, Shiraz, 71345, Iran

ABSTRACT

A high level of total homocysteine (tHcy) is one 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): 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 (BandE) 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, tHcy level of plasma 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 BandE group increased considerably (p<0.05). In contrast to the E group, significantly high

¹ Corresponding author: Department of Biochemistry, School of Veterinary Medicine, University of Shiraz, Shiraz, Iran. Dr. Masoud Alirezaei. E-mail address: Alirezaei_m54@yahoo.com. Tel.: +987112286950; Fax: +987112286940.
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 BandE group on the 90th day compared to the control group, the serum concentrations of folate on the 90th day differed. 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.

**Keywords:** Betaine; Hyperhomocysteinemia; 5-hydroxyindoleacetic acid; Ethanol; Vitamin B₁₂; Folate.

**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: methylenetetrahydrofolate 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-synthetase;
- GSH: glutathione;
- DMG: dimethyl glycine;
- MS: methionine synthase;
- MAT: methionine adenosyl transferase.

**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 β-synthase, a pyridoxine-dependent enzyme, to form cysteine (Supplementary file) (Kruman et al., 2000).
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S. 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-synthetase; GSH, glutathione; DMG, dimethyl glycine; MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; MTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; THF, tetrahydrofolate; MAT, methionine adenosyltransferase; SAMe, S-adenosyl methionine; SAH, S-adenosyl homocysteine; SAHH, S-adenosyl homocysteine hydrolase. (Bottiglieri, 2005). T. Bottiglieri, Homocysteine and folate metabolism in depression, Progress in Neuropsychopharmacology & Biological Psychiatry 29 (2005) 1103-1112.

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). It is interesting to note that the content of BHMT in rodent livers increased with both restriction and excessive dietary methionine, prompting the suggestion that this reaction could function to either conserve methionine or remove excess homocysteine (Finkelstein, 2007). 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 a decreased conversion of methionine to S-adenosylmethionine (SAMe) and homocysteine to methionine (Cravo et al., 1996; Bottiglieri, 2005). All tissues possess the methionine cycle
with methyltetrahydrofolate as the methyl donor, but the liver, kidney, pancreas, intestine and brain also contain the transsulfuration pathway (Finkelstein, 2007). 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 the methyl groups required in the synthesis of neuronal messengers and membranes (Papakostas et al., 2005). Folate and vitamin B$_{12}$ deficiency, hyperhomocysteinemia and the T677 allele of the methyltetrahydrofolate 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 proven 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). Effective treatment of depressive disorders being advanced by the precise delineation of the neurochemical basis of the disease as well as alterations in the catecholaminergic system during depression and upon antidepressant treatment have been reported (Zangen et al., 1999). 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 a 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 B$_{12}$ and folate.

**MATERIALS AND METHODS**

**Materials**

Alcohol (Ethanol 95%) and 1-octanesulfonic acid sodium salt were from the Merck Chemical Company (Merck, Darmstadt, Germany). Betaine (Betafin® 96%) was obtained from the Biochem Company (Lohne, Germany). 5-Hydroxyindoleacetic acid was purchased from Sigma (St Louis, MO, USA). SimulTRAC-SNB Radio assay kit vitamin B$_{12}$ [$^{57}$Co]/Folate [$^{125}$I] 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 Sciences. A total of 32 adult New
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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 a standard chow diet (average 50 g/kg), and water ad libitum in a 2-week acclimation period. The animals were divided into four equal groups (n=8); the first group (Control) received a standard chow diet plus 2ml water by using gavage daily. The second group (Ethanol) was administered ethanol at a dosage of 4 g/kg per day by using gavage plus the standard chow diet. The 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 the fourth group (Betaine and Ethanol) was administered with the betaine group diet. After one hour the rabbits received ethanol at a dosage of 4 g/kg per day by using gavage (pretreatment method) (Ji and Kaplowitz, 2003; Song et al., 2003). Each group consisted of 4 male and 4 female animals. The study period was 90 days. Weight gains and food consumption were determined at weekly intervals (data not shown).

**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 the day before treatment began (0.0 day) and on the 30th, 60th and 90th day of the treatment. 1.0 mL of whole blood was drawn into tubes of ethylenediamine tetra-acetic acid (EDTA), centrifuged, separated into plasma aliquots and the remaining whole blood placed in other tubes. The collection of serum was assessed in micro tubes. Serum and plasma aliquots were stored at -70°C until analysis. Serum folate and vitamin B12 levels were determined using a radioimmunoassay; (SimulTRAC-SNB Radiolooxay kit vitamin B12/Folate) (Ferrucci et al., 2007; Golbahar et al., 2005) and the total plasma homocysteine level was determined by Axis® homocysteine EIA kit (Golbahar et al., 2005; Karthikeyan et al., 2007).

**Determination of 5-HIAA**

Plasma 5-hydroxyindoleacetic acid concentrations were measured 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-μl loop, and a 3 mm particle size (250×4.6 mm, I.D.) with a 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 KH2PO4 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 measured normally. The concentration of 5-HIAA was calculated by the 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 was a combination of a protein precipitation step via acetonitrile and centrifugation at 14500 g for 5.0 minutes at 4 °C. 20 μl of the supernatant was injected normally 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 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 and control groups 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 Figure 1 and 2 respectively.

![Graph showing 5-HIAA levels of plasma between the control and treatment groups, on the 90th day of the treatment. Values represent mean ± SD of tHcy; *, **, *** indicate the statistical difference (P < 0.05) between the groups.](image)
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**Figure 2.** 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 the statistical difference (P < 0.05) between the groups.

**Figure 3.** Effect of the administration of betaine and ethanol on the serum folate. Values represent mean ± SD of Folate; *, **, ***, **** indicate the statistical difference (P < 0.05) between the groups on the same days.
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 BandE 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 3 and 4 show the folate and vitamin B$_{12}$ concentrations of both the control and the treatment groups in 1 month intervals. While vitamin B$_{12}$ showed no significant difference in the BandE group on the 90$^{th}$ day compared to the control group, the serum concentrations of folate on the 90$^{th}$ day differed.

Significant differences were observed for folate on the 30$^{th}$, 60$^{th}$ and 90$^{th}$ days of the treatment with regard to the 0.0 day for the all treatment groups (Figure 3). Also, significant differences for vitamin B$_{12}$ on the 30$^{th}$, 60$^{th}$ and 90$^{th}$ day of the treatment in the B and E groups and on the 30$^{th}$ and 60$^{th}$ day in the BandE group were observed with respect to the 0.0 day (Figure 4). The plasma total homocysteine levels in the E group are not significantly higher in male (16.32µmol/l) versus female rabbits (14.75µmol/l), however, no significant difference was observed between tHcy and gender.

**DISCUSSION**

Our data support the hypothesis that betaine reduces hyperhomocysteinemia (Finkelstein, 2007) and enhances 5-hydroxyindoleacetic in ethanol-induced hyperhomocysteinemia in rabbits. To the best of our knowledge, this study is the first ethanol-induced hyperhomocysteinemia investigation of associations between folate, vitamin B$_{12}$, total...
homocysteine and the serotonergic system to determine the therapeutic effects of betaine. The protective effects of betaine were confirmed by higher folate and vitamin B$_{12}$ 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. There is a negative association between the 5-HIAA (metabolite of serotonergic system) and ethanol treatment as well.

While vitamin B$_{12}$ showed no decrease in the E group on the 90$^{th}$ day with regard to the 30$^{th}$ day of the treatment, the concentration of folate differed, and folate seems to be associated with an increased risk of hyperhomocysteinemia. Furthermore, vitamin B$_{12}$ showed no significant difference in the Band E group on the 90$^{th}$ day compared to the control group, whereas the serum concentration of the folate differed significantly. Thus, the important role of folate rather than vitamin B$_{12}$ is established and confirmed by previous studies (Bottiglieri, 2005; Kim et al., 2008; Papakostas et al., 2005). However, plasma total homocysteine levels were higher in the male versus the females rabbits, although not significantly. 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 α-amino acid formed during methionine metabolism through the demethylation of methionine (Nasir et al., 2007). In recent years increasingly more evidence supports 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; Ziemsinska and Lazarewicz, 2006). High levels of homocysteine are associated with cerebrovascular disease, abnormality of monoamine neurotransmitters, and depression of mood. A plausible hypothesis for these associations is that high homocysteine levels cause cerebral vascular disease and neurotransmitter deficiency, causing depression of mood (Folstein et al., 2007).

Traditional explanations of the mechanism of Hcy neurotoxicity point to the key role of a disturbance in the methylation and remethylation process. SAMe accumulation in cells in hyperhomocysteinemia is a very strong competitive antagonist of many transferase (Bottiglieri et al., 2000; Ziemsinska 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 the neurotransmitter metabolism. Low concentrations of cerebrospinal fluid (CSF) 5-hydroxyindole acetic acid, a metabolite of serotonin (5-HT) that reflects the global central nervous system tissue levels, have been reported in folate-deficient patients with various neuropsychiatric illnesses and severe depression (Bottiglieri, 2005). The observation for vitamins, tHcy and 5-HIAA concentrations in the treatment groups of the present study supports the idea that vitamin B$_{12}$ and folate are associated with 5-HIAA through their involvement in homocysteine remethylation. The role of folate in the central nervous system function has been established because of the essential role of folate in the one-carbon cycle that furnishes SAMe, the principal methyl donor for a broad range of reactions involving the synthesis of neuroactive substances, the formation of membrane phospholipids, and the metabolism of nucleic acids (Atmaca et al., 2005).

A possible mechanism linking folate deficiency and perturbed monoamine neurotransmitter function may involve tetrahydrobiopterin (BH4) metabolism, a co-factor 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 the 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). Low cerebrospinal fluid levels of the serotonin metabolite 5-hydroxyindoleacetic acid and the dopamine metabolite homovanillic acid (HVA) have been reported in several studies among folate-deficient patients with epilepsy, other neuropsychiatric disorders, and congenital folate-deficiency states (Atmaca et al., 2005). Plasma homocysteine levels have been found to increase with age, and it has been reported that the extent of increase is much greater in patients with Alzheimer’s disease and Parkinson’s disease (Chandra et al., 2006).

With regards 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 are 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 an antidepressant efficacy greater than the placebo and comparable to that of tricyclic antidepressants (Atmaca et al., 2005; Hofmann et al., 1996). In contrast, in some cases the causative treatment of hyperhomocysteinemia and depression to reduce the levels of Hcy in human body fluid include supplementing the diet with folic acid and/or vitamin B12 and vitamin B6, which has been unsuccessful (Zieminska and Lazarewicz, 2006). Therefore, we designed the present investigation to determine the preventive effect of betaine on ethanol-induced hyperhomocysteinemia in rabbit.

The results of the present study demonstrated that betaine supplementation to alcohol-fed rabbits promotes the generation of hepatic S-adenosyl methionine due to the stimulation of methionine synthesis by the alternate BHMT pathway (Bottiglieri, 2005; Finkelstein, 2007). Betaine, a methyl-donor that continuously generates S-adenosyl 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 SAMe is the likely gauge for the availability of methionine, either excess or insufficiency. 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.

SAMe is an allosteric inhibitor of MTHFR, the sole source of the methyltetrahydrofolate (MTHF) substrate for methionine resynthesis via the methionine synthase (MS) reaction (Finkelstein, 2007). Thus, SAMe, which is provided from the 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 (Supplementary file).

With regard to some limitations, the 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 were no instruments or program to define the 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 the excessive accumulation of cytosolic calcium, N-methyl D-aspartate
(NMDA) receptor overstimulation, generates oxidative stress and the activation of the apoptotic pathway (Chandra et al., 2006).

According to the monoamine hypothesis of affective disorders, depression is due to a deficiency of 5-HT, norepinephrine, or both of 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 the 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, while independent of folate, the dietary intake of choline and betaine are inversely associated with plasma homocysteine, a putative cardiovascular and neurodegenerative disease risk factor (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 concentration in both the Betaine and the 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 systems which have also been suggested for the etiopathogenesis of depression (Atmaca et al., 2005).

The present study provides evidence 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 declined in the Betaine and Ethanol group of rabbits with long-term betaine therapy. Therefore, these relationships have probably resulted in the elevation of serotonin, which plays a major role in the configuration of mood and depression (Bleich et al., 2004; Bottiglieri, 2005; Kim et al., 2008).

In summary, the results of the present study may have important implications in understanding the possible role of betaine on the treatment of 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 a brain BHMT pathway would explain the 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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Reviewed by
Sang Yoon
Professor JS Yoon, Department of Psychiatry and Depression
Clinical Research Centre, Chonnam National University Medical School, Kwang
Republic of Korea.
Email: jsyoon@chonnam.ac.kr