

Effects of Theanine on Alcohol Metabolism and Hepatic Toxicity

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We previously showed that theanine, is a major amino acid in green tea, enhanced doxorubicin (DOX) induced antitumor activity. Besides, theanine induced the elevation of glutathione (GSH) level attributable to the increase of glutamate in the liver of mice, namely theanine would reduce the adverse reaction of DOX. Consequently, theanine was thought to be effective against the tissue changes with GSH level reduction. On the other hand, it is suggested excessive uptake of alcohol causes a production of free radicals, a decrease of GSH level, and an increase in the amount of lipid peroxide (LPO) in liver, and shifting to an alcoholic liver injury. Then, aiming at the prevention and medical treatment of a hepatic toxicity by the food components with little toxicity, we have studied the effect of theanine (i.p.) on ethanol metabolism and hepatic toxicity using ethanol (*p.o.*) single-administered mice. On the 1st hour after ethanol administration, the ethanol concentrations in blood of the theanine combined groups decreased compared with the ethanol-alone group. The alcohol dehydrogenase and aldehyde dehydrogenase activities in the liver increased by combined theanine. Since the elevation of cytochrome P450 (CYP) 2E1 activity was controlled in the theanine-combined groups, it was considered that these disorders attributable to CYP2E1 in ethanol long-term uptake might be avoidable by theanine. Although LPO increased in 3 h after by single-administration of ethanol, the increase was controlled by theanine-administration and was improved until the normal level. In conclusion, it was indicated that theanine was effective against alcoholic liver injury.

Key words theanine; glutathione; alcohol; alcohol dehydrogenase; aldehyde dehydrogenase; cytochrome P450 2E1

Theanine (Fig. 1), a major amino acid in green tea, is a *umami* (the fifth taste sensation)-component of tea and is contained 2–3% in tea leaves.^{1,2)} Theanine is a glutamate derivative and is used extensively as supplements and beverages, because it has a brain nerve function such as a relaxing effect due to inducing alpha waves.^{3–6)} But there are few reports about pharmacological action of theanine in comparison with catechin or caffeine, major tea components, and its effects on living individuals have not revealed closely. In recent studies, we showed that theanine enhanced doxorubicin (DOX), an anthracycline derivative, induced antitumor activity, and confirmed that this action contributed to the increase in the concentration of DOX in tumor with inhibition of the efflux of DOX from tumor cells.^{7–13)} Besides, theanine induced the elevation of glutathione (GSH) level attributable to the increase of glutamate in the liver of mice, namely, theanine would reduce the adverse reaction of DOX. Although GSH in tissues are kept constant by homeostasis, whereas was decreased by disease or medicine or age.¹⁴⁾ The usefulness of theanine was shown when the GSH level in liver decreased by the hepatic load of DOX. Furthermore, it was suggested that GSH level would increase by the metabolism of theanine. Thus, theanine was thought to be effective against tissue changes with GSH level reduction.

On the other hand, it is suggested excessive uptake of alcohol causes a production of free radicals, a decrease of GSH level, and an increase in the amount of lipid peroxide (LPO) in liver, and shifting to an alcoholic liver injury.^{15–18)} In the

liver, theanine is metabolized, converted glutamate, and induced to increase GSH level.¹⁹⁾ Namely, theanine may be expected to affect on to an alcoholic liver injury. Then, aiming at the prevention and medical treatment of a hepatic toxicity by the food components with little toxicity, we have studied the effect of theanine on ethanol metabolism and hepatic toxicity using ethanol single-administered mice. To evaluate the effect of theanine on ethanol metabolism and its toxicity, theanine was intraperitoneally injected and ethanol was orally administered.

MATERIALS AND METHODS

Materials Alcohol Dehydrogenase (ADH, EC 1.1.1.1) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The other chemicals used in this study were of the highest purity available.

Animals Male CDF₁ mice (5 weeks of age and weighing 20–25 g) were obtained from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed in a room maintained at 25±1 °C and 55±5% relative humidity, and were given free access to regular chow pellets and water.

Change of Ethanol Distribution in the Blood after Theanine Administration Subjects were assigned to one of three groups. One group was the ethanol alone group, which was administered only 50% ethanol (3.0 g/kg, *p.o.*). The second group was the theanine pre-administered group, which was administered theanine (100 mg/kg, i.p.) before 1 h of ethanol administration. The third group was the theanine post-administered group, which was administered theanine at 30 min after ethanol administration. After each administration, blood was collected from the heart at definite time, then, the liver was removed and weighed.

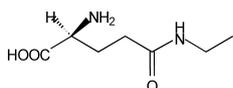


Fig. 1. Structure of Theanine

The alcohol-concentration in blood was measured by the enzyme-method by Bonnichsen, *et al.* (ADH-method).^{20,21} Perchloric acid (0.33 N, 0.8 ml) was put into the centrifuging tube and 0.1 ml of blood was added, and mixed. After removal of pellet by centrifugation (1200×g, 5 min), 0.1 ml of the supernatant in the centrifuging tube diluted by adding 4.8 ml of semicarbazide-plus buffer (pH 8.7). After addition of 0.1 ml of 0.48 mM nicotinamide adenine dinucleotide (NAD) and 0.02 ml of ADH (≥ 32 IU/ml), incubated at 37 °C for 25 min, and then determined the absorbance of generated nicotinamide adenine dinucleotide, reduced form (NADH) (wavelength, 340 nm).

Effect of Theanine on the Activities of Alcohol Metabolic Enzymes after Ethanol Administration The ADH activity was determined by the modified method of Haseba *et al.*²² The liver samples were homogenized in 10 volumes (w/v) of 0.5 mM Tris-HCl buffer (pH 8.5) and then centrifuged (3000×g, 20 min). The supernatant was centrifuged at 105000×g for 1 h, 0.1 ml of this supernatant was diluted by adding 2.0 ml of 0.1 mM glycine-sodium hydrate buffer (pH 10.7), then added 0.1 ml of 39 mM NAD and pre-incubated at 37 °C for 1 min. 0.1 ml of 2% ethanol was added to it as substrate, incubated at 37 °C for another 3 min, and the absorbance was measured (wavelength, 340 nm). The enzymatic activity was obtained by the difference of the absorbances.

The aldehyde dehydrogenase (ALDH) activity was determined by the method of Manthey's *et al.*²³ Homogenized liver samples in 20 volumes of 0.1 mM phosphate buffer (pH 7.0) were centrifuged at 15000×g for 30 min. The reaction-mixture (1.7 ml) was added to 0.2 ml of the supernatant, and pre-incubated at 37 °C for 3 min. After the addition of 0.1 ml of 80 mM acetaldehyde, the mixture kept incubation, then determined the absorbance at 340 nm for 5–10 min. The reaction-mixture was confected to mix 1.0 ml of 64 mM sodium pyrophosphate, 0.08 ml of 100 mM NAD, 0.02 ml of 10 mM pyrazole, 0.2 ml of 10 mM ethylenediaminetetraacetate (EDTA) and 0.4 ml of distilled water.

P450 level was calculated by the carbon monoxide difference spectrum method according to Omura-Sato.²⁴ The drug-metabolizing enzyme activity was determined using the HPLC method by Umegaki's *et al.*²⁵ Substrate and the diluted microsomes were mixed and the generated metabolite was measured by the HPLC method using the fluorescence detector.

The cytochrome P450 (CYP) 2E1 activity (*p*-nitrophenol hydroxylase activity) was determined by measuring *p*-nitrocatechol, generated by the enzymatic reaction, using the HPLC method.^{26,27}

The glutathione *S*-transferase (GST) activity was determined according to the Habig-Jakoby method.²⁸ The cytosol solution was diluted in 400 volumes with saline. Substrate solution (40 mM 1-chloro-2,4-dinitrobenzene, 50 μ l) was added to 1.85 ml of assay buffer (0.1 M potassium phosphate buffer, pH 6.5), 50 μ l of 40 mM GSH, and 50 μ l of the diluted cytosol solution. The mixture was incubated at 25 °C, the absorbance at 340 nm for 2–3 min was measured, and the GST activity was calculated by the difference of the absorbances.

The Effect of Combined Theanine on the Hepatic Toxicity of Mice after Ethanol Administration The LPO concentration as thiobarbituric acid reactive substance (TBARS)

was measured by the thiobarbituric acid-fluorometry,²⁹ and the GSH concentration was determined according to the Hissin-Hilf method.³⁰ The glutathione peroxidase (GSHPx) activity was determined by the modified method of Hafeman's *et al.* method.³¹ The γ -glutamyltranspeptidase (γ -GTP) activity in blood was measured using γ -GTP C-test WAKO® (Wako Pure Chemical Industries Ltd. (Tokyo, Japan)).

Statistical Analysis Statistical analysis was performed with Student's *t*-test and ANOVA.

RESULTS

Change of Ethanol Distribution in Blood after Theanine Administration The effect of theanine on the alcohol concentration in blood after ethanol administration is shown in Fig. 2. In the theanine pre-administrated group, we could observe change in the ethanol concentration in blood at 1 h after ethanol administration and significantly decreased to 38.8% ($p < 0.01$) compared to the ethanol alone group (0.108 \pm 0.039 mg/ml), and showed 43.7% in 3 h later. Moreover, in theanine pre-administrated group, area under the curve (AUC)_{0–3h} was 75% that of ethanol alone group. On the other hand, in theanine post-administration group, alcohol-concentration in blood decreased in 32.7% ($p < 0.01$) of the ethanol alone group in 1 h later.

Effect of Theanine on the Activities of Alcohol Metabolic Enzymes after Ethanol Administration The effect of theanine on the ADH activity in the liver is shown in Fig. 3. The ADH activity transiently increased after ethanol administration (Fig. 3A) whereas, in the theanine pre-administrated group, showed 1.7-fold higher ($p < 0.05$) at 30 min after administration of ethanol compared with the ethanol alone group. Furthermore, at 2 h later, the both ADH activities in theanine pre-administration and post-administration groups were tendency to increase and showed on 1.3-fold and 1.5-fold of that in ethanol alone group, respectively (Fig. 3B).

Next, change of the ALDH activity in the liver of mice is shown in Fig. 4. Although ALDH activity was decreased by ethanol administration, this decrease was prevented by theanine combination. At 3 h after administration of ethanol, each ALDH activity in theanine pre-administration and post-administration groups increased by 1.2-fold and 1.5-fold

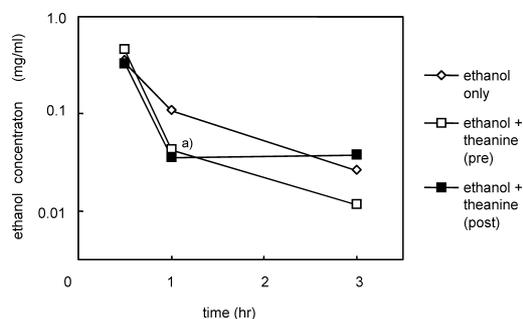


Fig. 2. Effect of Theanine on Blood Alcohol Concentration by Oral Administration of Ethanol to Mice

Male CDF₁ mice were administrated theanine (100 mg/kg) at 1 h prior to oral administration of ethanol (3.0 g/kg) (theanine pre) and administrated ethanol at 0.5 h prior to administration of theanine (theanine post). Each point is the mean for 4–5 mice. The S.D. of each value is less than 10%. Significant difference from the concentration of ethanol group (1 h) is indicated by a) $p < 0.01$.

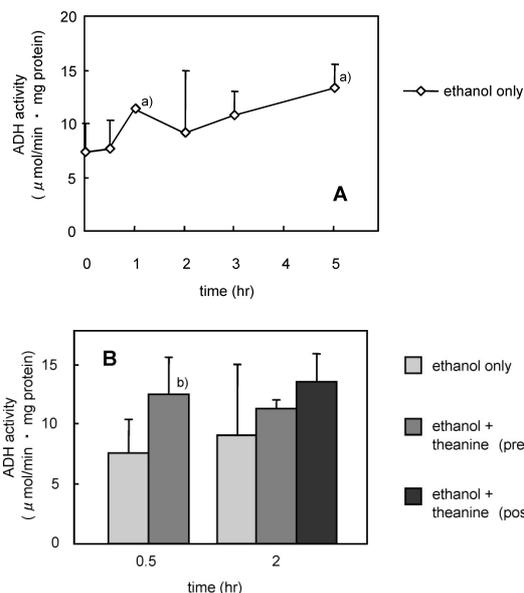


Fig. 3. Changes in the Activity of ADH in Liver of Mice after Ethanol and Theanine Administration

Each point represents the mean of 3–5 mice. Significant difference from the activity of normal is indicated by a) $p < 0.05$. Significant difference from the activity of ethanol is indicated by b) $p < 0.05$.

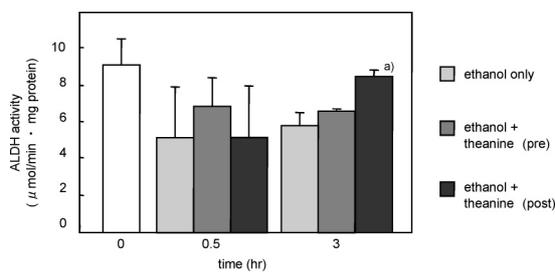


Fig. 4. Changes in the Activity of ALDH in Liver of Mice after Ethanol and Theanine Administration

Each point represents the mean of 3–5 mice. Significant difference from the activity in ethanol treated group is indicated by a) $p < 0.01$.

($p < 0.01$) compared to that of ethanol alone group, respectively. Moreover, in total ALDH activity for 3 h, the theanine administration showed 1.2-fold and 1.4-fold higher of that of the ethanol-alone ($1.47 \mu\text{mol/g protein}$), respectively.

The effect of theanine on CYP2E1 activity after ethanol administration is shown in Fig. 5A. CYP2E1 activity was induced by ethanol administration, and was 1.3-fold higher than that of the normal group in 3 h later. On the other hand, the theanine pre-administration inhibited the ethanol induced CYP2E1 activity and 3 h after, 75.6% of the ethanol-alone group ($p < 0.001$) was indicated. Whereas, in the theanine post-administration, change was not observed in CYP2E1 activity, compared to normal level. The effect of theanine on P450 content after ethanol administration is shown in Fig. 5B. The P450 content showed the same pattern as the effect of theanine on the CYP2E1 activity. Moreover, in each group, change was not seen about GST activity (data not shown).

The Effect of Theanine on Ethanol Induced Hepatic Toxicity Change of the LPO level in the liver of mice after ethanol and theanine administration is shown in Fig. 6. At 3 h after, the LPO level in ethanol alone group increased to 1.7-

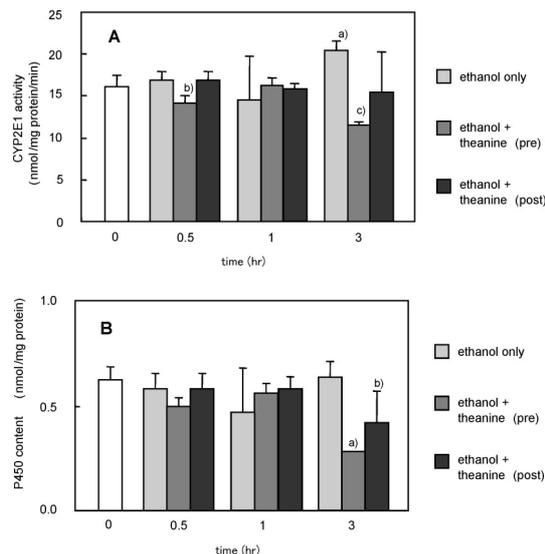


Fig. 5. Effects of Theanine on CYP2E1 Activity (A) and P450 Content (B) after Oral Administration of Ethanol to Mice

(A) Each point represents the mean of 4–5 mice. Significant difference from the activity of normal (0 h) is indicated by a) $p < 0.01$. Significant difference from the activity of ethanol (0.5 h) is indicated by b) $p < 0.01$. Significant difference from the activity of ethanol (3 h) is indicated by c) $p < 0.001$. (B) Each point represent the mean of 4–5 mice. Significant differences from the activity of ethanol (3 h) are indicated by a) $p < 0.001$ and b) $p < 0.05$.

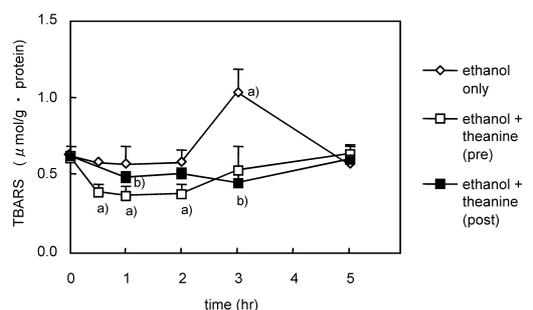


Fig. 6. Changes in the Lipid Peroxide Level in Liver of Mice after Ethanol and Theanine Administration

Each point represents the mean of 3–5 mice. Significant differences from the level of normal group (0 h) are indicated by a) $p < 0.01$ and b) $p < 0.05$.

fold of the normal level whereas the increased LPO level was suppressed by theanine combination, and it showed lower value significantly than the normal level within the measurement period. Especially, in the theanine pre-administration, the level in 3 h was continuously decreased to 60% of the ethanol alone group. There was no change of LPO level after theanine only treatment in the previous study.⁷⁾

γ -GTP, used as an index of alcoholic liver injury, was measured. In all groups, γ -GTP activity was within normal range (0–20 IU/l), and not being affected by ethanol single-administration was observed in this dosage and measurement period (data not shown).

The GSH concentration in the liver of mice after ethanol and theanine administration is shown in Fig. 7. The GSH concentration in the liver gradually decreased by ethanol administration, and decreased to 65.5% of the normal level ($p < 0.001$) at 5 h after. Contrary, the theanine post-administration group was maintaining the level in normal range. Whereas, the GSH concentration in mitochondria of the liver did not decrease compared to the normal level by ethanol ad-

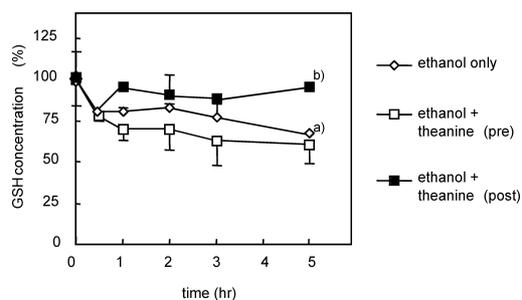


Fig. 7. Changes in the Concentration of GSH in Liver of Mice after Ethanol and Theanine Administration

Each point represents the mean of 3–5 mice. Significant difference from the level of normal is indicated by a) $p < 0.05$. Significant difference from the level of ethanol (5 h) is indicated by b) $p < 0.001$.

ministration but increased with time. Furthermore, in theanine pre-administration group, the mitochondrial GSH increased to 1.5-fold of the ethanol alone group in 30 min after ethanol administration (data not shown).

DISCUSSION

The alcoholic liver injury can be categorized by its grade of condition and there are fatty liver, alcoholic hepatitis, alcoholic fibroid liver, and liver cirrhosis. Although the fatty liver, generated in early stages, recovers by temperance, extensive and continued drinking goes on to alcoholic hepatitis or alcoholic fibroid liver and finally, shifts to liver cirrhosis. With chronic liver injury, necrosis and fibrosis of liver cells advance gradually and there will be a danger of going on to liver cirrhosis. An excessive intakes of alcohol causes a production of free radicals, a decrease of GSH level, and an increase in the amount of LPO in liver, and then shifting to an alcoholic liver injury is suggested.^{15–18}) Acetaldehyde, the reactive oxygen species (ROS) and malondialdehyde (MDA), generated along with the metabolism process of alcohol, are considered as the cause.³²) Some biochemical changes of liver cells are as follows: an intergradation of coenzyme system of liver cells to reduced form (redox shift), an increase of the amount of oxygen consumption, an induction of CYP activity, a functional and organic change of membrane, a production of acetaldehyde and an accumulation of LPO.³³)

In experiments on ethanol-induced liver injury, ethanol had been administrated by various dosages and routes, and it was common that the oral administration of ethanol was performed by 0.6–6.0 g/kg.^{34–36}) When oral administration of 0.6, 3.0, and 6.0 g/kg ethanol in CDF₁ mice was carried out, the concentration in blood of ethanol was low in 0.6 g/kg, and actual drinking was not reflected. On the other hand, the mice died about 5 min after 6.0 g/kg ethanol administration. Since the ethanol concentration (0.5–1.0 mg/ml) in blood of ebrious condition was obtained by oral administration of 3.0 g/kg ethanol, we administrated 3.0 g/kg of ethanol in this experiment. In theanine pre-administration, theanine was intraperitoneally injected 1 h before ethanol oral administration so that theanine might be distributed within liver at the time of ethanol administration. Moreover, since the concentration in blood might reach its peak in 30 min after ethanol administration, theanine was intraperitoneally injected 30 min after ethanol administration so that theanine might be absorbed

after the ethanol distribution. Since the ethanol concentrations in blood of the both combined theanine groups decreased compared with the ethanol-alone group at 1 h past administration (Fig. 2), it was shown that the combination of theanine (i.p.) with ethanol drinking effectively reduces the ethanol concentration in blood. Namely, as theanine (i.p.) proved to be effective on ethanol acute condition after ethanol single-administration, it was thought that theanine could be effective also by *p.o.* and moreover against a chronic condition.

Since it became clear that the ethanol concentration in blood was decreased by combined theanine, the enzyme activity of alcohol metabolism was examined subsequently. The ADH and ALDH activities were increased by combined theanine, and this efficacy was strong in the theanine post-administration group. It is not clear on the mechanism of the increased activities of these enzymes by theanine. It is expected that increased activities by theanine were caused by both increase in its protein level and elevation of its sensitivity. However, it is expected that the elevation of these enzymes activity by theanine promoted the oxidative metabolism of ethanol and acetaldehyde, and induced clearance and excretion of ethanol. Both oxidation by ADH and ALDH require NAD as coenzyme. Since NADH accumulated with ethanol oxidation reaction, re-oxidation of NADH in mitochondria will behave as a rate-controlling factor, and the metabolism of the acetaldehyde which is the second reaction of ethanol oxidation will be affected.^{37–39}) Although theanine did not only increase ADH activity but also ALDH activity, it was thought that metabolism of acetaldehyde was also performed smoothly. The first step of ethanol metabolism is oxidation reaction, and is usually performed with ratio of ADH; 80% and CYP2E1; 20%. The K_m value of CYP2E1 is 4 to 5 times as high as ADH. CYP2E1 induces by ethanol of over-intake or chronic ethanol intake.^{17,32}) Although CYP2E1 was induced by single-administration of ethanol in this study, whereas this increase was suppressed in the theanine pre-administration group. The post-administrated theanine did not increase CYP2E1 activity, too. The CYP2E1 activity increases over a long period of alcohol intake, and it is reported that this metabolism ratio enhancement brings about toxic increases, such as elevation in the amount of oxygen consumption, increase in aldehyde or oxidation and LPO accumulation.³⁷) Since toxicity of acetaldehyde is higher than that of ethanol in ethanol metabolism, it is ideal that acetaldehyde oxidization is faster than ethanol oxidization. Theanine decreased the CYP2E1 activity in this ethanol single-administration, therefore, it was considered that CYP2E1 induced disorder by ethanol long-term intake might be avoidable by theanine. In P450 content after ethanol administration, the same pattern as the effect of theanine on CYP2E1 activity was shown, and it was thought that P450 content correlated with CYP2E1 activity.

Although LPO increased after 3 h of single-administration of ethanol, the increase was suppressed by theanine combination and was improved until the normal level (Fig. 6). Thus, it was considered that the theanine induced decrease in the ethanol level in blood lead the normalization of LPO level, and that theanine was effective against alcoholic liver injury.

As theanine improved the liver damage by DOX, based on the inhibition of GSH reduction, it was expected that thea-

nine mediated GSH generation on the ethanol-induced LPO induction. In the liver, theanine is metabolized, converted glutamate, and induced to increase GSH level.¹⁹⁾ A glutamate concentration in the liver is about 50 nmol/g tissue, and it increased to 90 nmol/g tissue by theanine administration.¹⁹⁾ In this study, GSH level in the liver decreased by ethanol administration whereas was maintaining the normal level by theanine combination.

It became apparent that the combination of ethanol with theanine reduced the concentration in blood of ethanol due to the increase in ethanol metabolic enzyme activity by theanine (i.p.). Furthermore, it was shown that theanine combination decreased LPO leading to fatty liver which was the initial liver injury by alcohol. It was suggested that the improvement of GSH reduction by theanine, and the decrease of the ethanol concentration in blood by theanine were considered to prevent decrease of liver function. It is considered that the connection with theanine conversion mediated effects and ethanol metabolism is very complication. Thus, it is expected that each administration schedule had different effects on some parameters.

Because GSH, has antioxidant properties, does not transfer into a cell from the outside of the cell, its clinical effect is weak. Then, there are many reports on the increase of the cell membrane permeability by GSH derivatives.^{40,41)} Since theanine has the increase effect of GSH in a liver cell as well as them, the effect on a chronic alcohol-induced liver injury will be also expected. Moreover, it is shown that the reduction of GSH in a mitochondria raises sensitivity to tumor necrosis factor- α (TNF- α), and an ethanol-induced liver injury advances.⁴²⁾ Since theanine increased the GSH in a mitochondria, theanine is also expected to be effective on this point. In conclusion, although theanine itself does not have antioxidant properties,⁷⁾ it is predicted that intake of theanine accompanied by the increase in effective GSH on the oxidative stress and the disease.

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