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1'-Acetoxychavicol Acetate Ameliorates Age-related Spatial Memory Deterioration by Increasing Serum Ketone Body Production as a Complementary Energy Source for Neuronal Cells

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Abbreviations: ACA, 1'-Acetoxychavicol acetate; SAMP8, senescence-accelerated mice prone 8; SAMR1, senescence-accelerated resistant/1; AD, Alzheimer's disease; TCA, tricarboxylic acid; PDHC, H & E, hematoxylin and eosin; pyruvate dehydrogenase complex; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT-enhancer-binding protein α; HFD, high-fat diet; AMPK, AMP-activated protein kinase.

Abstract

1’-Acetoxychavicol acetate (ACA) is naturally obtained from the rhizomes and seeds of *Alpinia galangal*. Here, we examined the effect of ACA on learning and memory in senescence-accelerated mice prone 8 (SAMP8). In mice that were fed a control diet containing 0.02% ACA for 25 weeks, the learning ability in the Morris water maze test was significantly enhanced in comparison with mice that were fed the control diet alone. In the Y-maze test, SAMP8 mice showed decreased spontaneous alterations in comparison with senescence-accelerated resistant/1 (SAMR1) mice, a homologous control, which was improved by ACA pretreatment. Serum metabolite profiles were obtained by GC-MS analysis, and each metabolic profile was plotted on a 3D score plot. Based upon the diagram, it can be seen that the distribution areas for the three groups were completely separate. Furthermore, the contents of β-hydroxybutyric acid and palmitic acid in the serum of SAMP8-ACA mice were higher than those of SAMP8-control mice and SAMR1-control mice. We also found that SAMR1 mice did not show histological abnormalities, whereas histological damage in the CA1 region of the hippocampus in SAMP8-control mice was observed. However, SAMP8-ACA mice were observed in a similar manner as SAMR1 mice. These findings confirm that ACA increases the serum concentrations of β-hydroxybutyric acid and palmitic acid levels and thus these fuels might contribute to the maintenance of the cognitive performance of SAMP8 mice.

Keywords: Morris water maze test; Y-maze test; 1’-Acetoxychavicol acetate; Senescence-accelerated mouse prone 8 (SAMP8); metabolite profile; β-hydroxybutyric acid.

1. Introduction

Twenty-four million people currently suffer from dementia, and this amount will double every 20 years to 42 million by 2020 and 81 million by 2040 [1]. Most of these people will suffer from Alzheimer's disease (AD). AD is the most common age-dependent neurodegenerative disorder and causes a progressive decline in cognitive function. Therefore, it has been highly desirable to find a method of preventing and treating AD.

Brain energy demands are among the highest of all organs, and its principal energy substrate is glucose. Many studies have suggested that an impairment of glucose metabolism may contribute to the cognitive deficits that are observed in AD [2, 3]. Especially, the hippocampus is vulnerable to glucose insufficiency [4], and in AD, cerebral glucose metabolism is reduced by 20-40% [5]. Blass and Gibson reported that even slight reductions in brain glucose metabolism reduce brain function [6]. Experimental results suggest that abnormal glucose metabolism may be critical in the pathophysiology of AD. The reduction in the glucose metabolism rate correlates with senile plaque density [7], one of the neuropathological hallmarks of AD. The main pathway for glucose metabolism in the brain is the tricarboxylic acid (TCA) cycle. The oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase complex (PDHC) provides acetyl CoA which feeds TCA cycle. Thus the PDHC is important to brain health and survival. however, many studies have reported that PDHC activities are decreased in AD [8-11]. To compensate for the decline in glucose-driven ATP generation in AD ketone bodies (acetoacetate/β-hydroxybutyrate) are used as a complementary source of acetyl-CoA [12, 13].

1'-Acetoxychavicol acetate (ACA) occurs naturally in rhizomes and the seeds of Zingiberaceae plants, such as *Languas galangal* and *Alpinia galangal.* The plants are used as a spice or Chinese medicine in Southeast Asia. ACA exhibits various biological activities [14, 15]. Furthermore, in normal intestinal epithelial cells, ACA induces phase II enzyme activities, which detoxify xenobiotics [16]. However, the effect of ACA on neuronal cells death remains unclear. In this study,

we studied whether ACA might increase the serum ketone bodies and thereby maintained the brain functions.

A senescence-accelerated mouse (SAM) was established as a model of accelerated aging by Takeda et al., wherein SAM prone 8 (SAMP8) spontaneously shows age-related behavioral disorders, including a shortened life span, cognition impairment, and loss of hair [17]. SAMP8 has been recognized as a suitable model of AD. Wang *et al*. found that metabolic mechanisms of cognitive impairment in SAMP8 mice are related to multiple pathways, such as dysfunctional lipid metabolism and altered levels of amino acids and energy-related metabolism [18].

In this study we investigated the effects of ACA on learning and memory in SAMP8 mice using the Y maze and the Morris water maze. We also utilized a GC-MS-based metabolomics approach to screen for altered cognition-related metabolic profiles and investigated the differences in metabolite levels in the serum of SAMP8 mice.

2. Materials and Methods

2.1 Materials

Racemic-ACA was supplied by Dr. H. Azuma, Graduate School of Engineering of Osaka City University. It was prepared according to the previously reported method [19] as following. Starting from 4-, 3-, and 2-hydroxybenzaldehyde, racemic-ACA was synthesized via a Grignard coupling reaction with vinylmagnesium bromide and further acetylation of the produced hydroxyl intermediate.

Mayer's hematoxylin solution and 1% eosin Y solution were purchased from Muto Pure Chemicals Co., LTD. (Tokyo, Japan). 2-Isopropylmalic acid was obtained from Sigma-Aldrich, Tokyo, Japan. Other chemicals used in this study were special-grade commercial products purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Animals

Eight age-matched SAM resistance/1 (SAMR1) mice (8 weeks old) and 24 SAMP8 mice (8 weeks old) were provided by Japan SLC (Shizuoka, Japan). They were housed in a temperature-controlled (23 ± 1°C) room with 12 h light/12 h darkness. All mice were acclimated in this environment for two weeks prior to initiating the feeding protocols. After the interval of acclimation, SAMR1 mice (n=8) were used as homologous controls and 24 SAMP8 mice were assigned randomly to the control group (n=12) and the 0.02% ACA group (n=12) and maintained for 25 weeks (Fig 1). Composition of diets was shown in Table 1. Food and tap water were provided *ad libitum*. Mice were monitored visually every 2 days to assess animal health and body condition. After 25 weeks, mice were anesthetized with isoflurane, and then performed euthanasia. All efforts were made to minimize suffering.

2.3 Ethics Statement

This experiment was approved by the Osaka City University animal experiment committee (#S0041) and complied with the Regulations on Animal Experiments from Osaka City University.

2.4 Y-maze Test

The Y-maze is used as a measure of immediate spatial memory, which is a form of short-term memory. The Y-maze is a three-arm horizontal maze (40 cm long and 3 cm wide with walls 12 cm high) in which the arms are symmetrically disposed at 120° to each other. The mice were initially placed in the center, and the sequence (i.e., ABCCAB, etc.) and the number of arm entries were recorded manually for each mouse during a 5-min period after acclimation for 3 min. The proportion of alternation was subsequently computed by dividing the number of alternations by the

total number of alternation plus non-alternations. Perseverative behavior was defined as subjects making significantly fewer alternations than would be expected at a chance level (50% in the absence of arm biases).

2.5 Morris Water Maze Test

The Morris water maze was used to monitor spatial learning and memory in rodents. The Morris water maze is a circular pool (120 cm in diameter and 45 cm in height). The pool was filled with tap water (25°C, 30-cm deep) and divided into four quadrants (NE, NW, SE, and SW) by two imaginary lines crossing the center of the pool. In the center of one quadrant was a removable escape platform (10 cm in diameter) 1 cm below the water level that was covered with black ink. The room had adjustable indirect illumination, and a camera was fixed to the ceiling. Swimming paths were tracked by the camera and stored in a computer with an ANY-maze video tracking system version 4.96 (Stoelting Co., Wood Dale, IL, USA). Training was conducted four times a day for nine consecutive days. One day after the training trial sessions, mice were subjected to a trial session in which the platform was removed from the pool, allowing the mice to swim for 60 sec to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed.

2.6 Histologic Procedure

Brain samples were collected from each mouse, fixed in 10% buffered formalin fixative and dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks and cut into 5-μm sections. Consecutive sections were stained with hematoxylin and eosin (H&E). The pathologist was blinded to the groups of mice.

2.7 GC-MS Analysis

GC-MS analysis of serum was performed according to the method of Tamai *et al*. [20]. Twenty-five microliters of serum was mixed with 5 μl of 1 mg/ml 2-isopropylmalic acid as an internal standard (IS), and 900 μl of 70 % ethanol was added. GC-MS analysis was performed using a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) with a DB-5 column (Length: 30 m, Film: 1.00 μm, Inside diameter: 0.25 mm, Agilent Technologies, Santa Clara, CA, U.S.A.). The flow rate was 24.5 ml/min. The GC column temperature was programmed to increase from 100 to 300°C at a rate of 4°C per min. The ion source temperature was 230°C. Chromatogram acquisition, detection of peaks and data processing were performed using Shimadzu GCMS solution software ver. 2.71 (Shimadzu). Low-molecular-weight metabolites were identified using the NIST library (NIST08) with peaks assigned based upon a similarity index >75. The peak area of each metabolite was divided by that of the IS to give a relative value. The serum levels of metabolites were shown as a fold ratio. GC-MS data analysis was performed using MetaboAnalyst 3.0 [21]. MetaboAnalyst 3.0 (www.metaboanalyst.ca) is a web server designed to permit comprehensive metabolomic data analysis, visualization and interpretation.

2.8 Statistical Analysis

The data are presented as the mean ± SD. Statistical analyses were performed using Statcel3 the usefull add-in forms on excel statistical softwere (OMS publishing Inc., Saitama, Japan). Significant differences in assay values were evaluated using ANOVA followed by Tukey's test. A value of $p < 0.05$ indicated a statistically significant difference.

3. Results

3.1 Effect of ACA on the Y-maze Test

Short-term memory was examined by monitoring spontaneous alternation behaviors in the Y-maze. After 24 weeks of a control diet or 0.02% ACA, the Y-maze test was performed. We observed that untreated SAMR1 exhibited a spontaneous alternation percentage of 74.4±26.2 (n =8), whereas the SAMP8-control group showed a decreased alternation percentage of 49.5±14.9 (n = 12). Pretreatment of the SAMP8-ACA group increased the alternation percentage to the level of the SAMR1 group (Fig 2).

3.2 Effect of ACA on the Water Maze Test

Fig 3 shows the performance in the Morris water maze test. All mice improved their performance during the nine learning sessions. In comparison to animals in the SAMR1 group, the escape latency of the SAMP8 group was significantly longer until 5 days after training. The total latency of the SAMP8-ACA group was reduced significantly in comparison with that of the SAMP8-control group and was reached to that of SAMR1 group (Fig 3A).

On the day following the final day of the training trial sessions, the mice were subjected to a 60-second prove test without the goal platform. In the test, the numbers of platform crossing quadrants (platform quadrant) were analyzed. The mice in the SAMP8-control group exhibited a significant decrease in the number of platform crossings in comparison with those in the SAMR1 group. However, the mice in the SAMP8-ACA group increased the number of platform crossings to the level of the SAMR1 group (Fig 3B).

3.3 Effect of ACA on Histopathological Changes in the Brain Tissues

We performed a histopathological evaluation of the brain tissues using H&E. Abnormal morphological features were observed in pyramidal neurons in the hippocampal CA1 of SAMP8-control mice, including neuronal cell loss and nuclei shrinkage. However, the cells in the hippocampus of the SAMP8-ACA mice had almost normal structures (Fig 4A). The ratio of the

damaged pyramidal cells in the hippocampus of the SMAP8-control group was significantly higher than those of the SAMR1 and SAMP8-ACA groups (Fig 4B).

3.4 Effect of ACA on Metabolite Profile

The serum metabolite profile was obtained by GC-MS analysis and each metabolite profile was plotted on a 3D score plot (Fig 5). Based upon the score plot and 3D distribution diagram, it can be seen that the distribution areas for the three groups were completely separate, thereby showing that metabolite profiles of SAMP8-ACA group (red) are different from those of the SAMP8-control group (green) and the SAMR1 group (blue). There were nine metabolites that were changed significantly among the three groups (Fig 6). The relative contents of β-hydroxybutyric acid, phosphoric acid, aspartic acid, serine, valine, urea, palmitic acid increased, and the citric acid content decreased in SAMP8-ACA group. The increase in the concentrations of β-hydroxybutyric acid and palmitic acid in serum of SAMP8-ACA group shows that the lipid metabolism in SAMP8-ACA group is more enhanced than those in SAMR1 group and SAMP8-control group.

4. Discussion

We examined the effect of ACA on age-related learning and memory impairments in senescence-accelerated mice. SAMP8 mice contain a natural mutation resulting in an age-related amyloid-β protein-mediated model of cognitive impairment. In this study, we evaluated the effect of ACA on learning and memory in SAMP8 mice using the Morris water maze test and the Y-maze test. The Morris water maze test is a classic test that is widely used to study hippocampal-dependent spatial learning and memory. The present study showed that SAMP8 mice displayed significant memorial and cognitive defects in the Morris water maze test at 25 weeks. ACA supplementation significantly enhanced the behavioral performance in a probe trial session in which the platform was removed from the pool. In the Y-maze test, SAMP8 mice tended to decrease spontaneous

alterations in comparison with that of SAMR1 mice, which was improved by ACA pretreatment. These behavioral studies suggest that ACA improves short- and long-term memory in SAMP8 mice.

Glucose is the principal energy substrate in brain. Even a slight depletion in brain energy affected judgment, memory, orientation, and other higher brain functions within seconds. More severe and prolonged impairments of brain glucose metabolism produce severe brain damage, including permanent dementia [22]. Diminished brain glucose metabolism in patients with AD precedes the appearance of overt clinical manifestations of the disease [23]. In parallel with the decline in glucose metabolism in AD, there is a generalized shift away from glucose-derived energy production, which is associated with a decrease in the expression of glycolytic enzymes that were coupled to a decrease in the activity of the PDHC [24]. The highest activity of PDHC in brain was observed in the hippocampus, and the lowest was observed in the medulla [25, 26]. Inhibition of PDHC activity impairs mitochondrial oxidation and promotes its cytoplasmic reduction to lactate or transamination to alanine. Alterations in brain metabolic profile in AD are further evidenced by concomitant activation of compensatory pathways that promote the usage of alternative substrates, such as ketone bodies, to compensate for the decline in glucose-driven ATP generation. The ketone bodies are oxidized and release acetyl-CoA, which enters the TCA cycle. In the present study, we found that ACA increased the serum β-hydroxybutyric acid and palmitic acid.

Some studies have investigated the effect of diets rich in saturated fat on the development of Alzheimer's disease [27-29]. Furthermore, high-fat diets increase the deposition of amyloid β peptides in a transgenic mouse model [30-32]. The brain does not oxidize fatty acids, because fatty acids except for omega-3 fatty acid docosahexaenoic acid cannot across the blood-brain barrier [33]. In liver, fatty acids are oxidized to acetyl CoA by β oxidation. Very little of the acetyl CoA generated in the liver is oxidized completely by the TCA cycle and provides most of the ATP needed for gluconeogenesis and other metabolisms. Therefore, most of acetyl CoA is converted into ketone bodies by liver mitochondria. The ketone bodies (acetoacetate and β-hydroxybutyrate) are

released into the blood and are a source of energy for many tissues. The ketone bodies can cross the blood-brain barrier and function as an alternative fuel for the brain.

Some recent studies using a transgenic mouse model of Alzheimer's disease have found that a ketogenic diet improved Alzheimer's pathology [34]. Pan *et al.* have shown that supplementation of medium-chain triacylglycerol in aged Beagle dogs can significantly increase blood ketone body concentrations under fed conditions and improve age-related decline in cognitive function by providing an alternative source of brain energy in old healthy dogs [35]. In addition, β-hydroxybutyric acid appears to protect cultured mesencephalic neurons from the heroin analogue 1-methyl-4-phenylpyridinium toxicity and hippocampal neurons from β-amyloid$_{1\text{-}42}$ toxicity [36]. β-Hydroxybutyric acid substituted for glucose as an energy substrate and preserved neuronal integrity and stability in rat hippocampal slices [37, 38]. Cerebral activity of 3-hydroxybutyric acid dehydrogenase, the gateway enzyme for ketone body utilization, is constitutive and is not significantly influenced by nutritional state or dietary composition [39]. Therefore elevation of plasma ketone body levels with diets or dietary supplements is now therapeutically exploited for improving brain function in seizure [40], epilepsy [41] and AD [42]. Kim *et al*. studied the effects of both the ketogenic diet and ketone body in spontaneously epileptic *Kcna*1-null mice. Then they reported that ketone bodies alone were sufficient to exert antiseizure effects and restore intrinsic impairment of hippocampal long-term potentiation and spatial learning-memory defects in *Kcna*1 mice [40]. Furthermore, Reger *et al*. reported that AD patients without apolipoprotein E allele showed cognitive improvements in response to acute elevation of β-hydroxybutyric acid levels and found that there was a significant correlation between the increase in serum β-hydroxybutyric acid concentration and the improvement of memory [43, 44]. Henderson *et al*. also showed that treatment with AC-1202 (containing 20 g medium chain triglyceride) in patients with mild-to-moderate AD improved memory and cognition and suggested that the significant improvements in cognition with the treatment of AC1202 were correlated with blood levels of

β-hydroxybutyric acid [45]

The most frequently used is the traditional ketogenic diet that was originally developed in the 1920's by Wilder, which is based upon long-chain fatty acid [46]. Then a medium-chain triglyceride diet was introduced, which produces greater ketosis [47]. However, this modification has not been widely accepted because it is associated with bloating and abdominal discomfort. A third variation of the diet is known as the Radcliffe Infirmary diet and represents a combination of the traditional and medium-chain triglyceride diets [48]. Its efficacy is also similar to that of the traditional ketogenic diet. Such a ketogenic diet produces ketone bodies by sequestering lipids from the outside but is disadvantaged by causing a bad mood at the time of energy oversupply and eating. Therefore, the development of a novel treatment strategy to increase the blood ketone bodies without having ketone diets is important. In this study, we found that the metabolite profile of the SAMP8-ACA group is different from those of the SAMP8-control group and the SAMR1 group and showed that the contents of β-hydroxybutyric acid and palmitic acid increased in the serum of SAMP8-ACA mice. These results suggest that treatment with ACA enhanced the effective management of learning and memory of SAMP8 mice and was dependent on the different metabolic pathway from that of SAMR1 mice.

In lipid metabolism, we have shown that ACA causes a significant decrease in the activity of glycerol 2-phosphate dehydrogenase, a key enzyme in triglyceride synthesis, in 3T3L1 adipocytes, and it inhibits cellular lipid accumulation through the down-regulation of transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) [15]. We also showed that the visceral fat mass in rats that were fed a high-fat diet (HFD) containing 0.05% ACA tended to be lower than that in rats fed an HFD alone. Furthermore, a histological examination of livers from rats fed an HFD showed steatohepatitis. However, rats fed an HFD containing 0.05% ACA showed no histopathological changes in the liver tissue.

We observed that palmitic acid-increased in serum of SAMP8-ACA group. However, it is

reported that saturated free fatty acid such as palmitic acid induces endothelial insulin resistance and inflammation via production of NADPH oxidase-generated superoxide [49]. As described above, rats fed a HFD containing 0.05% ACA showed no histopathological change in the liver tissue. Recently, Liu et al. have shown that resveratrol inhibits palmitic acid induced-inflammation and ameliorates insulin resistant endothelial dysfunction via regulation of AMP-activated protein kinase (AMPK) and sirtuin 1 activities [50]. We have also shown that ACA induces a dose-dependent phosphorylation of AMPK in various cells [15, 51, 52]. AMPK monitors intracellular status and regulates the uptake and metabolism of glucose and fatty acids, as well as the synthesis and oxidation of fatty acids, cholesterol, glycogen and protein, to meet energy demands [53]. Activated AMPK has several metabolic effects. It increases fatty acid oxidation by inactivating acetyl-CoA carboxylase and by up-regulating the genes that are involved in fatty acid oxidation. It also inhibits lipid synthesis by down-regulating sterol regulatory element-binding protein-lc and phosphorylating PPARγ [54, 55]. Furthermore, McCarty *et al.* reported that AMPK may suppress NADPH oxidase activation in vascular tissues [56]. These results suggest that ACA inhibits palmitic acid-induced inflammation via regulation of AMPK.

In the present study, we examined the effect on the cognitive performance of ACA at a dose of 0.02% in the diet. About the doses of ACA, there are several reports on the chemopreventive effect of ACA at doses of 0.01-0.05% in the diet. ACA exhibited strong chemopreventive effects on 4-nitroquinoline 1-oxide-induced oral carcinogenesis [57] and on azoxymethane-induction of colone aberrant crypt foci [58] in rat model at doses of 100-500 ppm in the diet. However, Tanaka et al showed ACA suppressed the growth of adenocarcinomas by treatment during either initiation or promotion stages at 500 ppm in the diet, but there was a weak effect in the promotion stage at 100 ppm in an azoxymethane-induced rat colon carcinogenesis model. On the other hand, ACA did not inhibit GST-P positive foci development in the post-initiation stage of diethylnitrosamine-induced hepatocarcinogenesis [59]. These results suggest that the effect of ACA depend on the dose level,

organ site and stage of carcinogenesis. In the present study, we examined the effect of ACA on learning and memory at a dose of 0.02% in the diet and found that pretreatment of the SAMP8-ACA group maintained the cognitive performance to the level of the SAMR1 group. Furthermore, histological damage in the CA1 region of the hippocampus in SAMP8-control mice was observed, but was not observed in SAMP8-ACA mice. However, it is necessary to further examine the effect of various doses of ACA in this experimental system.

In conclusion, these findings confirm that ACA increased the plasma concentrations of β-hydroxybutyric acid and palmitic acid levels; thus, these fuels might contribute to the maintenance of the cognitive performance of SAMP8 mice.

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Figure legends

Fig. 1. Experimental schedule

Fig. 2. Effect of ACA on short-term memory in the Y-maze test in SAMP8 and SAMR1 mice.

After 24 weeks, the spontaneous alteration of behavior and the number of arm entries were recorded manually for each mouse during a 5-min period after 3 min of conditioning. The results are presented as the mean ± S.D. of eight or 12 mice. *$p<0.05$ compared with the SAMP8-control. n.s. means not significant.

Fig. 3. Effect of ACA on spatial learning and memory in the Morris water maze test in SAMP8 and SAMR1 mice.

Mice were fed a control diet or a 0.02% ACA diet. After 22 weeks, the mice were tested four times per day for nine consecutive days. (A) Learning curve for latency to find the platform. ○: SAMR1; ●: SAMP8-control; ▲: SAMP8-ACA. The results are presented as the mean ±S.D. of eight or 12 mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the SAMP8-control. (B) The number of cross platform areas was used as a measure of spatial learning in the hidden platform task. The results are presented as the mean ±S.D. of 8 or 12 mice. **$p<0.01$ compared with the SAMR1, † $p<0.05$ compared with the SAMR8-control.

Fig. 4. Effect of ACA on histopathological changes in the brain tissues

(A) Hippocampal sections were stained with H&E. (a, d) SAMR1; (b, e) SAMP8-control; (c, f) SAMP8-ACA. The hippocampal CA1 region in each group was (d), (e) or (f). SAMR1 did not show histological abnormalities, while histological damage in the CA1 region of the hippocampus in SAMP8-control was observed. However, SAMP8-ACA was observed in a similar manner as

SAMR1. Original magnification; x4.2 (a, b, c) and x40 (d, e, f). Scale in (a), (b) and (c) indicates 200 μm. Scale in (d), (e) and (f) indicates 20 μm. (B) Ratio of damaged pyramidal cells in hippocampus. The results are presented as the mean ±S.D. of 8 or 12 mice. **$p<0.01$ compared with the SAMR1.

Fig. 5. Scores plot of the metabolite profile of SAM mice obtained by GC-MS.

The metabolic profile of each mouse was plotted on a 3D scores plot. The SAMP8-control was indicated by green circles, The SAMP8-ACA was indicated by red circles, and the SAMR1 was indicated by blue circles.

Fig. 6. Metabolites significantly changed in serum among SAM mice groups.

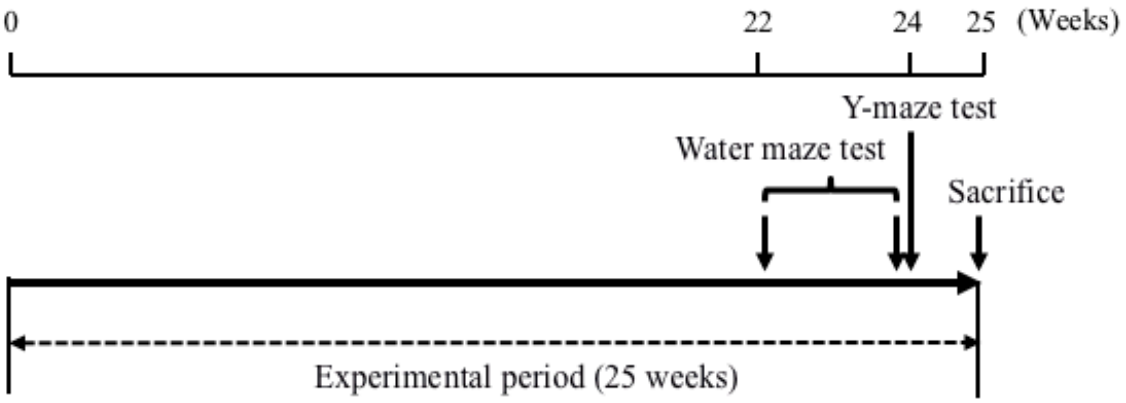
Metabolic contents significantly changed among the sera of SAM mice, as shown in box plots.

Table 1. Composition of diets

Components (g)	Control	0.02% ACA
Casein	200	200
L-Cystine	3	3
Cornstarch	397.486	397.286
α-Cornstarch	132	132
Sucrose	100	100
Soybean oil	70	70
Cellulose powder	50	50
Mineral mix (AIN-93G-MX)[1]	35	35
Vitamin mix (AIN-93VX)[2]	10	10
Choline hydrogen tartrate	2.5	2.5
t-Butylhydroquinone	0.014	0.014
ACA	0	0.2
Total	1000	1000

[1] Composition in g/kg diet: Calcium Carbonate, 357; Potassium Phosphate, Monobasic, 196; Potassium Citrate · H_2O, 70.78; Sodium Chloride, 74; Potassium Sulfate, 46.6; Magnesium Oxide, 24; Ferric Citrate, 6.06; Zinc Carbonate, 1.65; Manganese Carbonate, 0.63; Cupric Carbonate, 0.324; Potassium Iodate, 0.01; Sodium Selenate, 0.01025; Chromium K Sulfate • $12H_2O$, 0.275; Ammonium Molybdate • $4H_2O$, 0.00795; Sodium Silicate • $9H_2O$, 1.45; Lithium Chloride, 0.0174; Boric Acid, 0.0815; Sodium Fluoride, 0.0635; Nickel Carbonate • $4H_2O$, 0.0306; Ammonium Vanadate, 0.0066; Sucrose, 221.0032.

[2] Composition in g/kg diet: Vitamin A Acetate (500,000 IU/g), 0.8; Vitamin D3 (400,000 IU/g), 0.25; Vitamin E Acetate (500 IU/g), 15; Phylloquinone, 0.075; Biotin, 2; Cyanocobalamin, 2.5; Folic Acid, 0.2; Nicotinic Acid, 3; Calcium Pantothenate, 1.6; Pyridoxine-HCl, 0.7; Riboflavin, 0.6; Thiamin HCl, 0.6; Sucrose, 974.655.



Experimental groups

- SAMR1 : Control diet
- SAMP8 control : Control diet
- SAMP8 ACA : 0.02% ACA diet

Fig. 1

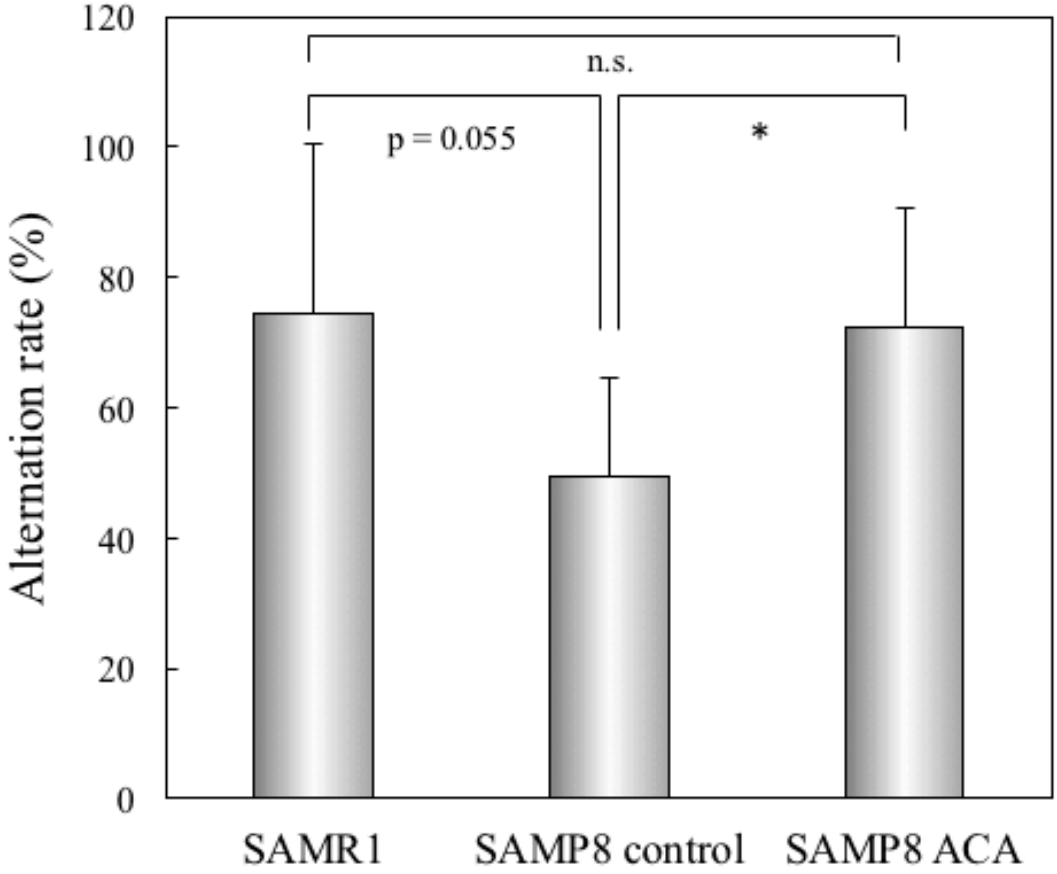

120
100
80
60
40
20
0
Alternation rate (%)
n.s.
p = 0.055
*
SAMR1
SAMP8 control
SAMP8 ACA

Fig. 2

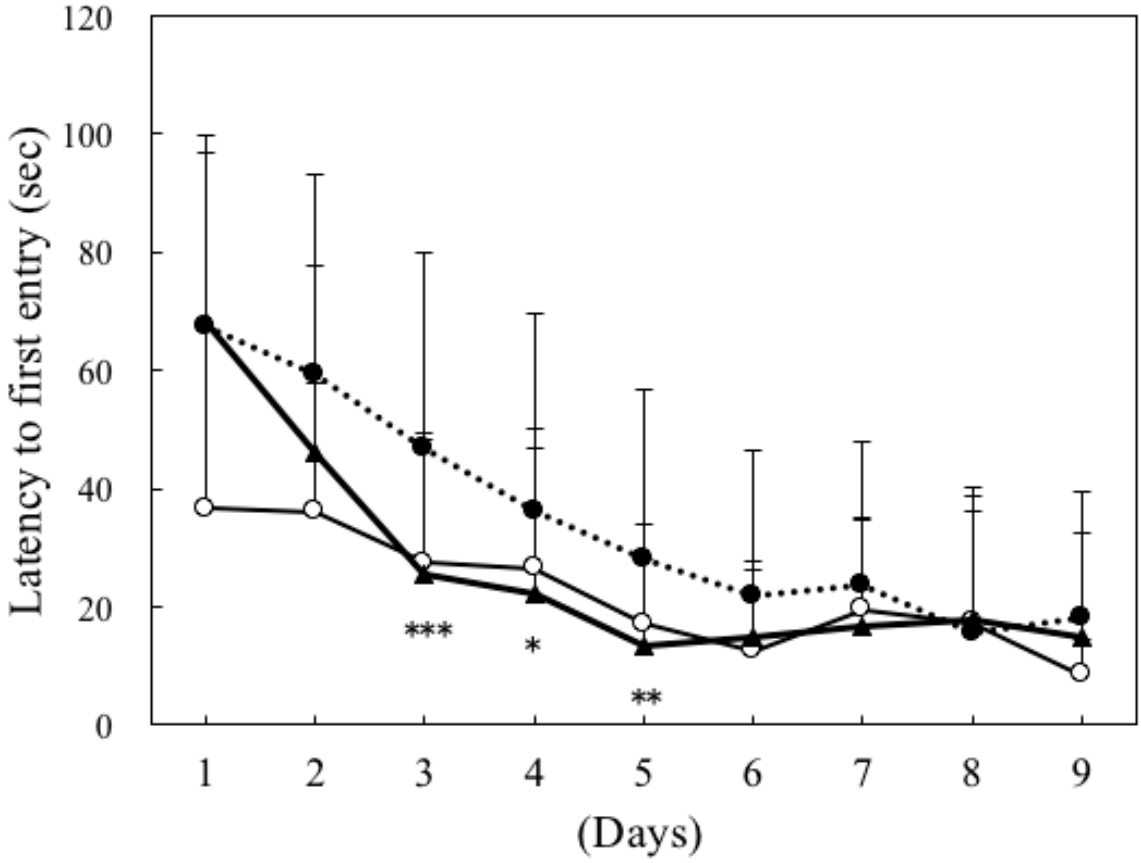

Latency to first entry (sec)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
(Days)
***
*
**

Fig. 3A

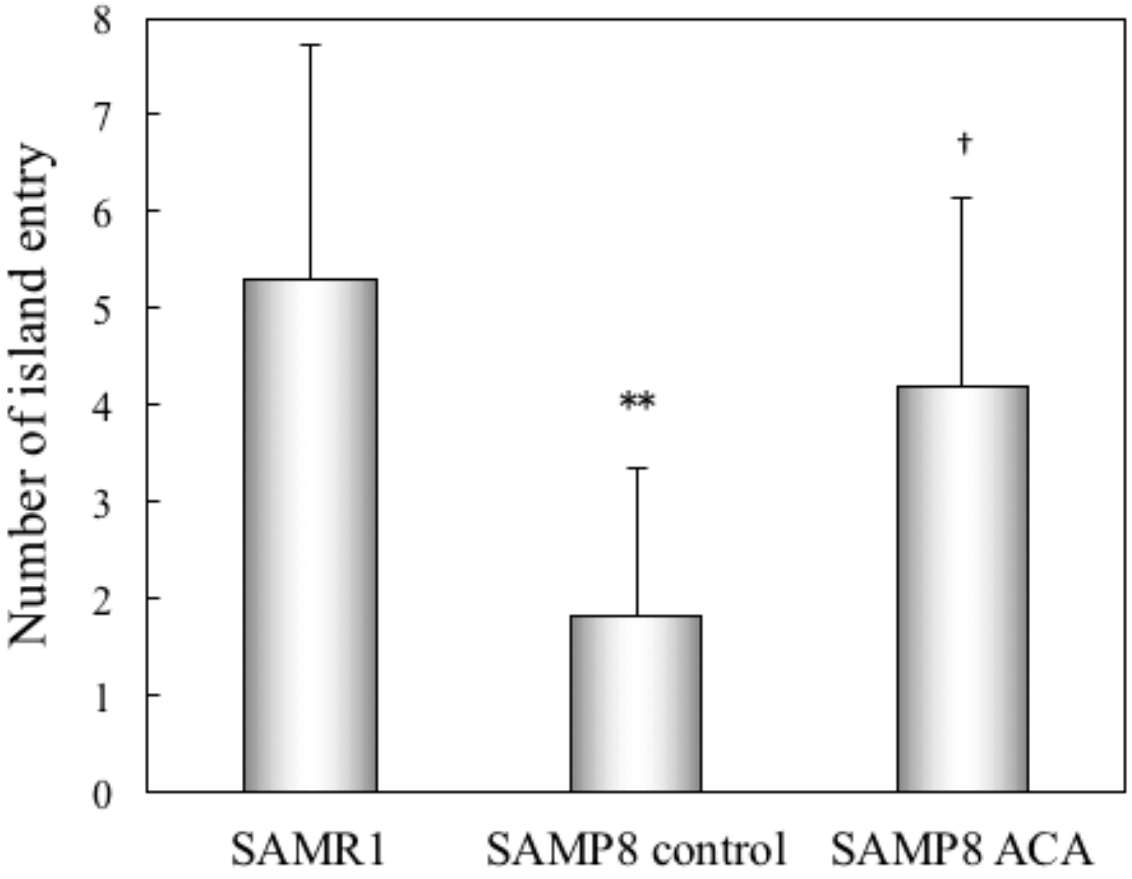

Number of island entry
0
1
2
3
4
5
6
7
8
**
†
SAMR1
SAMP8 control
SAMP8 ACA

Fig. 3B

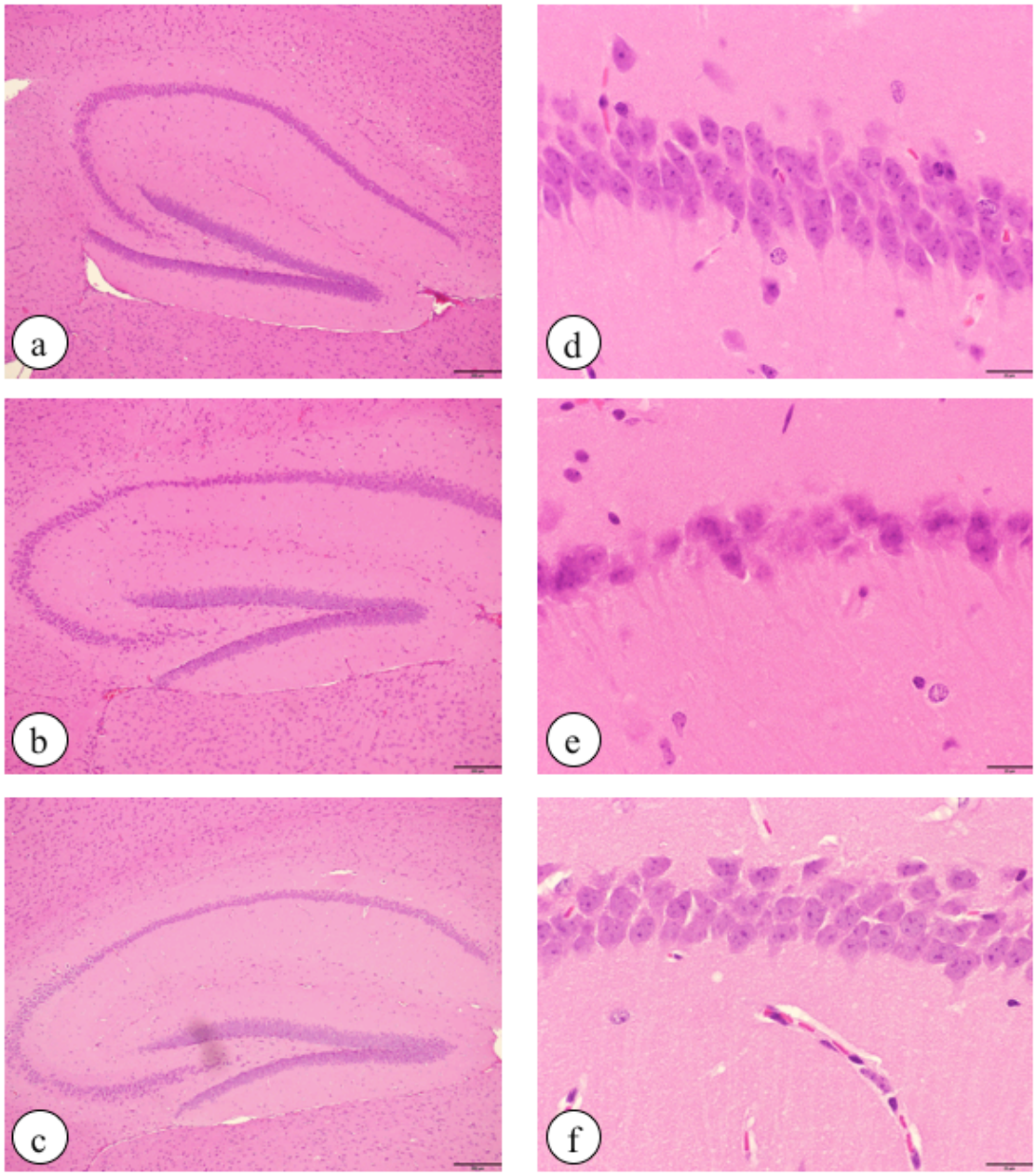


Fig. 4A

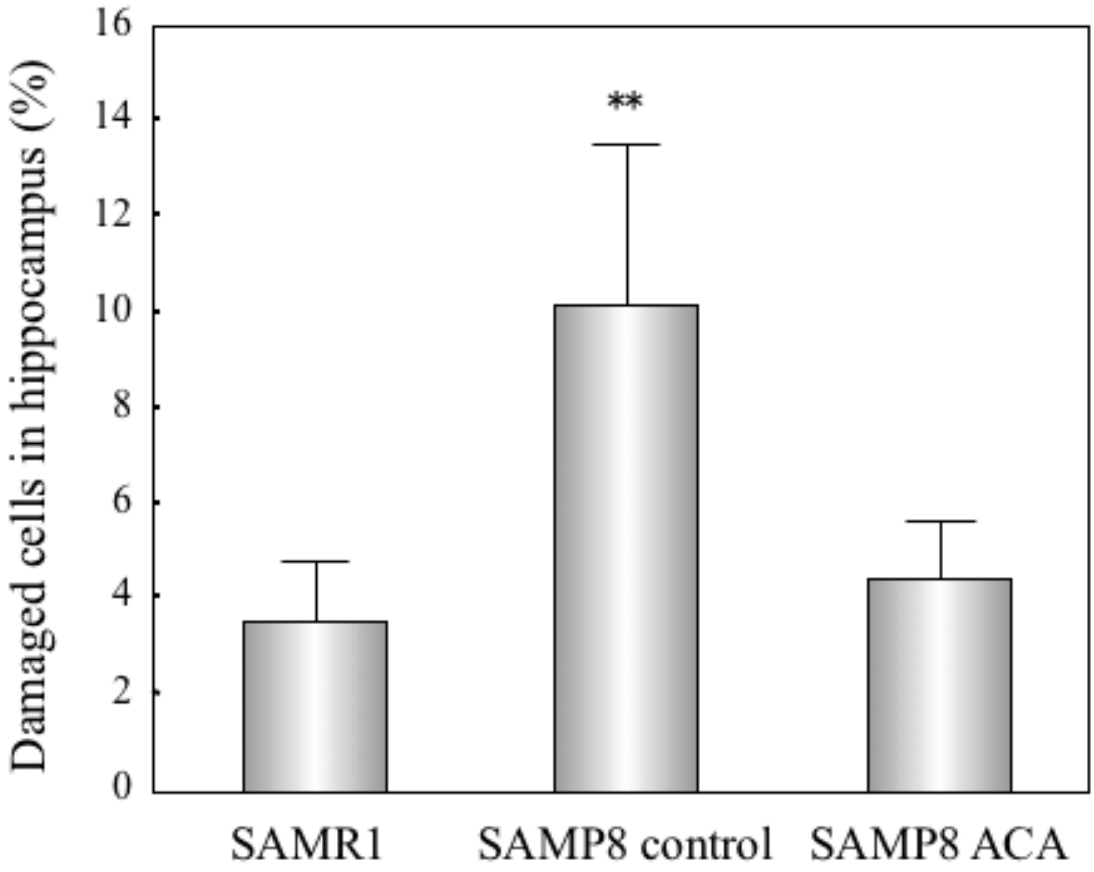

Damaged cells in hippocampus (%)
16
14
12
10
8
6
4
2
0
**
SAMR1
SAMP8 control
SAMP8 ACA

Fig. 4B

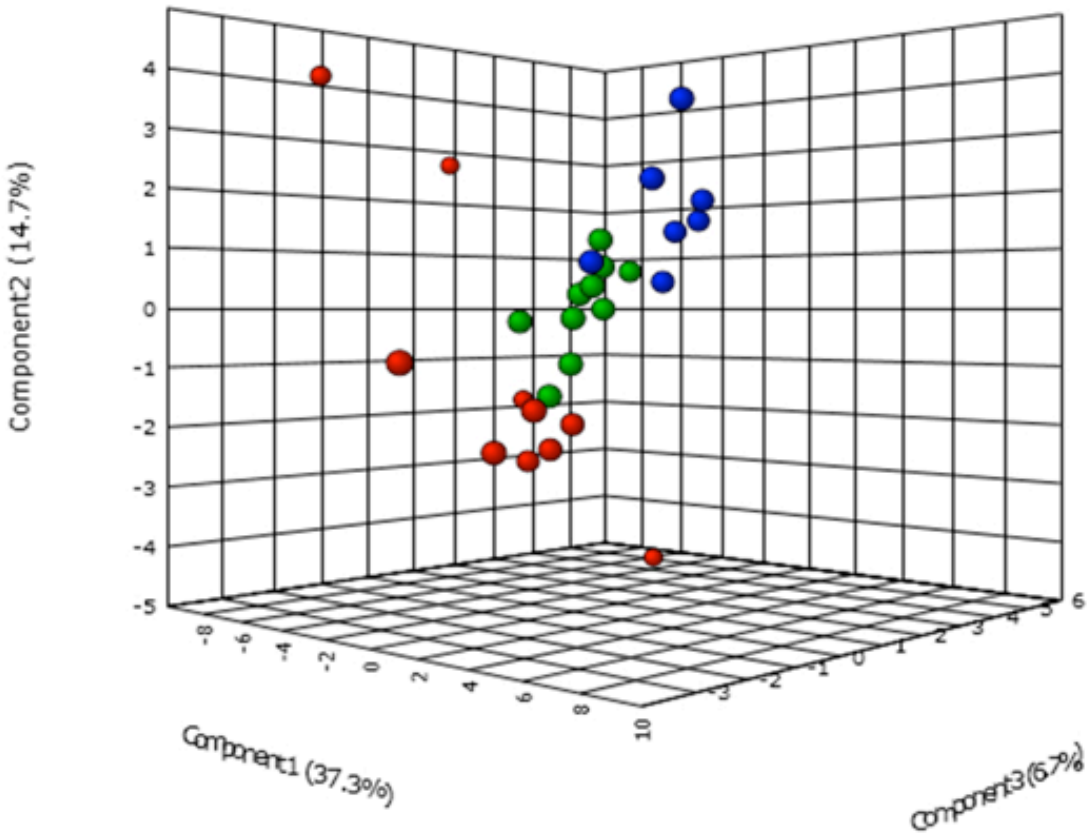


Fig. 5

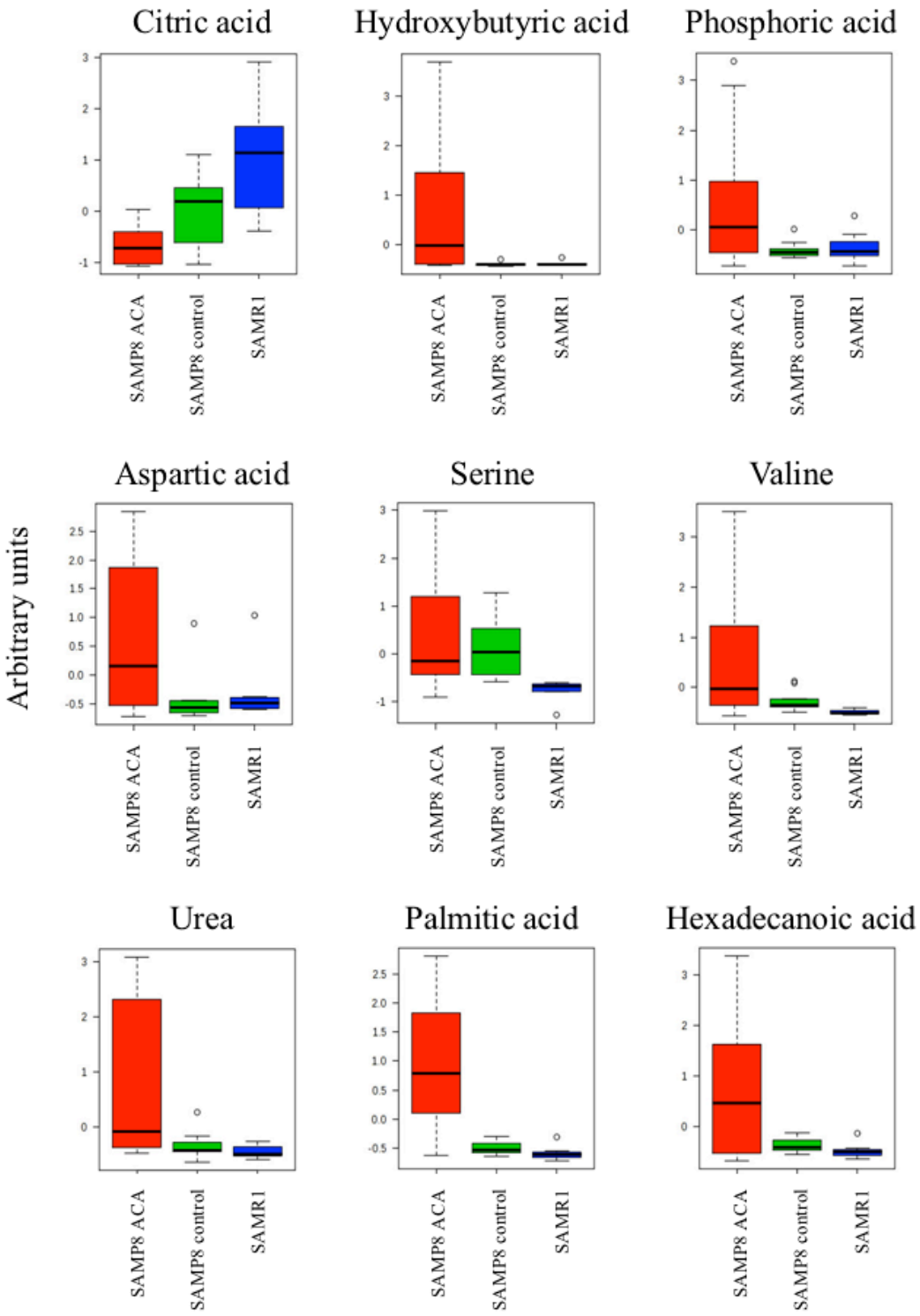

Citric acid
Hydroxybutyric acid
Phosphoric acid
Aspartic acid
Serine
Valine
Urea
Palmitic acid
Hexadecanoic acid
Arbitrary units
SAMP8 ACA
SAMP8 control
SAMR1

Fig. 6