Dissociation of Alzheimer's morphological pathology from cognitive impairment

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Abstract
We observed the Alzheimer's morphological pathology, amyloid production induces Alzheimer's cognitive impairment, was dissociated from the cognitive impairment. The earlier Alzheimer's pathological changes can be induced in normal C57BL mice, by B6 deficient feeding 4 months with no amyloid, and this cognitive and memory impairments were completely inhibited by anti-homocysteic acid antibody. According to Koch's postulate, if a pathogen of Alzheimer's disease is administrated to the normal animal, we would observe the Alzheimer's cognitive impairment in the normal animal. We actually have observed this cognitive impairment in normal C57BL male mice with no amyloid. From our observations, it is suggested the dissociation of Alzheimer's morphological pathology may be possible from the cognitive impairment.

Introduction
Recently our research team has found (1) that anti-homocysteic acid antibody could show the strong cure and preventive effect on 3xTg-AD mice with normal feeding or with vitamin B6 deficient feeding, which was intended to increase homocysteic acid. Homocysteic acid (HA) was already shown to have a neurodegenerative toxicity and its toxicity was increased by the presence of amyloid (2). And HA is well known as an agonist of glutamic acid receptor such as NMDA and metabotropic receptor (3). Our findings show that the pathological process of 3xTg-AD mice was induced by glutamic acid receptor-bound HA. If HA was a real pathogen of Alzheimer's disease, we can observe the Alzheimer's pathology (cognitive and memory impairment) in normal animal, when HA is administrated to normal animal.

Here we show that vitamin B6 deficient feeding in normal C57BL mice whose age was 4 month-old showed the earlier Alzheimer's pathological changes such as cognitive and memory impairment with no amyloid. And these earlier Alzheimer's pathological process were completely inhibited and recovered to normal cognitive ability such as good memory formation by anti-HA antibody. These our findings have submitted the very important dogma for Alzheimer's pathological process. That is, HA is one of some real pathogens of Alzheimer's disease according to Koch's postulate. And amyloid pathology of Alzheimer's disease is possible dissociated from the cognitive impairment.

Methods
Vitamin B6 deficient feeding
C57BL male mice (n=10), whose age were 4 month-old, were feeding with vitamin B6 deficient food for 4 months. The vitamin B6 deficient food was same as that described in reference (1).

Anti-HA antibody
Anti-HA antibodies were purchased from MoBiTec Co. (Germany). Polyclonal antisera were raised in rabbits after immunization with a glutaraldehyde-containing HA
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conjugate, following which antibody specificity was determined by performing ELISA

with competition experiments involving HA-G-BSA (compound cross-reactivity ratio 1:1), cysteine-G-BSA (1:85), and homocysteine-G-BSA (1:231)

Morris water maze test

The apparatus used for all Morris water maze tasks comprised a circular aluminum tank (1.5 m in diameter) painted white and filled with water maintained at 26–29°C. The maze was located in a room containing several simple, visual extramaze cues. To reduce stress, mice were placed on a platform in both the hidden and cued versions of the task for 10 s prior to the first training trial.

Spatial reference Morris water maze training

Mice were trained to swim to a 14-cm circular clear Plexiglas platform placed 1.5 cm beneath the water surface that was invisible to the mice while swimming. The platform location was randomly selected for each mouse, but was kept constant for that mouse throughout the training period. In each trial, the mouse was placed in the tank at one of the four designated start points in a pseudorandom order. Mice were allowed to search for and escape to the submerged platform. If a mouse failed to find the platform within 60 s, it was manually guided to it and allowed to remain there for 10 s. Then, each mouse was placed in a holding cage under a warming lamp for 25 s until the start of the next trial. To ensure that memory differences were not due to the lack of task learning, the mice were trained for four trials a day for as many days as required to meet the criterion, which was defined as <20-s mean escape latency before the first probe trial was run. To prevent overtraining, probe trials were run for each group as soon as they met the group criterion and stopped after all the groups met the criterion. Retention of spatial training was assessed 1.5 and 24 h after the last training trial. Both probe trials consisted of a 60-s free swim in the pool without the

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platform. Mice were monitored by a camera mounted on the ceiling directly above the pool, and all trials were stored on videotape (burnt onto a DVD.)or subsequent analysis. The parameters measured during the probe trial comprised (1) initial latency time to reach the platform.

HA level measurement

HA was extracted from mouse brain (hippocampus and cortex) with an acid of trichloroacetic acid. Brain samples were prepared by a modification of the method of Reed and Bellerche (8). Brains (1.50–2.00 g) were isolated from 4-month-old 3xTg-AD homozygous male mice. The mice were killed by rapid decapitation and their brains were quickly excised and placed on an ice-cold Petri dish. For the gradient highperformance liquid chromatography (HPLC) method, tissue samples were weighed and homogenized using a sonicator for 10 s in ice in 4 ml of ice-cold 10% (w/v) trichloroacetic acid per 100 mg tissue (wet weight). HA (4 µg) was added as the internal standard. For isocratic HPLC, tissue samples were divided into six aliquots. The samples were homogenized as described above. The homogenates for isocratic or gradient HPLC were left on ice for 1 h and centrifuged at 20,000 ×*g* for 25 min. The supernatant was washed five times with an equal volume of diethyl ether and the aqueous phase was maintained. Residual ether was evaporated under nitrogen at room temperature for 5 min. Immediately thereafter, 20 µL was injected into the HPLC system. OPA method was applied. (HPLC-ECD method)

Ventricular cannula

The mice were anesthetized, 2-mm-wide incisions were made in the left hemisphere,

and a guide cannula was inserted into the left ventricular space using a Teflon tube (1 mm in diameter). This operation did not impair learning and memory performance, and the abilities of the operated mice were similar to those of mice that did not undergo surgery.

Behavior record
Mice were put into new 50 cm x 50 cm box type cage. Their behavior were recorded with DVD. Mice were fed with B6 deficient food for 4 months (control)(n=20). Anti-HA antibody was administrated to control (n=10) at 3 months feeding and every 4 day, anti-HA antibody was administrated

ELISA method for amyloid beta 40, 42 was described in S.Oddo et al (4)

Results
Table 1 shows the HA level in mice brain. It clearly shows that vitamin B6 deficient food induced higher level of HA compared with normal control, and anti-HA antibody treatment decreased this higher level of HA induced by B6 deficient food.

Also amyloid production was measured. ELISA method and immunohistochemical staining with 4G8 anti-amyloid antibody could not detect any increase in amyloid production.(Table 2)

Now we observed these mice's behavior in open-space cage. As shown in Fig. 1, B6 deficient feeding control showed abnormal behavior. That is, anti-HA antibody and control with normal food showed the anxiety behavior. But B6 deficient control showed no anxiety behavior, which indicated their cognitive abilities were impaired. And this behavior abnormality was consisted with the result of Table 1.

We observed the memory performance in Morris water maze test. Fig. 2 shows the result. It clearly shows that B6 deficient control showed the strong memory impairment, but control with normal food and anti-HA antibody treatment showed the good memory performance.

From C57BL mice's results, we were interested in HA level in down syndrome and Alzheimer's patients. Because the down syndrome is well known that their pathology will proceed 100% Alzheimer's disease. We collected 24 urine of down syndrome and measured urinal HA level. The result is shown in Table 3. Down syndrome showed the strong higher level of HA compared with that of normal. And also we measured urinal HA level of Alzheimer's patients. (Table 4). And HA level is very strong related to their cognitive abilities. From the Fig. 3, the strong negative association between HA level and MMSE score was observed, which indicates that HA is related to cognitive impairment.

Discussion
According to Koch's postulate, (a)the pathogen will be found in disease organ. (b) If this pathogen can be taken off from the disease animal, the disease can be cured. (c) The pathogen will be added to normal sensitive animal, we can observe the same disease pathological change in normal sensitive animal.

Now HA is the real pathogen of Alzheimer's disease according to above Koch's postulate.

(a)We can observe the high level of HA in model mice, when the memory impairment started and amyloid accumulation into neuron was observed in all area of amygdale, cortex and hippocampus.
(b)We can observe the cure effect of anti-HA antibody or anti-HA vaccine in model mice with normal feeding or with B6 deficient feeding.
(c) normal young mice (4 month-old and final 8 month-old) did show the cognitive and memory impairment and these impairments were completely inhibited by anti-HA antibody, which suggests HA is an etiological compound for these impairment. And these impairment were observed with no amyloid. We confirmed with amyloid antibody of 4G8 with immunohistochemical staining and ELISA method. Also we published that HA has no effect on amyloid production in previous paper (2). From these results and our published HA effect, it is concluded that amyloid production was not induced by vitamin B6 deficiency.

And we observed the HA level in C57BL mice brain after vitamin B6 deficient feeding for 4 months. (Table 1). It clearly shows that HA level was increased after B6 deficient feeding and anti-HA antibody decreased these increased HA level. It is already published that HA itself shows the neurodegenerative toxicity (5, and our data of Fig.7 in reference 1)

Then it is interesting in whether these impairment were related to Alzheimer's disease or not. In human case, some papers reported that down syndrome patient showed an abnormal vitamin B6 metabolism, lower level of B6 compared with normal control (6-9). It is well-known that down syndrome patient will proceed 100% Alzheimer's disease. And B6 deficiency induces the pre-Alzheimer's cognitive and memory impairment of human case also.

B6 deficiency in down syndrome is explained by genetic abnormality. It is well-known that vitamin B6 is a cofactor for cystathionine beta-synthase(CBS) (10) and CBS level in down syndrome is higher than that of normal by 1.5 times, then B6 deficiency is appeared by genetic abnormality. And our unpublished observation showed HA is produced by CBS. Then down syndrome shows essentially HA abnormal increased level. Indeed we observed the urinal HA level of down syndrome (Table 2). Then the cognitive impairment observed in C57BL mice is strongly related to Alzheimer's cognitive impairment.

Why does down syndrome proceed to 100% Alzheimer's pathology? Because abnormal higher level of amyloid (APP gene is located in 21 chromosome) and also abnormal higher level of HA work together to induce Alzheimer's pathology, whose situation is completely same as that of 3xTg-AD mice (APP transgenic and higher level of HA which was reported in our previous paper (1))

Now we have observed the urinal HA level in Alzheimer's patients (Table 3). And the strong association between HA level and their cognitive impairment, which indicates that HA induced the cognitive impairment. This human observation is very strongly related to the cognitive impairment of C57BL mice induced by HA.

From our observation, it is clearly shown that HA induced the Alzheimer's cognitive and memory impairment in normal C57BL mice and HA is a real etiological compound for Alzheimer's disease.

Now we can submit the big unsolved question about the Alzheimer's disease. That is, Why did many normal brains show the normal cognition with amyloid plaques? Why did some people show the cognitive impairment with no amyloid? Especially we know Sister Mary's case(10). She showed normal cognitive ability even with the strong pathology of Alzheimer's disease. Why could she do? We also know the Japanese case.

The senior ladies Twins (their name are Kin and Gin) acted normal behavior at 100 years old, but after their death, their brain showed abnormal Alzheimer's pathology. And recent unsatisfactory clinical trails of amyloid treatment also submitted the question why these trails failed.

Our observations indicates that HA induced the cognitive impairment with no amyloid. Then if someone's brain does produce the higher level of HA, his or her cognitive ability shows impairment even with no amyloid plaque. On the contrary, if someone's brain does not produce the higher level of HA, his or her cognitive ability shows normal even with amyloid plaque. Because it is reported that normal cognitive brain has the amyloid plaque. In general it is believed that amyloid induces the Alzheimer's pathology (Amyloid cascade hypothesis), which means that amyloid induces the cognitive impairment. However our observations did not support this hypothesis. That is, the pathology induced by amyloid is dissociated from the impaired cognition. HA with no amyloid induced the cognitive and memory impairment.

Usually it is theoretical reasonable that the pathology is always associated with the impaired function. However the brain sometimes shows the strong the compensation action. Then it is suggested when the pathology is small, the compensation action will be strong, and sometimes the pathology is not associated with the impaired function. For example, our anti-HA antibody cured the 3xTg-AD mice memory impairment (1) with the presence of amyloid. Usually the presence of amyloid might induce the memory impairment. But our observations did not show this impairment. That is, amyloid toxicity may be not so stronger than that of HA toxicity, then the cognitive ability with amyloid can show the normal by the compensation action after HA destroyed by anti-HA antibody.

However, HA toxicity is so strong that the cognitive ability can not show the normal at all. Our observations in C57BL mice indicates that HA destroyed the compensation action and consequently induced the cognitive and memory impairment.

Finally our answer is for the unsatisfactory clinical trails of amyloid treatment Human and mouse's results have not matched for the acknowledgment of cognitive function recovery by amyloid treatment, but amyloid treatment has matched between on human and on mice. The thing will have not matched between amyloid treatment and the cognitive recovery. For instance, the cognitive dysfunction without amyloid and amyloid with normal cognitive function in human were reported. This shows the necessity for assuming the causative agent who causes the cognitive dysfunction besides amyloid.

From a certain thing to our assumption of toxicity of HA report that NMDA receptor suggests thing that is relation in amyloid development of toxicity in it, the reason for HA is that it has already been established as an agonist of NMDA. And, it explains from the viewpoint of the development of toxicity of HA.

Though HA demonstrates the neurodegenerative action for the mouse by 40-50μM, one μM of HA is sufficient enough to kill the neurons in the presence of amyloid (please refer Fig. 6 of reference 2 to compare with Fig. 7 of reference 1). Therefore, Alzheimer's disease recovers in the mouse when the amyloid was taken off by the vaccine treatment. Because the change of the pathology of Alzheimer's disease doesn't occur, by which HA level is still one μM and not enough to induce the neurodegeneration.

Well, it is comparison of HA level in urine (Table 3) between a patient with Alzheimer's disease and healthy person, and the HA level in patient groups was 960nM, and its level in healthy person was 300nM. The toxicity of HA will appear in the human when an amyloid is removal, because the HA level is still near the toxic concentration,

then Alzheimer's disease cannot be recovered.

Why did we use B6 deficient feeding in our experiment? It is best way to circulate homocysteic acid itself in model mice, but homocysteic acid may induce the seizure of model mice. So since we had to increase HA level and circulate constantly in mice brain, we used the B6 deficient feeding which was reported to increase HA level (12).

In conclusion, our findings present that HA is one of some real pathogens of Alzheimer's disease according to Koch's postulate, and HA will show its toxicity with no amyloid.

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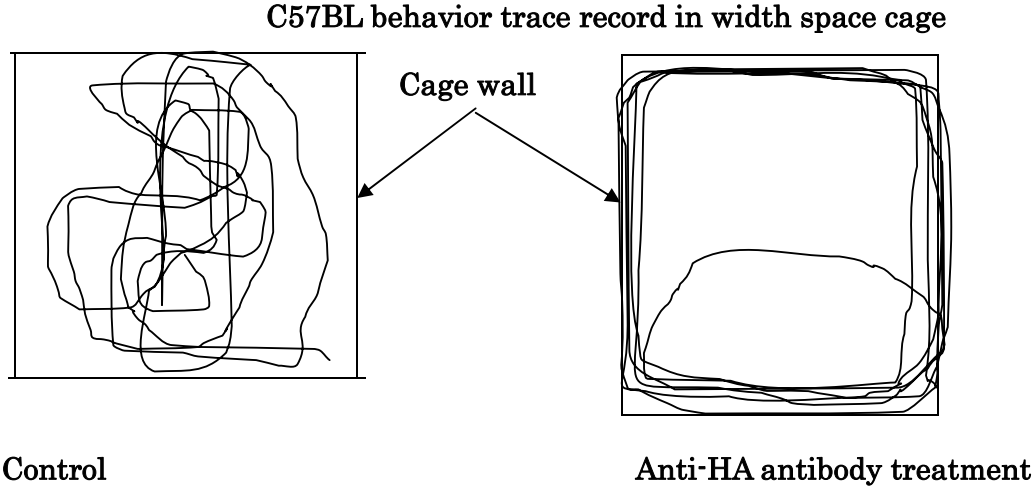
Figure legends

Fig. 1**C57BL male mice (4 month-old) behavior trace record.**
Mice were put into new 50 cm x 50 cm box type cage. Their behavior were recorded with DVD. Mice were fed with B6 deficient food for 4 months

Control: C57BL male mice +B6 deficient food for 4 months.
Anti-HA antibody: C57BL male mice +B6 deficient food for 3 months+anti-HA antibody
Antibody treatment was same as described our manuscript (3) after B6 deficient feeding for 3 months. For one month, antibody treatment was conducted.
Also normal control which were fed with normal food showed same behavior as that of anti-HA antibody treatment.

Fig. 2 **Morris-water maze test of C57BL mice**
Figure shows the average of 5 male mice. Control and antibody treatment were same as that of Fig. 4a. Normal control: normal C57BL mice were fed with normal food.

Fig. 3 **The association between urinal HA level and MMSE score**.
The data were collected from Table 3. $r=-0.758$, $p<0.006$



Control: no anxiety = cognitive ability impairment

Anti-HA antibody treatment = anxiety

Fig. 1

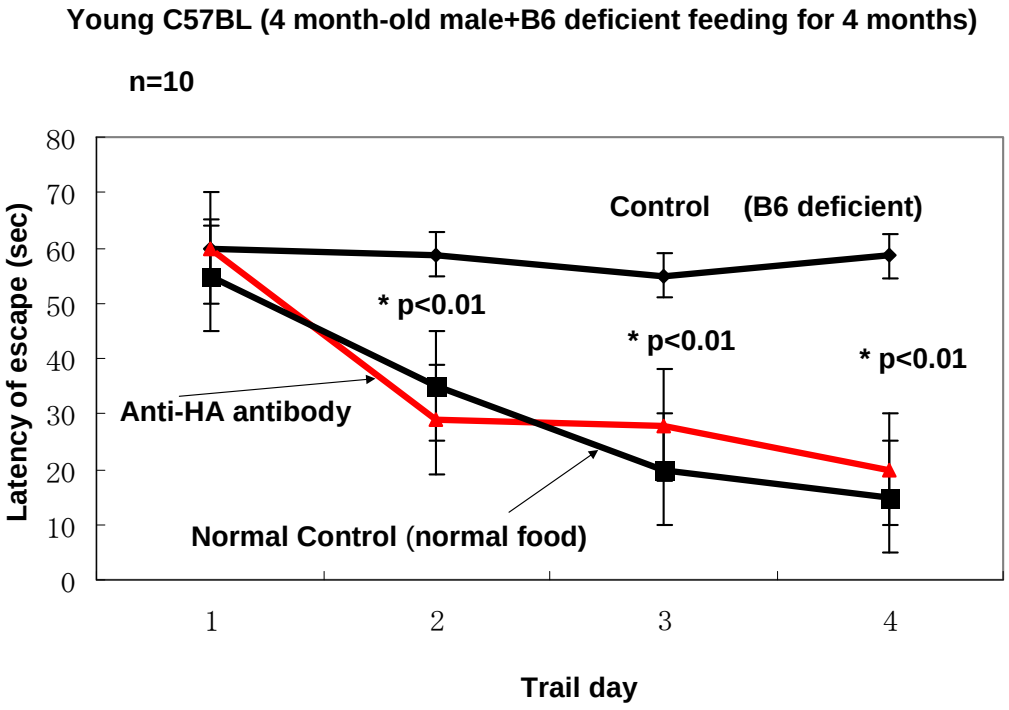

Young C57BL (4 month-old male+B6 deficient feeding for 4 months)
n=10
Latency of escape (sec)
80
70
60
50
40
30
20
10
0
Control (B6 deficient)
* p<0.01
* p<0.01
* p<0.01
Anti-HA antibody
Normal Control (normal food)
1
2
3
4
Trail day

Fig. 2

Table 1 HA level in C57BL brain after vitamin B6 deficient food for 4 months

Control (n=10)	25.6 ± 8.5 pmoles/mg brain
	P<0.001
B6 deficient (n=10)	45.2 ±15.0 pmoles/mg brain
	P<0.01
Anti-HA antibody Treatment (n=10)	20.1 ± 7.1 pmoles/mg brain

HA level was measured by the method described in reference 1.
HA level was measured after memory task.

Table 2 Amyloid beta 40 and 42 level in brain after B6 deficient feedings

	Detergent soluble (pg/mg brain)		Detergent insoluble	
	Aβ40	Aβ42	Aβ40	Aβ42
Control	0.5 ± 0.1	0.8 ± 0.2	1 ± 0.2	1 ± 0.3
B6 deficient Feeding	0.6 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	1.1 ± 0.2

ELISA method for amyloid beta 40,42 were described in reference (11)

N=10, Detergent was SDS.

Table 3 Urinary HA level in Down syndrome

Normal control	0.2 + 1.1	
(male age, (36.3 ± 5.5), n=16)		p<0.001
Down syndrome	11.6 ± 4.2	μM
(male age (34.3 ± 7.8) n=18,)		

Urine was collected for 24 hrs. HA was measured according to the method described in previous paper (1)

Table 4, Urinary HA level in Alzheimer's patients

Normal control	0.3 ± 0.2 μM
(Femal (n=5)and male (n=6) age, (71.4 ± 6.0), n=11)	
MMSE score	
28.5 ± 3.0	
	p<0.002
Alzheimer's patients	0.96 ± 0.6 μM
(Femal (n=7)and male(n=4) age (73.3 ± 6.0) n=11)	
MMSE score	
17.4 ± 4.0	

First urine was collected immediately after getting up in the morning. Urinal specific gravity of first urine was adjusted to 1.020. HA was measured according to the method described in previous paper (1)

Association between urinal HA level and MMSE score

R=-0.758, p<0.006

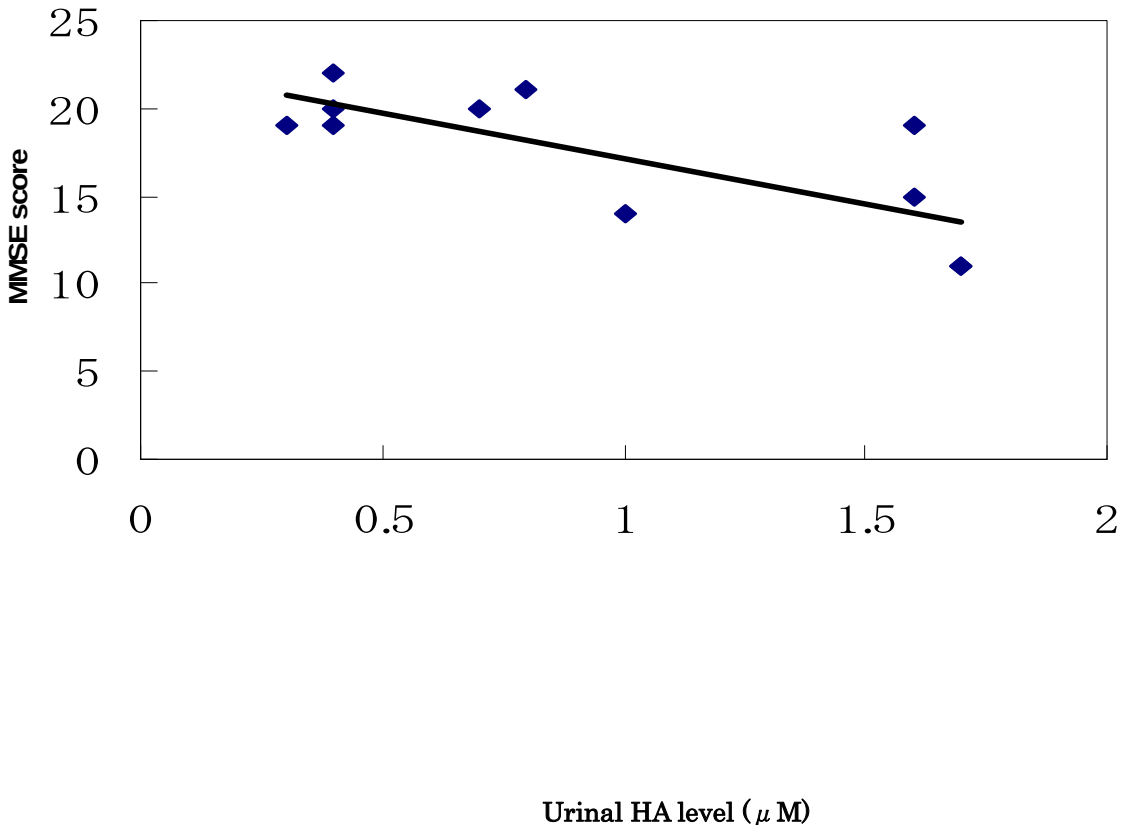


Fig. 3