Journal of Alzheimer's Disease 31 (2012) 59–64
DOI 10.3233/JAD-2012-120022
IOS Press

Urinary Homocysteic Acid Levels Correlate with Mini-Mental State Examination Scores in Alzheimer's Disease Patients

Tohru Hasegawa[a,*], Masayoshi Ichiba[b], Shin-ei Matsumoto[c], Koji Kasanuki[d], Taku Hatano[c], Hiroshige Fujishiro[d], Eizo Iseki[d], Nobutaka Hattori[c], Tatsuo Yamada[e] and Takeshi Tabira[c]
[a]*Saga Woman Junior College, Saga, Japan*
[b]*Social Medicine, Faculty of Medicine, Saga University, Saga, Japan*
[c]*Department of Neurology, Graduate School of Medicine, Juntendo University, Bunkyo-ku, Tokyo, Japan*
[d]*PET/CT Dementia Research Center, Juntendo Tokyo Koto Geriatric Medical Center, Juntendo University School of Medicine, Koto-ku, Tokyo, Japan*
[e]*Department of Neurology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan*

Accepted 22 March 2012

Abstract. Homocysteic acid (HA) has been suggested as a pathogen in a mouse model of Alzheimer's disease (AD), 3xTg-AD. However, it is not established whether HA is involved in humans. We investigated the relationship between urinary HA levels and Mini-Mental State Examination (MMSE) scores in AD patients ($n = 70$) and non-AD controls ($n = 34$). We found a positive, statistically significant relationship between the two variables (the urinary HA level and MMSE score) ($r = 0.31$, $p = 0.0008$, $n = 70$). This relationship was stronger in females than males ($r = 0.43$, $p = 0.005$, $n = 44$ in females; $r = 0.48$, $p = 0.02$, $n = 22$ in males). The urinary HA levels were significantly different in AD patients than controls (AD: 8.7 ± 7.5, $n = 70$; non-dementia control: 13.3 ± 9.4, $n = 34$, $p < 0.01$). In addition, aging and smoking were found as lowering factors for urinary HA levels. Our preliminary study showed a negative, statistically significant relationship between blood HA (micromole) and urine HA levels ($r = -0.6$, $p = 0.007$, $n = 19$), and between blood HA levels and MMSE scores ($r = -0.79$, $p = 0.0000518$, $n = 19$). On the basis of these results, we speculate that reduced urinary excretion induces elevated HA levels in blood, resulting in cognitive dysfunctions. This study also suggests that HA may be a candidate of neurotoxins for uremic encephalopathy. Since amyloid-β increases HA toxicity and HA is an agonist of N-methyl-D-aspartic acid (NMDA) receptor, we speculate that elevated blood HA affects the brain cognitive function through NMDA receptor-mediated toxicity in AD.

Keywords: Aging, Alzheimer disease, homocysteic acid, MMSE, smoking, uremia

Supplementary data available online: http://dx.doi.org/10.3233/JAD-2012-120022

INTRODUCTION

We have recently shown that vitamin B6 deficiency induces elevated levels of homocysteic acid (HA) in the brain in association with accumulation of amyloid-β (Aβ) and cognitive dysfunctions in C57BL/6 mice, which were reversed by immunotherapy targeting HA [1]. This was confirmed in an Alzheimer's disease (AD) mouse model, 3xTg-AD mice carrying human amyloid-β protein precursor, presenilin 1, and tau gene mutations, which were successfully treated with anti-HA antibodies [2]. These results suggest that HA might be involved in the pathological mechanism of AD. In

*Correspondence to: Tohru Hasegawa, Social Medicine, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 840-8502, Japan. Tel.: +81 952 34 2289; E-mail: hasegawa@yumekobo-shizuka.com.

the present study, we measured urine HA levels in humans and found that urine HA levels correlate well with Mini-Mental State Examination (MMSE) scores.

MATERIALS AND METHODS

The study protocol was approved by the Juntendo University Ethics Committee, and all individuals agreed with the procedure by providing informed written consent. In the case where demented patients did not understand the procedure, the main family care giver provided informed written consent. The individual profiles are shown in Table 1. Control individuals consisted of normal healthy individuals and individuals with other diseases such as hypertension ($n=5$), cerebral infarction ($n=4$), Parkinson's disease ($n=3$), amyotrophic lateral sclerosis ($n=1$), spastic paraplegia ($n=1$), epilepsy ($n=1$), cervical spondylosis ($n=1$), myositis ($n=1$), polyneuropathy ($n=1$), hyperlipidemia ($n=1$), diabetes mellitus ($n=1$), ovarian cyst ($n=1$), prostatic hypertrophy ($n=1$), and frontotemporal dementia ($n=1$). All AD cases including 2 cases with mild cognitive impairment (MCI) met the NINCDS-ADRDA criteria, and the diagnosis was assisted by CT, MRI, and SPECT findings. Urine samples were collected at Juntendo Hospital and Juntendo Tokyo Koto Geriatric Medical Center, and kept frozen without preservatives at -20°C until use.

In addition, as a preliminary study for blood HA, patients were recruited at the Memory Clinic of Fukuoka University. Those patients were composed of normal ($n=2$, male), MCI ($n=6$, male), and AD ($n=11$) (female, $n=11$ 75 ± 5 years; male, $n=8$, 73 ± 6 years). They all agreed with this observation.

Measurement of HA

The specific gravity of each urine sample was measured, and the urinary HA level was adjusted to that of 1.020 specific gravity. High performance liquid chromatography (HPLC) with an ECD detector was performed using a previous method with modifications [3]. Urine was diluted 10 times with water,

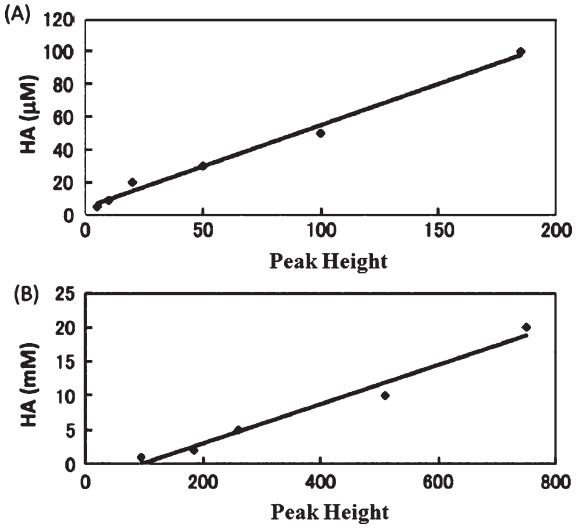

Fig. 1. The titration curve of authentic homocysteic acid (HA). This is to show the titration curve of authentic HA by ECD-HPLC chromatograms.

4 mg of HA was added to 1 ml of each urine sample as an internal standard, and 20 μl of the urine sample was added to the measurement solution, composed of 150 μl o-phthaldialdehyde reagent and 150 μl mercaptoethanol. Fifteen minutes after mixing the diluted urine sample with the measurement solution, the sample was injected into the HPLC system. The urine HA measurement was done blind. Blood HA levels were measured as previously described [3]. Typical chromatograms of authentic HA, urine, and blood are shown (supplementary Figure 1A–C; available online: http://www.j-alz.com/issues/31/vol31-1.html#supplementarydata02), and a titration curve is shown in Fig. 1. The retention time of HA was 3.9–4.1 min in urine and 3.6–3.8 min in blood. When the urine samples were measured, HPLC-ECD detector sensitivity was modified 10 times lower than that of blood.

Statistical significance was estimated by Student's *t* test, and we analyzed the correlation by Spearman analysis.

Table 1
Profiles of AD patients and controls

	AD patients			Controls		
	n	Age (years)	Body weight (Kg)	*n*	Age (years)	Body weight (Kg)
Total	71	77.9 ± 7.5	52.2 ± 10.7	36	70.9 ± 7.5	55.1 ± 11.0
Male	22	75.4 ± 8.7	60.0 ± 8.1	14	70.7 ± 8.0	61.9 ± 7.2
Female	44	79.7 ± 6.2	47.9 ± 9.5	21	70.8 ± 7.3	51.0 ± 10.8
GND*	5	74.2 ± 7.2	49.5 ± 5.7	1	74	67

*GND, gender not described.

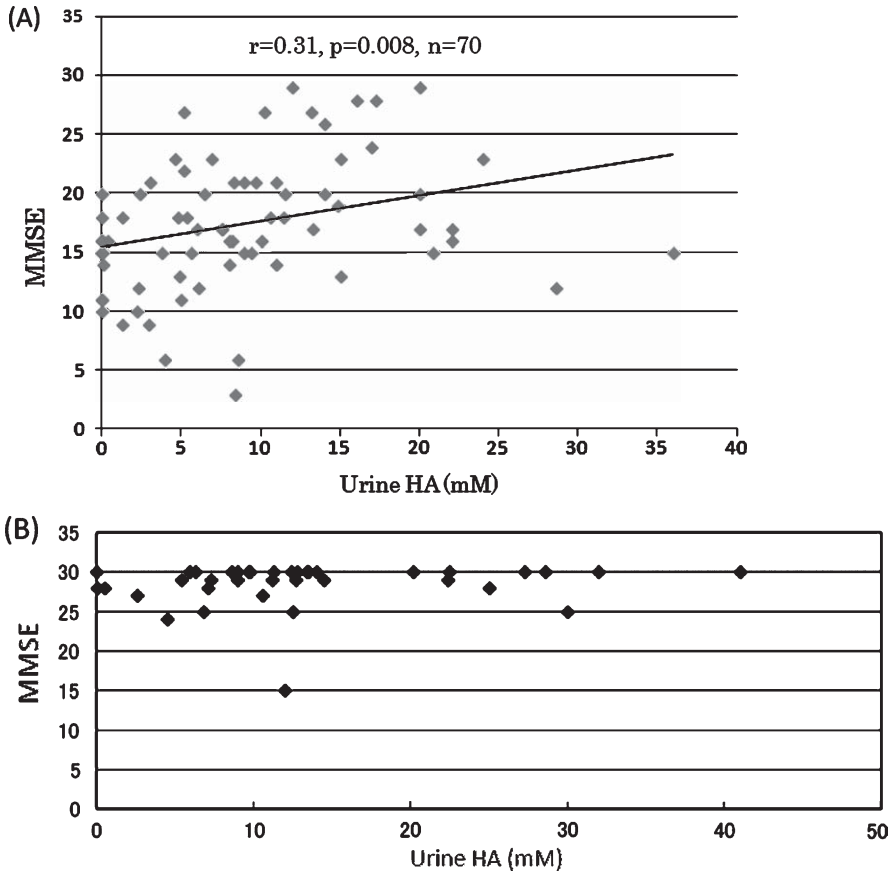

Fig. 2. Relationship between urinary homocysteic acid (HA) levels and Mini-Mental State Examination (MMSE) scores. All urine samples were collected at Juntendo University Hospital and Juntendo Tokyo Koto Geriatric Medical Center. Illustrated are relationships between urinary HA levels and MMSE scores in AD patients (A) ($n = 70$; females, $n = 44$; males, $n = 22$; age, 75.8 ± 8 years) and non-AD controls (B) ($n = 34$, 72.4 ± 7.9 years).

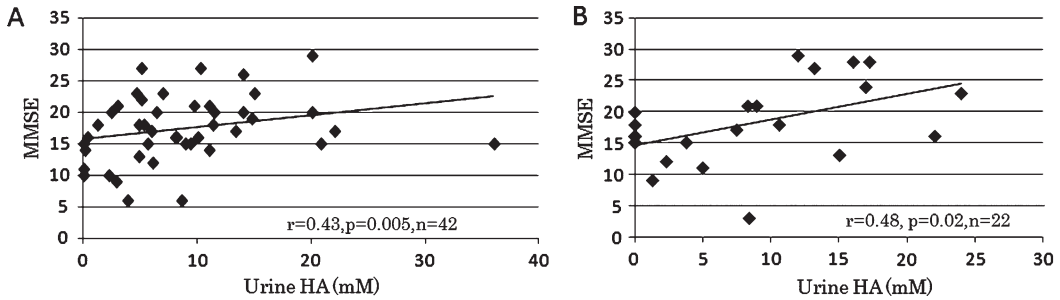

Fig. 3. Relationship between urinary homocysteic acid (HA) levels and Mini-Mental State Examination (MMSE) scores in females ($n = 44$; age, 79.7 ± 6.2 years) (A) and in males ($n = 22$; age, 75.4 ± 8.7 years) (B). As shown, the correlation is stronger in females than males.

RESULTS

Urinary HA levels were positively correlated with MMSE scores in AD patients. In other words, the lower the urinary HA levels, the lower the MMSE scores (Fig. 2). In controls, we could not observe a relationship between urinary HA levels and MMSE scores. This correlation was stronger in females than in males

Table 2
Urine HA levels in AD patients and controls

	AD patients		Controls		Significance
	n	Urinary HA (mM)	*n*	Urinary HA (mM)	
Male	22	8.02 ± 8.0	14	9.4 ± 5.9	
Female	44	8.5 ± 7.0	21	15.7 ± 11.0	AD versus control $p < 0.01$
GND*	5	12.82 ± 10.16			
Total	71	8.74 ± 7.11	35	13.34 ± 9.53	AD versus control $p < 0.01$

*GND, gender not described.

Table 3
Effect of smoking on urinary HA levels

	Urinary HA (mM)
Without smoking	15.2 ± 9.4 ($n = 27$)
With smoking	7.7 ± 4.2 ($n = 8$)*

*$p < 0.05$.

(Fig. 3A, B). When urinary HA levels were compared between AD and non-AD controls, the difference was statistically significant ($p < 0.01$) (Table 2).

We looked for factors that may correlate with the reduced HA in urine. In addition to cognitive dysfunction, aging was found to be a significant factor for reducing urine HA levels (Fig. 4A, B). In controls, there was no relationship between age and urinary HA levels. Further, smoking was also found to be a suppressing factor for the urinary HA excretion (Table 3).

We are interested in the relationship between urinary HA levels and blood HA levels. Our preliminary study shows an inverse relationship between urinary HA levels and blood HA levels ($r = -0.6$, $p = 0.007$, $n = 19$) (Fig. 5). As shown in Fig. 6, there was a strong negative relationship between blood HA levels and MMSE scores ($r = -0.79$, $p = 0.0000518$, $n = 19$). The 19 cases include 2 normal individuals and 6 MCI patients.

DISCUSSION

Our results clearly indicate that urinary HA levels were positively correlated with MMSE scores. When comparing AD patients and non-AD controls, urinary HA levels were significantly lower in AD patients (Table 2). However, there was a strong discrepancy in the relationship between urinary HA levels and MMSE scores. As shown in Fig. 2B, some patients with other diseases and normal controls showed lower urinary HA levels, but their MMSE scores were within normal range, except for a case with frontotemporal dementia. These discrepancies between AD and non-AD controls are based on the fact that Aβ increases HA toxicity [20].

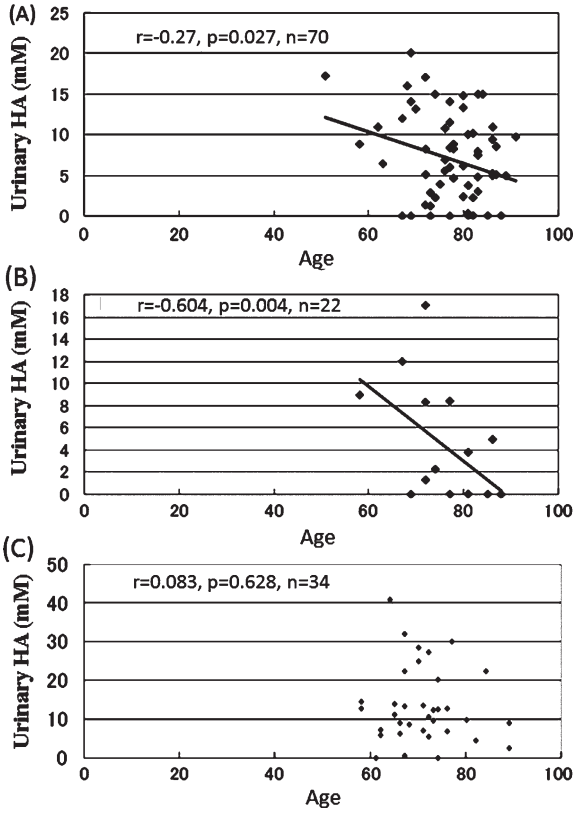

Fig. 4. Effect of aging on urinary homocysteic acid (HA) levels. As shown, a significant inverse correlation between urinary HA levels and age is seen in all individuals (A) and males (B) in AD. In case of females, a similar tendency was observed, but the correlation was not statistically significant. There was no correlation in controls (C).

Since HA is excreted into urine, the results suggest that those patients with lower urinary HA levels may have higher HA levels in blood. Indeed, our preliminary study showed an inverse correlation between urinary and blood HA levels ($r = -0.6$, $p = 0.007$, $n = 19$) (Fig. 5). Further, there was a strong negative relationship between blood HA levels and MMSE scores, which indicated that blood HA might induce cognitive impairment in AD. Recently many papers reported that Aβ oligomers induced synaptic damage through stimulation of N-methyl-D-aspartic acid

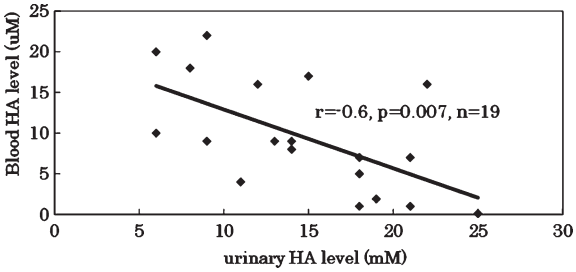

Fig. 5. The relationship between urinary homocysteic acid (HA) levels and blood HA levels. 19 AD patients submitted their urine and blood at Fukuoka University Hospital. They all agreed with the collection of urine and blood (female, $n = 11$ 75 ± 5 years; male, $n = 8$, 73 ± 6 years). As shown, levels of urinary HA and blood HA are inversely correlated ($r = -0.6$, $p = 0.007$).

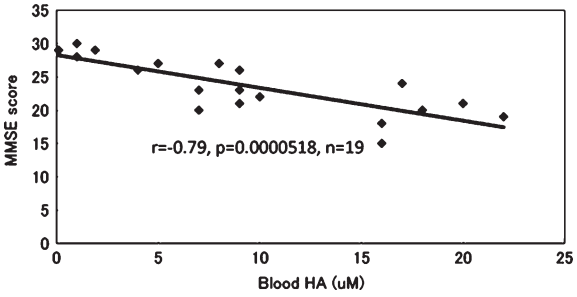

Fig. 6. The relationship between blood homocysteic acid (HA) levels and Mini-Mental State Examination (MMSE) scores. These data were obtained from samples in Fig. 4, and MMSE scores were measured at Fukuoka University Hospital.

(NMDA) receptor [4, 5]. Since HA is known as an agonist of NMDA receptor, blood HA might induce cognitive impairment through activation of NMDA receptor and augmentation of NMDA toxicity.

It is well known that homocysteine (HC) is a risk factor for AD and vascular dementia [6]. However, it has not been clearly shown that HC is neurotoxic. It is known that HA is formed from HC by cystathionine-β-synthase at renal tubules, which is secreted into urine and reuptake of HA does not occur in a normal condition, because HA was detected in the blood of normal controls by 0.2 μM [11], while the urinary HA level was at mM. Thus, urinary HA reabsorption does not seem to occur. In AD patients, excretion of HA is reduced or reuptake of HA is increased for some reason. Some studies have recently reported that kidney functions of patients with AD are impaired [7, 8]. Other papers showed that exogenous NMDA receptor agonists including HA disrupted blood brain barrier permeability [9, 10], so that HA could enter the brain and work as a neurotoxin. In uremic patients, several factors are suggested to induce brain dysfunction [10]. Our results suggest that HA is a candidate of neurotoxins in uremic patients.

It is interesting to know that brain HA levels are very low in mice [2]. The urinary HA levels in mice are also low at micromolar concentrations [2], whereas those in humans are at millimolar concentrations. This concentration of HA in urine seems to be concentrated from the blood HA (micromolar). A similar phenomenon is seen in a certain urine antigen [11, 12]. There is a report saying that blood HA in normal individuals was detected at 34 ± 7 ng/ml = 0.2 ± 0.04 μM by gas-chromatography-mass spectrometry [13]. Our blood result is concordant with this paper. From these observations, it may be one of the reasons why neuronal death is rare in mouse models of AD.

Our observations also provide other interesting insights that aging and smoking decrease the urinary HA levels. All these are well-known risk factors for AD [14, 15]. Our results suggest that aging and smoking might increase the risk of AD by decreasing urinary HA excretion. It is known that organic anion transporters in the kidneys play an important role in the excretion of toxic substances [16]. Perhaps aging and smoking may affect this transporter system.

There is much evidence suggesting the involvement of HA in the pathogenic processes of AD. First, Vlassenko and colleagues reported the possible link between regional aerobic glycolysis and amyloid deposition in normal brain [17]. We think that this phenomenon may be induced by HA, because HA is known to be a neurotransmitter in normal brain [18], and HA induces seizures in immature rat pups. In the pup brain, metabolism was changed to strong glycolysis [19]. Moreover, HA induces intraneuronal accumulation of $A\beta_{42}$ [20], and antibodies to HA attenuate AD pathology in 3X-Tg mice [2]. Second, it has been reported that lowering of HC by B vitamins slows down the rate of accelerated brain atrophy in MCI [21]. This report suggests that HC induces brain atrophy, but we think HA produced from HC acted as a neurotoxin. Indeed, HA induces neurodegeneration *in vitro* [22].

In conclusion, our findings indicate that when urinary HA excretion is decreased, HA rises to a toxic level in the body, particularly in the brain. This retention of HA may induce the AD pathogenic process and cognitive impairment. In the future, we should investigate a relationship with ApoE genotypes and examine HA levels in cerebrospinal fluid.

ACKNOWLEDGMENTS

Authors are grateful to Dr. Toshiki Nakahara, Dr. Heii Arai, and Ms. Yukako Hasegawa for their help in this study. This study was partially supported by a grant of Strategic Research Foundation Grant-aided Project for Private Universities from Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT), 2011–2015.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1234).

REFERENCES

[1] Hasegawa T, Mikoda N, Kitazawa M, LaFerla FM (2009) B6 deficient feeding or homocysteic acid induces the earlier Alzheimer's pathological change in normal C57BL male mice. *Nature Precedings*, http://hdl.handle.net/10101/npre.2009.2764.1.

[2] Hasegawa T, Mikoda N, Kitazawa M, LaFerla FM (2010) Treatment of Alzheimer's disease with anti-homocysteic acid antibody in 3xTg-AD male mice. *PLoS One* **5**, e8593.

[3] Quinn CT, Griener JC, Bottiglieri T, Hyland K, Farrow A, Kamen BA (1997) Elevation of homocysteine and excitatory amino acid neurotransmitters in the CSF of children who receive methotrexate for the treatment of cancer. *J Clin Oncol* **15**, 2800-2806.

[4] Klyubin I, Wang Q, Reed MN, Irving EA, Upton N, Hofmeister J, Cleary JP, Anwyl R, Rowan MJ (2011) Protection against Abeta-mediated rapid disruption of synaptic plasticity and memory by memantine. *Neurobiol Aging* **32**, 614-623.

[5] Bicca MA, Figueredo CP, Piemartin TC, Meotti FC, Bouzon Z, Tesca CI, Medeiros R, Calixto JB (2011) The selective and competitive N-methyl-D-aspartate receptor antagonist, (-)-6-phosphonomethyl-deca-hydroisoquinoline-3-carboxylic acid, prevents synaptic toxicity induced by amyloid-beta in mice. *Neuroscience* **192**, 631-641.

[6] Miller JW, Green R, Mungas DM, Reed BR, Jagust WJ (2002) Homocysteine, vitamin B6, and vascular disease in AD patients. *Neurology* **58**, 1471-1475.

[7] Tsai C-F, Wang S-J, Fuh J-L (2010) Moderate chronic kidney disease is associated with reduced cognitive performance in midlife women. *Kidney Int* **78**, 605-610.

[8] Madero M, Gul A, Sarnak MJ (2008) Cognitive function in chronic kidney disease. *Semin Dial* **21**, 29-37.

[9] Chi OZ, Hunter C, Liu X, Weiss HR (2009) Effect of exogenous excitatory amino acid neurotransmitters on blood-brain barrier disruption in focal cerebral ischemia. *Neurochem Res* **34**, 1249-1254.

[10] Liu X, Hunter C, Weiss HR, Chi OZ (2010) Effect of blockade of ionotropic glutamate receptors on blood-brain barrier disruption in focal cerebral ischemia. *Neurol Sci* **31**, 699-703.

[11] Liu M, Liang Y, Chigurupati S, Lathia JD, Pletnikov M, Sun Z, Crow M, Ross CA, Mattson MP, Rabb H (2008) Acute kidney injury leads to inflammation and functional changes in the brain. *J Am Soc Nephrol* **19**, 1360-1370.

[12] Selickman J, Paxos M, File T Jr, Seltzer R, Bonilla H (2010) Performance measure of urinary antigen in patients with Streptococcus pneumoniae bacteremia. *Diagn Microbiol Infect Dis* **67**, 129-133.

[13] Santhosh-Kumar CR, Deutsh JC, Kolhouse JC, Hassell KL, Kolhouse JF (1994) Measurement of excitatory sulfur amino acids, cysteine sulfuric acid, cysteic acid, homocysteine sulfuric acid, and homocysteic acid in serum by stable isotope dilution gas-chromatography-mass spectrometry and selected ion monitoring. *Anal Biochem* **220**, 249-256.

[14] Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, Iwatsubo T, Jack CR, Kaye J, Montine TJ, Park DC, Reiman EM, Rowe CC, Siemers E, Stern Y, Yaffe K, Carrillo MC, Thies B, Morrison-Bogorad M, Wagster MV, Phelps CH (2011) Toward defining the preclinical stages of Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* **7**, 280-292.

[15] Cataldo JK, Prochaska JJ, Glantz SA (2010) Cigarette smoking is a risk factor for Alzheimer's disease: An analysis controlling for tobacco industry affiliation. *J Alzheimers Dis* **19**, 465-480.

[16] Robertson EE, Rankin GO (2006) Human renal organic anion transporters: Characteristics and contributions to drug and drug metabolite excretion. *Pharmacol Therapeut* **109**, 399-412.

[17] Vlassenko AG, Neil Vaishnavi S, Couture L, Sacco D, Shannon BJ, Mach RH, Morris JC, Raichle ME, Mintun MA (2010) Spatial correlation between brain aerobic glycolysis and amyloid-β (Aβ) deposition. *Proc Natl Acad Sci U S A* **107**, 17763-17767.

[18] Coutinho MR, Menescal-de-Oliveira L (2010) Role of homocysteic acid in the guinea pig (Cavia porcellus) anterior cingulate cortex in tonic immobility and the influence of NMDA receptors on the dorsal PAG. *Behav Brain Res* **208**, 237-242.

[19] Folbergrová J, Haugvicová R, Mare P (2001) Attenuation of seizures induced by homocysteic acid in immature rats by metabotropic glutamate group II and group III receptor agonists. *Brain Res* **908**, 120-129.

[20] Hasegawa T, Ukai W, Jo DG, Xu X, Mattson MP, Nakagawa M, Araki W, Saito T, Yamada T (2005) Homocysteic acid induces intraneuronal accumulation of neurotoxic Abeta42: Implication for the pathogenesis of Alzheimer's disease. *J Neurosci Res* **80**, 869-876.

[21] Smith AD, Smith SM, de Jager CA, Whitbread P, Johnston C, Agacinski G, Oulhai A, Bradley KM, Jacoby R, Refsum H (2010) Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: A randomized controlled trial. *PLoS One* **5**, e12244.

[22] Ziemińska E, Stafiej A, Lstrokazarewicz JW (2003) Role of group I metabotropic glutamate receptors and NMDA receptors in homocysteine-evoked acute neurodegeneration of cultured cerebellar granule neurons. *Neurochem Int* **43**, 481-492.