Urine formaldehyde level is inversely correlated to mini mental state examination scores in senile dementia

Zhiqian Tong [a,1], Jinling Zhang [a,1], Wenhong Luo [b,1], Weishan Wang [c], Fangxu Li [a], Hui Li [b], Hongjun Luo [b], Jing Lu [a], Jiangning Zhou [e], You Wan [f], Rongqiao He [a,d,*]

[a] *State Key Lab of Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China*
[b] *The Central Laboratory, Shantou University Medical College, Guangdong 515041, China*
[c] *Beijing Hospital for the Elderly, Beijing, China*
[d] *Lab of Mental Health, Institute of Psychology, Chinese Academy of Sciences, China*
[e] *University of Science and Technology of China, Anhui, China*
[f] *Neuroscience Research Institute, Peking University, Beijing, China*

Abstract

It is widely known that exogenous formaldehyde exposure induces human cognitive impairment and animal memory loss; and recent studies show that formaldehyde at pathological levels induces Aβ deposition and misfolding of tau protein to form globular amyloid-like aggregates. Endogenous formaldehyde may be a marker for progressive senile dementia.

The aim of this study was to investigate the correlation of endogenous formaldehyde in urine of senile dementia and mini mental state examination (MMSE) scores.

Formaldehyde level was analyzed by high-performance liquid chromatography (with fluorescence detection) in human urine from dementia patients ($n = 141$), patients with hypertension ($n = 33$) or diabetes ($n = 16$) and healthy individuals ($n = 38$), autopsy hippocampus samples from Alzheimer's disease (AD) patients and brains of three types of AD animal model: namely, senescence accelerated mice (SAMP8), APP-transgenic mice and APP/PS1-transgenic mice.

In a double-blind study, there was marked elevation of urine formaldehyde levels in patients ($n = 91$) with dementia, and a slight increase in patients ($n = 50$) with mild cognitive impairment. Urine formaldehyde level was inversely correlated with mini mental state examination scores ($Rs = -0.441$, $p < 0.0001$). Furthermore, formaldehyde levels were significantly increased in the autopsy hippocampus from Alzheimer's patients ($n = 4$). In SAMP8 brains the formaldehyde level was significantly increased, suggesting that the endogenous formaldehyde is related to aging in mice. The brain formaldehyde level in APP/PS1-transgenic ($n = 8$) mice at age of 3 months and APP-transgenic ($n = 8$) mice at age of 6 months was increased (0.56 ± 0.02 mM), respectively, as compared with their respective age-matched controls, when these two types of AD-like animals, respectively, started to form Aβ deposits and memory loss obviously. According to the level of formaldehyde in the brain of the transgenic mice, we treated normal mice with formaldehyde (0.5 mM, intraperitoneal administration) and observed the memory loss of the animal in Morris water maze trial.

Cognitive impairments for the senile dementia are probably related to endogenous formaldehyde levels; and the mini mental state examination scores referred to the evaluation of urine formaldehyde level in dementia patients may be used as a non-invasive method for the investigation and diagnosis of senile dementia.

---

* Corresponding author at: State Key Lab of Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Sciences, 15 Da Tun Road, Chao Yang District, Beijing 100101, China. Tel.: +86 10 6488 9876; fax: +86 10 6485 3625.
E-mail address: herq@sun5.ibp.ac.cn (R. He).
[1] These authors contributed equally to this work.

1. Introduction

Senile dementia, for instance those related to Alzheimer's disease (AD), is the most common form of dementia. The presence of amyloid-containing senile plaques together with neurofibrillary tangles has been shown in many investigations to be hallmarks of Alzheimer's disease (Hardy and Selkoe, 2002). In clinical studies, neuropsychological tests such as the mini mental state examination (MMSE) are widely used to evaluate cognitive impairments for diagnosis. Although diagnostic sensitivity and specificity even in early disease stages are improved by cerebrospinal fluid (CSF) markers (Jellinger et al., 2008), more comprehensive test arrays are necessary to improve the reliability of results, particularly in the earliest stages of the disease (Pasquier, 1999; Tombaugh and McIntyre, 1992). Simple, non-invasive tests for early detection of degenerative dementia using biomarkers are urgently required (Corey-Bloom et al., 1995). However, at the present time, no extracerebral diagnostic markers such as urine, serum and connective tissue have been validated for the diagnosis of Alzheimer's disease (Butcher, 2007).

On one hand, it is known that exogenous formaldehyde is a ubiquitous compound with high cyto-toxicity and gene-toxicity. On the other hand, evidences show that endogenous formaldehyde is present in urine, blood and all cells including the brain. Endogenous formaldehyde content of rat brain approaches 0.1 mM/g wet weight as determined by mass spectrometry (Heck et al., 1982). Recent studies show that elevation of endogenous formaldehyde levels may be related to the pathogenic processes of neurodegenerative diseases (Kilburn et al., 1987; Perna et al., 2001). Formaldehyde at low concentrations results in amyloid-β deposition (Chen et al., 2006) and the structure of α-synuclein changes *in vitro* (Floor et al., 2006). Our previous study has also shown that excessive formaldehyde induces misfolding of tau protein and formation of globular amyloid-like aggregates that have high cyto-toxicity *in vitro* and *in vivo* (Nie et al., 2007a,b). As described previously (Kilburn et al., 1987), exposure to formaldehyde leads to memory loss in rats and decreases in acetylcholine and norepinephrine levels, and also induces many forms of human impairment and changes in neurofilament proteins in hippocampal neurons (Perna et al., 2001). These changes show similarity to the pathological features of severe dementia.

Epidemiological investigations have shown that over-expression of semicarbazide-sensitive amine oxidase (SSAO), one of the enzymes in the pathway producing formaldehyde (Ferrer et al., 2002) and deficiency of aldehyde dehydrogenase class 2 (ALDH2), one of the enzymes that degrade formaldehyde (Kamino et al., 2000; Teng et al., 2001) are associated with an increased risk of late-onset Alzheimer's disease. In clinical studies, an increase of endogenous formaldehyde in the blood and CSF has been found in multiple sclerosis (Khokhlov et al., 1989) which is often accompanied by gradual cognitive decline. Abuse of formaldehyde-laced marijuana induces a high occurrence of dysmnesia (Marceaux et al., 2008). These data suggest that elevated endogenous formaldehyde-induced brain lesions are involved in the pathogenesis of Alzheimer's disease. As the urinary system is one of the main ways for endogenous formaldehyde to be excluded from the blood, we postulate that urine formaldehyde may be a non-invasive biomarker for diagnosing dementia. In this work, we sought to determine whether formaldehyde levels in the urine and brain tissues were associated with the degree of senile dementia.

2. Patients and methods

2.1. Participants

Patients ($n = 141$) with varying degrees of dementia underwent a standardized clinical assessment, which included a medical history, and physical and neurological examination together with mini mental state examination (MMSE) laboratory tests, psychometric evaluation, electroencephalograph (EEG) and brain magnetic resonance imaging (MRI). All patients met NINCDS-ADRDA criteria for probable dementia on clinical grounds. The degree of dementia was scored as mild cognitive impairment (MCI), medium and severe as previously described (McKhann, 1984). Thirty-eight healthy controls and 49 patients with common aging-related diseases (hypertension or diabetes) were also evaluated. Informed consent was obtained for all participants, and for patients with dementia, informed consent was obtained from relatives. This clinical investigation was approved by the Ethics Committee for Beijing Hospital for the Elderly, China.

2.2. Brain tissues collection

Autopsy hippocampus tissues from 4 healthy controls and 4 AD patients were provided by the Netherlands Brain Bank (NBB). The age-related and AD-like animal models, senescence accelerated mice, SAMP8 and SAMP1, were provided by Beijing University (China). APP-transgenic mice and APP/PS1-transgenic mice were provided by the Chinese Academy of Medical Sciences (Beijing). All animal experimental procedures described below were approved by the Ethics Committee for the Chinese Academy of Sciences.

2.3. Urine samples collection

Patients with renal disease and urine abnormalities were excluded from the study. Morning urine samples of patients with MCI, medium and severe dementias were taken before breakfast. Urine samples from healthy controls with neither any neurological disorders nor known alcohol and drug abuse, were also taken (Ghanbari et al., 1998).

2.4. Formaldehyde level detected by Fluo-HPLC

Ten milliliters of urine was collected from each participant and immediately placed on ice and stored at −70 °C. To

avoid the samples being thawed too many times, the urine was freshly analyzed or divided into equal parts to be stored for only one time use. After centrifugation (3000 rpm, 4 °C, 10 min), supernatant fractions of urine samples and brain homogenates (weight of brain tissue:ultrapure water = 1:4) were analyzed by high-performance liquid chromatography with fluorescence detection (Fluo-HPLC) as described (Luo et al., 2001). Briefly, an aliquot of 0.2 ml human urine or brain homogenate was pipetted into a 2-ml glass vial, to which 0.8 ml water, 0.1 ml ampicillin solution (2.5 mg/ml, in water) and 0.25 ml TCA (20%, w/v, in water) were added. The vial was capped (air tight) and vortexed vigorously for 30 s and then heated in a 90 °C water bath for 1 h. After cooling to room temperature, the contents of the vial were transferred to a 10-ml glass centrifuge tube. The vial was rinsed twice with 1 ml diethyl ether which was also transferred to the centrifuge tube. After addition of a small amount of sodium chloride (about 0.5 g) the centrifuge tube was vortexed vigorously for 1 min. After centrifugation (1000 × g) for 5 min, the upper layer (organic phase) was transferred to a 5-ml test tube. The aqueous phase in the centrifuge tube was extracted with another 2 ml diethyl ether and the ether extracts were combined. The diethyl ether was evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 0.5 ml acetonitrile–water (50:50), ready for LC analysis. 0.2 ml water was used as a reagent blank instead of the 0.2-ml sample, and the same procedure was followed.

The formaldehyde derivative eluted from the HPLC column at a retention time of 8 min. Methanol, formic acid, acetaldehyde and other aldehyde compounds did not interfere with the analysis of formaldehyde. In fact, no specific chromatographic peak was detected when acetaldehyde was reacted with ampicillin under the same reaction conditions. The formaldehyde standard curve was made with analytic purified formaldehyde (see Fig. 1A). The peak area count (mV) of the fluorescent formaldehyde derivative was used to determine urine formaldehyde from patients with different degrees of dementia, healthy controls, and blanks (water). Laboratory analyses and clinical investigations were carried out in a double-blind manner.

To testify the reaction of resveratrol with formaldehyde, the two compounds (1 mM) were incubated in PBS for 40 min. Then, the solution was analyzed by Fluo-HPLC.

2.5. Behavioral assessment of cognitive functions by Morris water maze test

Spatial learning memory was assessed by the Morris water maze behavioral test, as described previously (Morris, 1984). Mice of four groups were intraperitoneally administrated with physiologic saline, formaldehyde (0.5 mM),
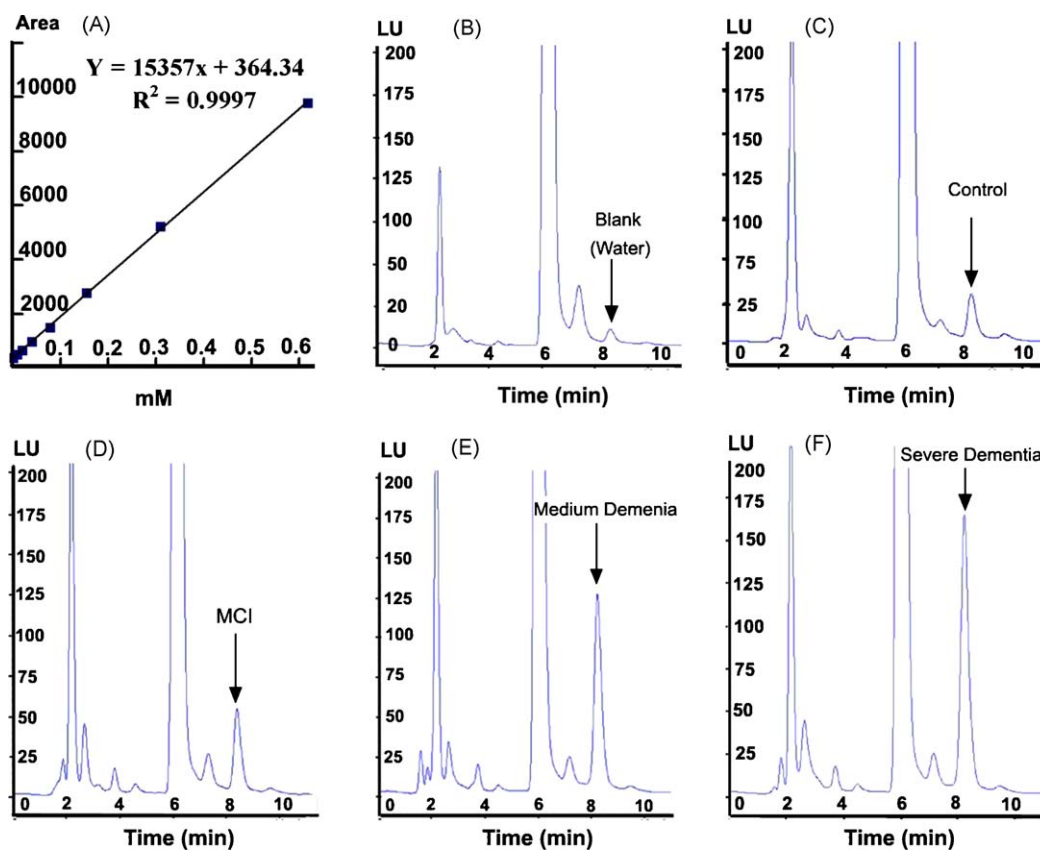

Fig. 1. Chromatogram of human urine samples using Fluo-HPLC. (A) The formaldehyde standard curve. (B) Typical peaks of the blank control—water. (C) Peaks of urine of healthy controls. (D) Peaks of patients with low-grade dementia. (E) Peaks of medium dementia. (F) Peaks of severe dementia.

Table 1
Correlation between urine formaldehyde level and MMSE score.

Group	Age, mean ± SEM (range)	Sex, F/M, (no.)	MMSE score, mean ± SEM (range)	FA level (mM)	Analysis of correlation between FA level and MMSE Score
Healthy control (n = 33)	67.373 ± 0.661 (60–85)	20/18	28.161 ± 0.142 (27–30)	0.083 ± 0.001	Spearman's rho = −0.441
Degrees of dementia (n = 139)					
MCI (n = 50)	69.729 ± 2.043 (54–89)	29/21	23.486 ± 0.270 (21–26)	0.196 ± 0.69	Number of obs = 179
Medium (n = 39)	74.566 ± 1.556 (54–06)	18/21	16.132 ± 0.461 (10–20)	0.293 ± 0.050	Test of Ho: x and y
Severe (n = 52)	76.363 ± 1.181 (57–91)	30/22	2.029 ± 0.384 (0–9)	0313 ± 0.046	independent p < 0.0001**

A severity score according to the clinic mini mental state examination (MMSE). Degrees of dementia: 27–30 as healthy control; 21–26 as MCI; 10–20 as medium dementia; and 0–9 as severe dementia. **$p < 0.000087$.

formaldehyde with resveratrol (0.5 mM), and resveratrol alone (0.5 mM), respectively, for 30 days. After that, they were tested in a pool filled with water. Mice were trained to mount a hidden/submerged (1.5 cm below water surface) escape platform in a restricted region of the pool. Spatial memory was assessed by recording the latency time for the animal to escape from the water onto a submerged escape platform as a function of the number of learning trials during the learning phase. Seven days after the learning phase, mice were subjected to a 60-s probe trial wherein the escape platform was removed. The water maze activity was monitored with the Instrument Poly-Track video tracking system (San Diego Instruments). After behavior assessment, formaldehyde levels in the brains of these four groups were detected by Fluo-HPLC.

2.6. Data analysis

Correlations between urine formaldehyde level and MMSE scores were assessed using the Spearman correlation coefficient. Statistical analysis (ANOVA) was conducted with SPSS for Windows (version 12.0.1). Data are expressed as means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control.

3. Results

3.1. Changes in urine formaldehyde of patients with senile dementia

First, in order to study the correlation between the degree of senile dementia and the level of urine formaldehyde, urine samples from different patients were analyzed as shown in Fig. 1. The fluorescent peak area of the formaldehyde derivative reacted with ampicillin exhibits positively with the degree of dementia (see Fig. 1). Table 1 shows that the level of urine formaldehyde was inversely correlated with MMSE scores of the patients ($Rs = −0.441$, $p < 0.001$). Meantime, we also found that urine formaldehyde level was not markedly correlated with age of patients ($Rs = 0.153$, $p = 0.068$). The urine formaldehyde levels in patients with mild cognitive impairment (MCI), medium and severe degrees of dementia exceed 42% (21/50), 82.05%

(32/39) and 88.46% (46/52) that of healthy controls, respectively (see Supplementary Figure 1). For patients with MCI, urine formaldehyde level was slightly elevated ($p = 0.098$) compared to that of healthy controls (see Fig. 2A). However, the concentration of urine formaldehyde from patients with medium and severe degrees of dementia was significantly higher than those of healthy controls ($p < 0.001$). Furthermore, urine formaldehyde level was markedly elevated in all types of patients with medium and severe dementia, including 41 patients with vascular dementia, 22 with senile dementia, 12 with mixed dementia and 16 patients with other types of dementia (see Fig. 2B).

3.2. Urine formaldehyde levels of dementia patients with age-related diseases

A recent epidemiological survey has shown that hypertension and diabetes play roles as vascular risk factors for senile dementia (Martinez et al., 2008). Thus, we paid attention to patients with these disorders. Our investigation included 54 patients with diabetes and 24 ones with hypertension (see Fig. 2C). Among them, 21 diabetes and 8 hypertension patients had cognitive disorders (dementia). The incidence of dementia was 38.89% for diabetic patients and 33.33% for those with hypertension, showing a much higher incidence than that (2.5–5%) of the 60–65 year old age bracket in an epidemiological survey of Alzheimer's disease in Chinese subjects (Yang, 2008). Urine formaldehyde in these patients was also significantly higher ($p < 0.01$) than that of healthy individuals. However, we did not detect an elevated level of urine formaldehyde in the patients with only hypertension or diabetes, i.e. no significant differences in urine formaldehyde levels were observed between healthy controls and patients with hypertension (or diabetes) whose MMSE scores were normal (see Fig. 2D). But, the levels of the diabetes (or hypertension) patients with and without dementia are significantly different from those of the control participants (see Fig. 2C)

3.3. Brain formaldehyde was increased in AD patients and AD animal models

To clarify the relationship between endogenous formaldehyde and dementia, we measured the formaldehyde in human
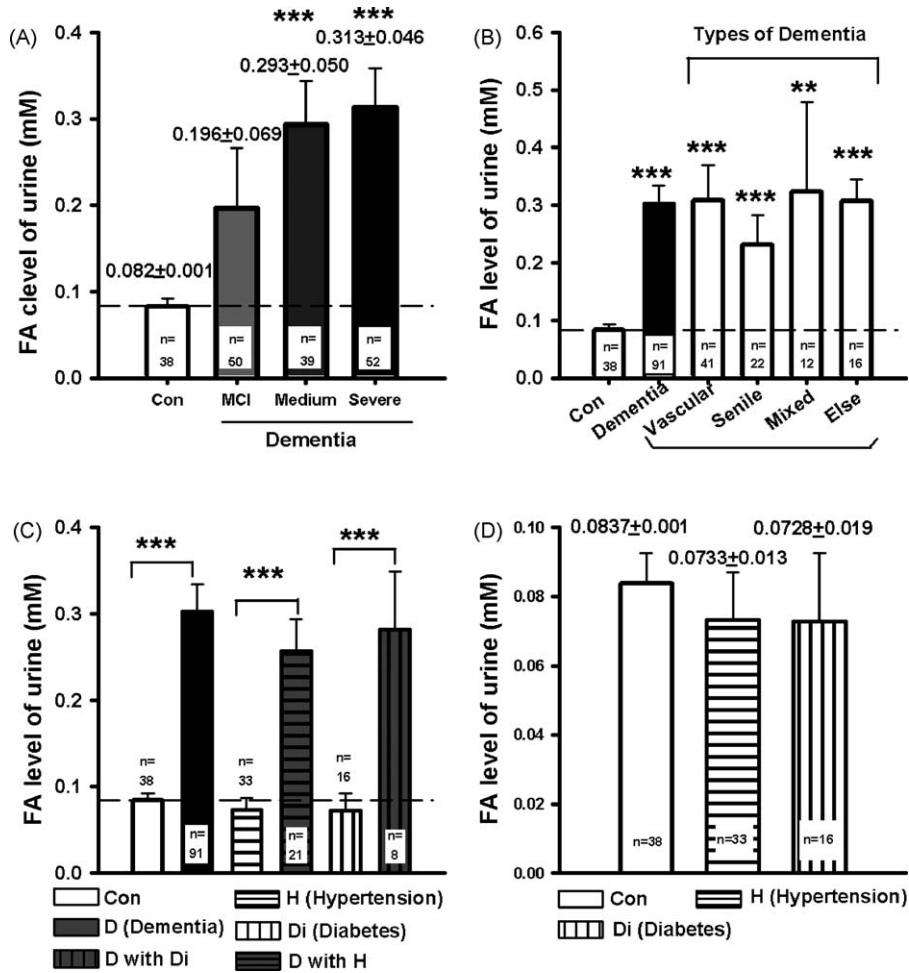
Fig. 2. Changes in formaldehyde levels of human urine. (A) Urine formaldehyde levels in patients with MCI ($n = 50$), medium ($n = 39$) and severe dementia ($n = 52$). (B) Urine formaldehyde levels in different types of dementia patients ($n = 91$). (C) Urine formaldehyde level in patients with dementias and hypertension (21 of 54) or diabetic (8 of 24). (D) Urine formaldehyde level in hypertension patients ($n = 33$) and diabetic patients ($n = 16$) without dementia. $**p < 0.01$; $***p < 0.001$.

and animal brains. As depicted in Fig. 3A, formaldehyde level from homogenates of autopsy hippocampus of AD patients ($n = 4$) showed a marked elevation ($p < 0.05$) as compared with those of healthy controls ($n = 4$).

Aging is the most important risk factor for senile dementia, and thus we investigated the correlation of endogenous formaldehyde with aging. Senescence accelerated SAMP8 mice which have deficiency in learning and memory and SAMP1 mice which have no distinct cognitive disorders were employed to test the endogenous formaldehyde levels in brain (see Fig. 3B). The formaldehyde level in brain homogenates from 5- and 10-month-old SAMP8 mice was higher than that for SAMP1 counterparts. This indicates that the concentration of cerebral endogenous formaldehyde is related to aging in SAMP mice.

Furthermore, to investigate whether endogenous formaldehyde levels are related to dementia, APP/PS1-transgenic mice were employed as an AD model because of their more robust amyloid-β (Aβ) deposition and dementia

(Trinchese et al., 2004). The brain formaldehyde of APP/PSI transgenic mice was significantly higher ($p < 0.01$) at 3 months than that of their respective age-matched controls (see Fig. 3C). It is known that this transgenic mouse starts to deposit Aβ in the brain at the age of 2 months and shows abnormal long-term potentiation (LTP) as early as the age of 3 months. It appears that the increase of brain formaldehyde accompanies the initiation of Aβ deposits and the onset of abnormal memory in these transgenic mice.

To confirm that the endogenous formaldehyde level of AD-like transgenic mice was higher than the control during the initiation of Aβ deposits, APP-transgenic mice were used. As Aβ deposits in the brain of these transgenic mice begin at 6–7 months (Kawarabayashi et al., 2001), we measured the formaldehyde level in the brain at 6 and 12 months. As shown in Fig. 3D, brain formaldehyde was significantly increased at 6 months, and became similar to that of the controls at 12 months. This suggests again that the onset of Aβ deposits is accompanied with an increase in brain formaldehyde.
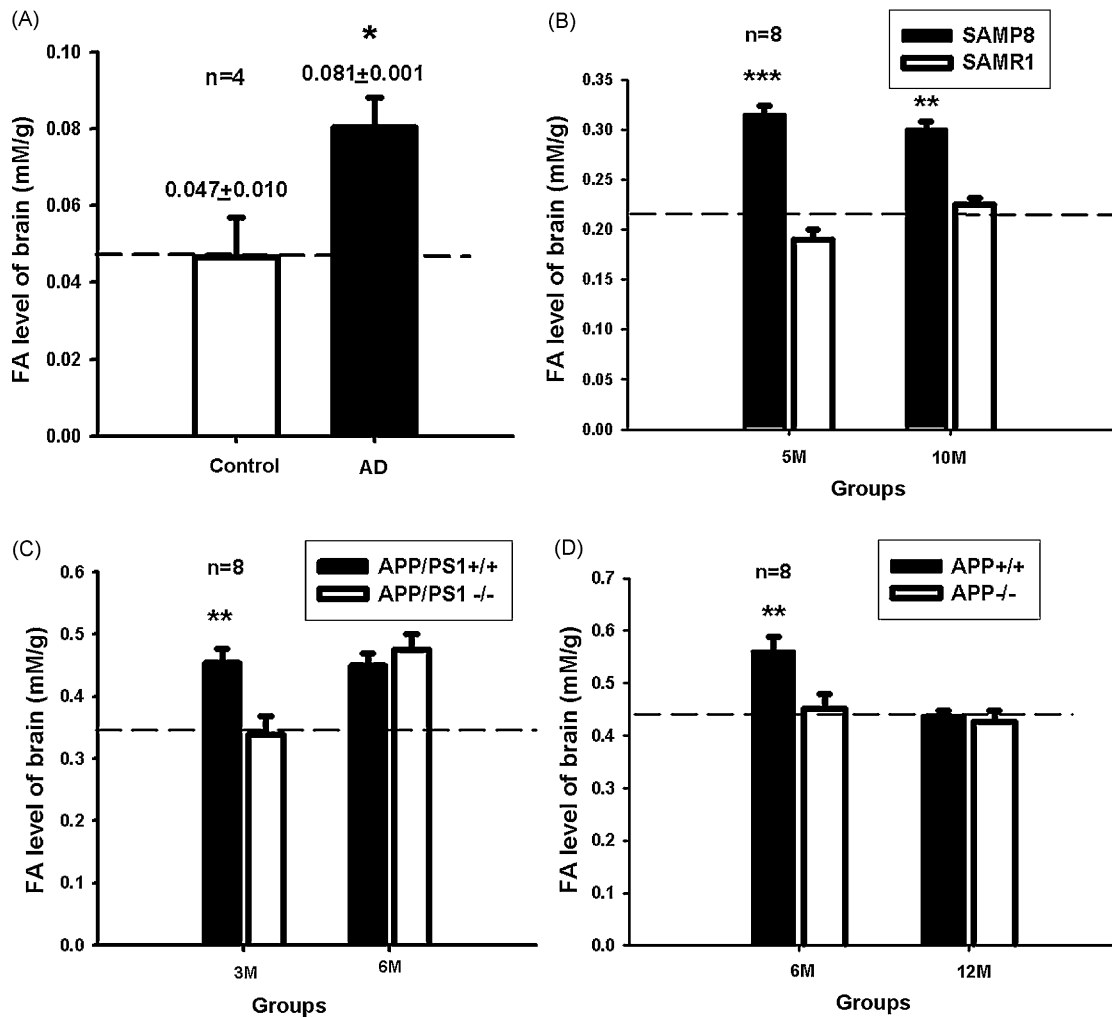
Fig. 3. Formaldehyde levels in the autopsy hippocampus from AD patients and the brains of AD-like animal models. (A) The autopsy hippocampus of AD patients and healthy controls. (B) SAMP8 mice at 5 and 10 months old and SAMP1 mice at age-matched. (C) APP-transgenic mice at 6 and 12 months old and controls. (D) APP/PS1-transgenic mice at 3 and 6 months old and controls. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

3.4. Formaldehyde-induced impairments of mice in spatial reference memory function

According to the level of formaldehyde in the brain of the transgenic mice as mentioned above, 0.5 mM formaldehyde was intraperitoneally administered to normal adult mice during learning trials in a Morris water maze test. We found that spatial reference memory function was significantly impaired after treated with formaldehyde for 30 days. Resveratrol, a natural formaldehyde scavenger (see Fig. 4C), obviously attenuated this damage, as reflected by reduced escape latency time as well as progressive learning trials (see Fig. 4A and B). The resveratrol could functionally remove formaldehyde in mice, as the formaldehyde level in brains of mice treated with reveratrol (0.5 mM) was significantly decreased (see Fig. 4D). Formaldehyde levels in brains of rats were inversely correlated to behavioral performance that time of staying in the target quadrant of the hidden platform (see Fig. 4E). This suggests

that excessive endogenous formaldehyde induces cognitive impairment.

4. Discussion

Formaldehyde is produced constitutively in the human body, and is present intracellularly and extracellularly in nuclei (Shi et al., 2004), cytoplasm (Lyles, 1996), blood (Andres et al., 2001) and CSF (Khokhlov et al., 1989). Several enzymes in the liver such as ALDH2, ADH1 and catalase can catalyze the reaction that oxidizes formaldehyde to formic acid (Teng et al., 2001). Catalysis of the conversion of formaldehyde to formate via glutathione-dependent formaldehyde dehydrogenase (FDH; also known as class III alcohol dehydrogenase, ADH3), takes place in all tissues of the human body as a consequence of the regulation of endogenous formaldehyde (Heck et al., 1982). By this pathway, endogenous or exogenous formaldehyde is elimi-
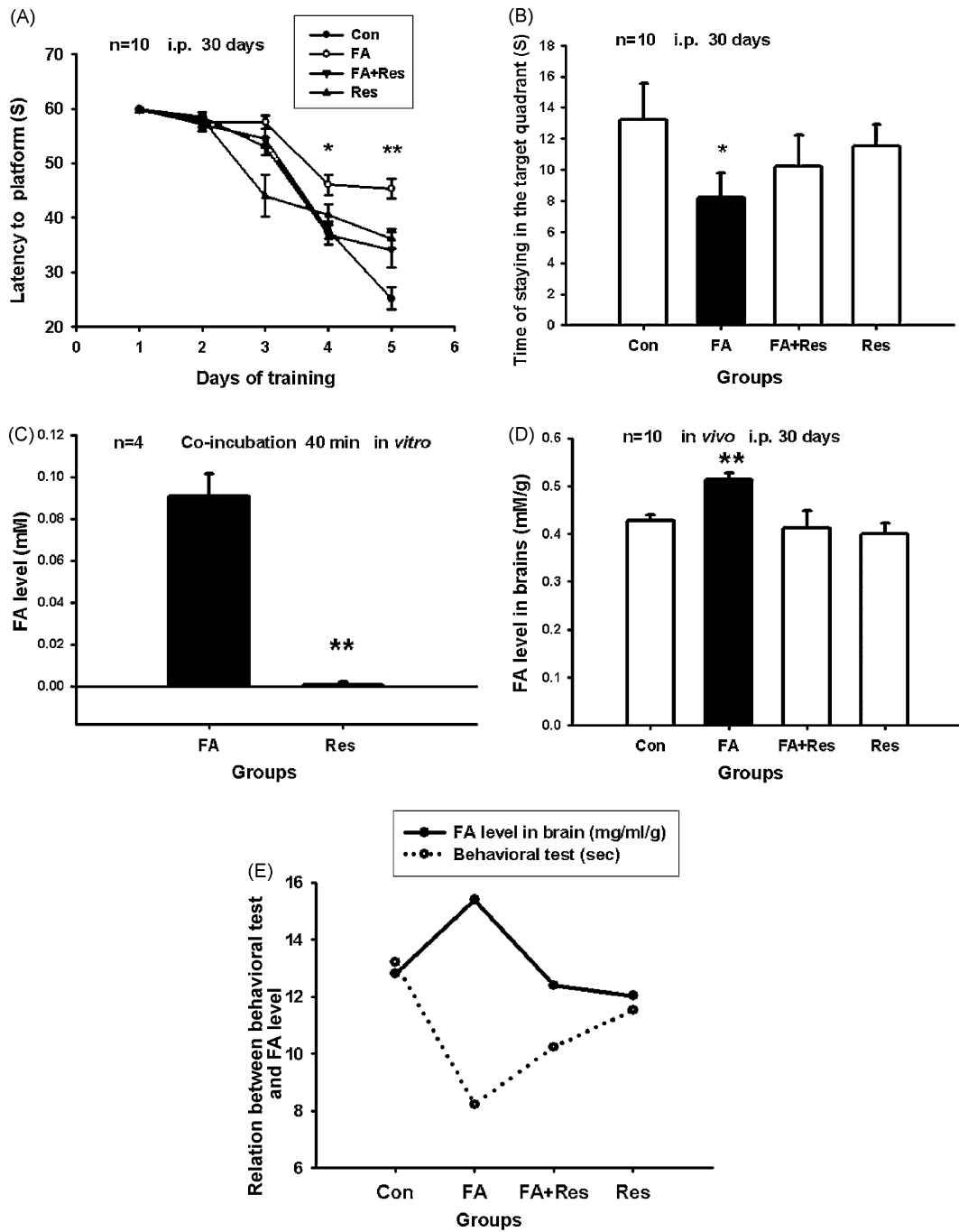
Fig. 4. The influence of formaldehyde at pathological level on spatial memory in adult mice by Morris water maze test. (A) Hidden platform acquisition. Latency score represents time taken to escape to the platform from the water. Mice of four groups were intraperitoneally administrated with physiologic saline, formaldehyde (0.5 mM), formaldehyde with resveratrol (0.5 mM), and resveratrol alone (0.5 mM), respectively, for 30 days. (B) Probe trial. Time is calculated as staying in the target quadrant of the hidden platform. (C) The reaction of resveratrol with formaldehyde in PBS for 40 min. (D) Formaldehyde level in brains of rats treated with resveratrol by intraperitoneal administration. (E) Relation between the FA level in brain and rat behavioral performance (time of staying in the target quadrant of the hidden platform). $*p < 0.05$; $**p < 0.01$.

nated from the body as metabolites, primarily as formate or CO_2. Endogenous formaldehyde accumulates in cells when FDH activity decreases. Excess levels of formaldehyde can be involved in cross-linkages between proteins and DNA or can enter the "one-carbon intermediary metabolic pool".

Formaldehyde is able to penetrate the blood–brain barrier (Shcherbakova et al., 1986), is transported in circulation and is eliminated from the body through urine and breath in addition to enzymic catalytic pathways (Estonius et al., 1996). Formaldehyde is much more rapidly dialyzed by the kidneys

and excreted through the urinary bladder than by respiration (Spanel et al., 1999). Laboratory tests can directly measure endogenous formaldehyde in urine, blood, and breath.

As an active organic molecule, formaldehyde reacts rapidly with protein α-/ε-amino groups. However, although serum proteins react rapidly with formaldehyde, there are few proteins in urine to react with formaldehyde. Thus measurement of urine formaldehyde by reacting with ampicillin (fluorescent reagent) is much less affected than that of serum formaldehyde. Urine is also much more convenient to sample and store than blood, CSF or brain tissue. Thus this study may provide a simple way for the diagnosis of senile dementia patients by using urine formaldehyde as a clinical criterion.

To guarantee the reproducibility of analytical results, aliquots of the first urine in the morning were taken before patients had breakfast. Dementia patients with renal disease and normal controls who had consumed alcohol within the previous week, or taken medicine within the previous two weeks were excluded. During trials, all subjects had a normal diet, except for any consumption of fatty and spicy foods. Patients with the peripheral bladder or prostate cancer were excluded because their urine formaldehyde was found to increase (2–8 folds higher than normal) as reported previously (Spanel et al., 1999). Proliferating cancer cells are known to secrete more formaldehyde (Tong et al., 2008). This is to say, tumor-derived formaldehyde is accumulated to a high level in urine, but it is not resulted from damage of kidney function. However, it is relatively easy to distinguish patients with these types of cancer by standard clinical diagnostic procedures. According to the reports of other authors who investigate dementia patients in clinical trials (Tombaugh and McIntyre, 1992), we used the mini mental state examination (MMSE) as an entry criterion for different degrees of aging dementia.

We found that MMSE scores were significantly correlated with urine formaldehyde (Rs = −0.441, $p < 0.001$), and urine formaldehyde levels of patients with medium and severe degrees of dementia were 3-fold higher than those of the controls. Furthermore, the urine formaldehyde concentrations of dementia patients were positively correlated with the degree of dementia (percentages of patients with enhanced formaldehyde levels were 42% (21/50), 82.05% (32/39) and 88.46% (46/52), for MCI, medium and severe dementia patients, respectively). This suggests that urine formaldehyde concentration can be used as a criterion to support MMSE scores in the diagnosis of patients with medium and severe dementia.

For MCI patients, however, urine formaldehyde concentrations were not altered as significantly as those for medium and severe dementia patients. The urine formaldehyde level for senile dementia patients with MCI was slightly elevated ($p = 0.098$) compared to that of healthy controls. However, in 42% (21/50) of patients, urine formaldehyde level exceeded the average level of healthy controls. Thus, endogenous formaldehyde level may also be useful for identifying incipient dementia.

At present, there are no clinical chemical methods for identifying MCI. Urine formaldehyde level is related to MMSE scores of MCI-like aging patients to a certain extent. This provides a chance for MMSE a widely and commonly used for screening cognitive function to be combined with the urine formaldehyde assay. In fact, 42% of MCI patients had significantly increased levels of urine formaldehyde. Patients with MCI at risk of developing dementia are usually followed clinically for 4–6 years, and the urine formaldehyde in the remaining 58% (29/50) of MCI-like patients in our study should be further consecutively investigated.

Urine formaldehyde concentration was markedly elevated in patients with different types of medium and severe dementia, for example 41 patients with vascular dementia, 22 with senile dementia, 12 with mixed dementia and 16 patients with other types of dementia all showed elevated levels of urine formaldehyde. Thus, urine formaldehyde and MMSE scores cannot be used on their own to distinguish between different types of dementia. Elevation of urine formaldehyde could be used to screen for cognitive impairments but does not help us to distinguish between vascular and senile dementia. However, the mechanism underlying the relationship between urine formaldehyde and cognitive impairment remains to be further investigated.

To support our clinical investigation data, we analyzed the formaldehyde content of the autopsy hippocampus from AD patients (Alzheimer's disease is a disease that results from brain lesion (Hardy and Selkoe, 2002)). As indicated in the results section, the concentration of endogenous formaldehyde was increased in the autopsy hippocampus relative to controls. A natural formaldehyde capturer (Szende et al., 1998), resveratrol, provides effective defense against cancer and neurodegenerative disease including Alzheimer's disease (Rocha-González et al., 2008; Saiko et al., 2008). Recent research has also shown that dietary supplementation with resveratrol reduces senile plaque (SP) pathology in a APP/PS1-transgenic model of Alzheimer's disease (Karuppagounder et al., 2008). This supports the hypothesis that excessive endogenous formaldehyde is involved in the pathogenesis of these diseases.

To provide indirect support that endogenous formaldehyde has potential for use as a clinical criterion, we first employed SAMP1 and SAMP8 mice (Banks et al., 2007) to test whether endogenous formaldehyde levels are related to aging. As reported previously, SAMP8 mice at 2–5 months old develop memory impairment which is associated with level of acetylcholine and activity of choline acetyltransferase (ChAT) decreases in the hippocampus (Kuo et al., 1996; Onozuka et al., 2002). Elevation of formaldehyde levels in the brain by formaldehyde injection also leads to memory loss with levels of neurotransmitters such as acetylcholine and norepinephrine decreases in rats (Kilburn et al., 1987; Perna et al., 2001). In our results, significant elevation of endogenous formaldehyde in the brain occurred simultaneously with the onset of cognitive impairment in this senile dementia animal model. Although over-expression of APP gene has

been found in brain, there are no typical senile plaques in the hippocampus and cortex in this senescence accelerated animal model (Banks et al., 2007). This hints that excessive formaldehyde directly induces cognitive impairments.

In transgenic AD animal model, increases in brain formaldehyde and the development of abnormal LTP levels concurred (from as early as 3 months of age) to in APP/PS1-transgenic mice (Trinchese et al., 2004), Interestingly, reduced LTP parallels plaque appearance, increased Aβ levels and abnormal short-term memory (working memory). In APP-transgenic mice, brain formaldehyde also increased at the early stage (6 months old) during which Aβ starts to deposit in the brain. Moreover, in both APP/PS1- (6 months old) and APP- (12 months old) transgenic mice, brain formaldehyde levels were not significantly different from those of their respective age-matched controls (see Fig. 3C and D), but more and more typical senile plaques were detected in the brain of these two transgenic types of AD mice (Trinchese et al., 2004; Karuppagounder et al., 2008). Previous studies have shown that excessive formaldehyde reacts with Aβ1–42 to form amyloid-β deposition (Chen et al., 2006), and formaldehyde at pathological level induces misfolding of tau protein to the formation of globular amyloid-like aggregates (Nie et al., 2007a,b). This suggests that excessive endogenous formaldehyde may play a role in the initiation of Aβ deposition in these transgenic mice.

Unexpectedly, although immunization with Aβ1–42 results in clearance of amyloid plaques in APP-transgenic mouse and patients with Alzheimer's disease, this clearance has not prevented progressive neurodegeneration. Thus, reducing total Aβ may be of limited therapeutic benefit to recovery of cognitive decline in a higher mammalian model of human brain aging and disease (Head et al., 2008; Holmes et al., 2008). We speculate that although Aβ1–42 plays a critical role in formation of senile plaques of AD; abnormal excessive formaldehyde not only results in Aβ deposition but also may directly induce cognitive impairments. Therefore, the role of endogenous formaldehyde in pathogeneses of Alzheimer's disease should be further investigated. These data also hint that these AD-like animal models partially simulate pathological characters of Alzheimer's disease in human beings.

To explore direct evidence that formaldehyde induces memory loss, normal adult mice were treated by formaldehyde (0.5 mM) for 30 days. Spatial reference memory function impairments of adult mice were significantly observed during learning trials in a Morris water maze test (see Fig. 4A and B). Previous research also indicates that memory behavioral changes in rats are due to chronic oral and systemic formaldehyde (60 days) (Bhatt and Panchal, 1992). Furthermore, other evidence shows that formaldehyde (i.p. 10 days) induces oxidative frontal cortex and hippocampal tissue damage of rats (Gurel et al., 2005). Formaldehyde is a small molecule which is capable of penetrating the blood–brain barrier (Shcherbakova et al., 1986). Exposure to exogenous gaseous formaldehyde leads to its accumula-

tion in brain of rats (Cui, 1996). In our study, both elevated formaldehyde level in the brain and memory loss were found after formaldehyde treatment (i.p.). Resveratrol, a natural formaldehyde scavenger (Szende et al., 1998), obviously attenuated the formaldehyde-induced damage of learning and memory by decreasing formaldehyde level *in vitro* and *in vivo* (see Fig. 4C–E). Deficiency of aldehyde dehydrogenase class 2 (ALDH2), one of the enzymes that degrade formaldehyde, has been found to be associated with an increased risk of late-onset Alzheimer's disease, and leads to memory loss (Kamino et al., 2000; Teng et al., 2001). These data further indicate that excessive endogenous formaldehyde directly induces cognitive impairment. Although Aβ1–42 plays a critical role in formation of senile plaques of AD, abnormal formaldehyde not only results in Aβ deposition but also directly induces memory loss. Therefore, endogenous formaldehyde probably participates in the pathogenesis of Alzheimer's disease.

As described previously (Tong et al., 2008), several mechanisms may lead to the elevation of formaldehyde levels *in vivo*, inducing a chronic imbalance between the production and degradation of endogenous formaldehyde with age. First, as hypothesized by Barker and coworkers (Barker et al., 1980), the excessive formaldehyde resulted from the disorder of "one-carbon units", leads to a genetically determined brain lesion. Deficiency of folate, B12, or 5,10-methylene THF-reductase (Reynolds, 2006; Stumpf, 1997), which is correlated with the risk of stroke and dementia, leads to formaldehyde accumulation (Kalász, 2003) via the "one-carbon-cycle". For example, injection (i.c.v.) of S-adenosylmethionine (SAM) induces an increase in formaldehyde to neurotoxic levels in the brain (Barker et al., 1980; Lee et al., 2008). Second, endogenous formaldehyde is produced by microsomal cytochrome P-450 and is dependent upon oxidation of xenobiotics, including various drugs and environmental pollutants such as neurotoxic compounds: formaldehyde, paraquat, and mercury (Retfalvi et al., 1998). N-demethylation, O-demethylation and S-demethylation result in the production of formaldehyde (Taranenko and Efimova, 2007). Third, over-expression or deficiency of genes involved in formaldehyde metabolism such as SSAO (Ferrer et al., 2002) and ALDH2 (Kamino et al., 2000; Teng et al., 2001), also induces formaldehyde accumulation. In addition, formaldehyde is also generated by lysine-specific demethylase 1 (LSD1), lipid peroxidation (LPO) and stress (Yu et al., 1997; Kang et al., 2007). These environmental and genetic factors related with formaldehyde metabolism may affect AD pathogenesis.

As described above, excessive endogenous formaldehyde leads to cognitive impairment. Urine formaldehyde level is positively correlated to the degree of aging dementia. Evaluation of endogenous formaldehyde in the urine has potential for use as a non-invasive and convenient method for the investigation and diagnosis of dementia. Our work also raises the possibility that urine formaldehyde could be used as a marker for the assessment of therapeutics.

Disclosure statement

The authors declare that they have no competing interests to disclose.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2009.07.013.

References

Andres, N., Lizcano, J.M., Rodriguez, M.J., Romera, M., Unzeta, M., Mahy, N., 2001. Tissue activity and cellular localization of human semicarbazide-sensitive amine oxidase. Journal of Histochemistry and Cytochemistry 49, 209–218.

Banks, W.A., Farr, S.A., Morley, J.E., Wolf, K.M., Geylis, V., Steinitz, M., 2007. Anti-amyloid beta protein antibody passage across the blood–brain barrier in the SAMP8 mouse model of Alzheimer's disease: an age-related selective uptake with reversal of learning impairment. Experimental Neurology 206, 248–256.

Barker, S.A., Carl, G.F., Monti, J.A., 1980. Hyperformaldehydism: a unifying hypothesis for the major biochemical theories of schizophrenia. Medical Hypotheses 6, 671–686.

Bhatt, H.V., Panchal, G.M., 1992. Behavioural change in rats due to chronic oral and systemic formaldehyde. Indian Journal of Physiology and Pharmacology 36, 270–272.

Butcher, J., 2007. Urine tests for Alzheimer's disease—are they fool's gold? Lancet Neurology 6, 106–107.

Chen, K., Maley, J., Yu, P.H., 2006. Potential implications of endogenous aldehydes in [beta]-amyloid misfolding, oligomerization and fibrillogenesis. Journal of Neurochemistry 99, 1413.

Corey-Bloom, J., Thal, L.J., Galasko, D., Folstein, M., Drachman, D., Raskind, M., Lanska, D.J., 1995. Diagnosis and evaluation of dementia. Neurology 45, 211–218.

Cui, Xiangli, 1996. Inhaled formaldehyde on the effects of GSH level and distribution of formaldehyde. China Journal of Preventive Medicine 30, 186.

Estonius, M., Svensson, S., Höög, J.O., 1996. Alcohol dehydrogenase in human tissues: localisation of transcripts coding for five classes of the enzyme. FEBS Letters 397, 338–342.

Ferrer, I., Lizcano, J.M., Hernández, M., Unzeta, M., 2002. Overexpression of semicarbazide sensitive amine oxidase in the cerebral blood vessels in patients with Alzheimer's disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Neuroscience Letters 321, 21–24.

Floor, E., Maples, A.M., Rankin, C.A., Yaganti, V.M., Shank, S.S., Nichols, G.S., O'Laughlin, M., Galeva, N.A., Williams, T.D., 2006. A one-carbon modification of protein lysine associated with elevated oxidative stress in human substantia nigra. Journal of Neurochemistry 97, 504.

Ghanbari, H., Ghanbari, K., Beheshti, I., Munzar, M., Vasauskas, A., Averback, P., 1998. Biochemical assay for AD7C-NTP in urine as an Alzheimer's disease marker. Journal of Clinical Laboratory Analysis 12, 285–288.

Gurel, A., Coskun, O., Armutcu, F., Kanter, M., Ozen, O.A., 2005. Vitamin E against oxidative damage caused by formaldehyde in frontal cortex and hippocampus: biochemical and histological studies. Journal of Chemical Neuroanatomy 29, 173–178.

Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353–356.

Head, E., Pop, V., Vasilevko, V., Hill M, Saing, T., Sarsoza, F., Nistor, M., Christie, L.A., Milton, S., Glabe, C., Barrett, E., Cribbs, D., 2008. A two-year study with fibrillar beta-amyloid (Abeta) immunization in aged canines: effects on cognitive function and brain Abeta. Journal of Neuroscience 28, 3555–3566.

Heck, H., White, E.L., Casanova-Schmitz, M., 1982. Determination of formaldehyde in biological tissues by gas chromatography/mass spectrometry. Biomedical Mass Spectrometry 9, 347–353.

Holmes, C., Boche, D., Wilkinson, D., Yadegarfar, G., Hopkins, V., Bayer, A., Jones, R.W., Bullock, R., Love, S., Neal, J.W., Zotova, E., Nicoll, J.A., 2008. Long-term effects of abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 372, 216–223.

Jellinger, K.A., Janetzky, B., Attems, J., Kienzl, E., 2008. Biomarkers for early diagnosis of Alzheimer disease: Alzheimer associated gene—a new blood biomarker? Journal of Cellular and Molecular Medicine 12, 1094–1117.

Kalász, H., 2003. Biological role of formaldehyde, and cycles related to methylation, demethylation, and formaldehyde production. Mini Reviews in Medicinal Chemistry 3, 175–192.

Kamino, K., Nagasaka, K., Imagawa, M., Yamamoto, H., Yoneda, H., Ueki, A., Kitamura, S., Namekata, K., Miki, T., Ohta, S., 2000. Deficiency in mitochondrial aldehyde dehydrogenase increases the risk for late-onset Alzheimer's disease in the Japanese population. Biochemical and Biophysical Research Communications 273, 192–196.

Kang, J.E., Cirrito, J.R., Dong, H., Csernansky, J.G., Holtzman, D.M., 2007. Acute stress increases interstitial fluid amyloid-beta via corticotropin-releasing factor and neuronal activity. Proceedings of the National Academy of Sciences of the United States of America 104, 10673–10678.

Karuppagounder, S.S., Pinto, J.T., Xu, H., Chen, H.L., Beal, M.F., Gibson, G.E., 2008. Dietary supplementation with resveratrol reduces plaque pathology in a transgenic model of Alzheimer's disease. Neurochemistry International (Epub ahead of print).

Kawarabayashi, T., Younkin, L.H., Saido, T.C., Shoji, M., Ashe, K.H., Younkin, S.G., 2001. Age-dependent changes in brain, CSF, and plasma amyloid {beta} protein in the Tg2576 transgenic mouse model of Alzheimer's disease. Journal of Neuroscience 21, 10.

Khokhlov, A.P., Zavalishin, I.A., Savchenko, I.N., Dziuba, A.N., 1989. Disorders of formaldehyde metabolism and its metabolic precursors in patients with multiple sclerosis. Zhurnal nevrologii i psikhiatrii imeni S. S. Korsakova 89, 45–48.

Kilburn, K.H., Warshaw, R., Thornton, J.C., 1987. Formaldehyde impairs memory, equilibrium, and dexterity in histology technicians: effects which persist for days after exposure. Archives of Environmental Health 42, 117–120.

Kuo, H., Ingram, D.K., Walker, L.C., Tian, M., Hengemihle, J.M., Jucker, M., 1996. Similarities in the age-related hippocampal deposition of periodic acid-Schiff-positive granules in the senescence-accelerated mouse (SAM P8) and C57BL/6 mouse strains. Neuroscience 74, 733–740.

Lee, E.S., Chen, H., Hardman, C., Simm, A., Charlton, C., 2008. Excessive S-adenosyl-L-methionine-dependent methylation increases levels

of methanol, formaldehyde and formic acid in rat brain striatal homogenates: Possible role in S-adenosyl-l-methionine-induced Parkinson's disease-like disorders. Life Sciences 83, 821–827.

Luo, W., Li, H., Zhang, Y., Ang, C.Y.W., 2001. Determination of formaldehyde in blood plasma by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography B: Biomedical Sciences and Applications 753, 253–257.

Lyles, G.A., 1996. Mammalian plasma and tissue-bound semicarbazide-sensitive amine oxidases: biochemical, pharmacological and toxicological aspects. International Journal of Biochemistry and Cell Biology 28, 259–274.

Marceaux, J.C., Dilks, L.S., Hixson, S., 2008. Neuropsychological effects of formaldehyde use. Journal of Psychoactive Drugs 40, 207–210.

Martinez, F., Flores, C., de las Heras, P., Lekumberri, M., Menocal, G., Imirizaldu, Z., Jose, J., 2008. Risk factors for dementia in the epidemiological study of Munguialde County (Basque Country-Spain). BMC Neurology 8, 39.

McKhann, G., 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease, vol. 34. AAN Enterprises, pp. 939–944.

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. Journal of Neuroscience Methods 11, 47–60.

Nie, C.L., Wang, X.S., Liu, Y., Perrett, S., He, R.Q., 2007a. Amyloid-like aggregates of neuronal tau induced by formaldehyde promote apoptosis of neuronal cells. BMC Neuroscience 8, 9.

Nie, C.L., Wei, Y., Chen, X., Liu, Y.Y., Dui, W., Liu, Y., Davies, M.C., Tendler, S.J.B., He, R.G., 2007b. Formaldehyde at low concentration induces protein tau into globular amyloid-like aggregates in vitro and in vivo. PLoS ONE, 2.

Onozuka, M., Watanabe, K., Fujita, M., Tomida, M., Ozono, S., 2002. Changes in the septohippocampal cholinergic system following removal of molar teeth in the aged SAMP8 mouse. Behavioural Brain Research 133, 197–204.

Pasquier, F., 1999. Early diagnosis of dementia: neuropsychology. Journal of Neurology 246, 6–15.

Perna, R.B., Bordini, E.J., Deinzer-Lifrak, M., 2001. A case of claimed persistent neuropsychological sequelae of chronic formaldehyde exposure clinical, psychometric, and functional findings. Archives of Clinical Neuropsychology 16, 33–44.

Retfalvi, T., Nemeth, Z.I., Sarudi, I., Albert, L., 1998. Alteration of endogenous formaldehyde level following mercury accumulation in different pig tissues. Acta Biologica Hungarica 49, 375–379.

Reynolds, E., 2006. Vitamin B12, folic acid, and the nervous system. Lancet Neurology 5, 949–960.

Rocha-González, H.I., Ambriz-Tututi, M., Granados-Soto, V., 2008. Resveratrol: a natural compound with pharmacological potential in neurodegenerative diseases. CNS Neuroscience Therapy 14, 234–247.

Saiko, P., Szakmary, A., Jaeger, W., Szekeres, T., 2008. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutation Research—Reviews in Mutation Research 658, 68–94.

Shcherbakova, L.N., Tel'pukhov, V.I., Trenin, S.O., Bashilov, I.A., Lapkina, T.I., 1986. Permeability of the blood–brain barrier to intra-arterial formaldehyde. Biulleten' eksperimental'noi biologii i meditsiny 102, 573–575.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119, 941–953.

Spanel, P., Smith, D., Holland, T.A., Singary, W.A., Elder, J.B., 1999. Analysis of formaldehyde in the headspace of urine from bladder and prostate cancer patients using selected ion flow tube mass spectrometry. Rapid Communications in Mass Spectrometry 13, 1354–1359.

Stumpf, D.A., 1997. The neurology of folate and vitamin B12. Northwestern University.

Szende, B., Király-Véghely, Z., Kátay, G., Németh, Z.I., Albert, L., Tyihák, E., 1998. Formaldehyde cycle and the natural formaldehyde generators and capturers. Acta Biologica Hungarica 49, 225.

Taranenko, N.A., Efimova, N.V., 2007. Biomonitoring of formaldehyde in the urinary samples from the pediatric population in the Irkutsk region. Gigiena i Sanitariia 4, 73–75.

Teng, S., Beard, K., Pourahmad, J., Moridani, M., Easson, E., Poon, R., O'Brien, P.J., 2001. The formaldehyde metabolic detoxification enzyme systems and molecular cytotoxic mechanism in isolated rat hepatocytes. Chemico-Biological Interactions 130, 285–296.

Tombaugh, T.N., McIntyre, N.J., 1992. The Mini-Mental State Examination: a comprehensive review. Journal of the American Geriatrics Society 40, 922–935.

Tong, Z.Q., Wan, Y., Luo, W.H., Rongq, He., 2008. Endogenous formaldehyde and some important human diseases. Progress of Natural science 11, 1201–1210.

Trinchese, F., Liu, S., Battaglia, F., Walter, S., Mathews, P.M., Arancio, O., 2004. Progressive age-related development of Alzheimer-like pathology in APP/PS 1 mice. Annals of Neurology 55, 801–814.

Yang, X.Q., 2008. Epidemiological features of senile dementia and its prevention. Occupational Health 24, 2.

Yu, P.H., Lai, C.T., Zuo, D.M., 1997. Formation of formaldehyde from adrenaline in vivo; a potential risk factor for stress-related angiopathy. Neurochemical Research 22, 615–620.