[Jpn. J. Toxicol. Environ. Health
衛生 化学
42 (3) 241-247 (1996)]

Comparative Hepatotoxicity of Aluminum Administered with Maltol and Kojic Acid to Mice

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(Received December 26, 1995)

Maltol is a food additive used worldwide, while it enhances the brain toxicity of aluminum (Al). Al injected with maltol is primarily transferred to the liver and so it may also cause hepatic injury. Kojic acid is used as a food additive in Japan and is structurally related to maltol. Therefore, the hepatotoxicity of Al with maltol was compared to that of Al with kojic acid in mice. Al injected intravenously with maltol in a 1:4 molar ratio at a dose of 0.25 mmol Al/kg caused an increase in the plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) despite lower hepatic Al concentration than those found after administration in a 1:2 molar ratio and with Al only. At the same time, hepatic phosphorus and sulfur (S) levels decreased. The decrease in the S level was ascribed to that in the taurine content. The increase in urinary taurine was also observed. Al alone, maltol alone, or Al: kojic acid at a ratio of 1:4 did not alter the plasma activities of AST and ALT or the hepatic element levels. These findings may suggest that tris(maltolato)aluminum(III) (Al(maltol)₃) is a hepatotoxin.

Key words — aluminum; maltol; kojic acid; hepatotoxin; taurine; HPLC-ICP

Introduction

Studies on aluminum (Al) toxicity during the past two decades have shown that Al in dialysates and in antacids causes encephalopathy.^{1,2)} A geographic correlation between the Al concentration in drinking water and the occurrence of Alzheimer's disease has also been demonstrated,³⁾ although the link is still controversial.^{4,5)}

Maltol (3-hydroxy-2-methyl-4-pyrone) is a food additive (flavoring agent) that is used worldwide.⁶⁾ The toxicity of Al in cultured neurons from rat brain hemispheres is enhanced in the presence of maltol.⁷⁾ Furthermore, neuronal lesions induced by Al-maltol complex have also been reported after intravenous and intraventricular administration.⁸⁻¹⁰⁾ Thus, much interest has been taken in the brain toxicity of Al in the pres-

ence of maltol.

We reported that Al injected with maltol was primarily transferred to the liver,¹¹⁾ as it is after administration of the Al-maltol complex.⁸⁾ The Al accumulated in the liver may cause hepatic injuries. Namely, Al with maltol may be toxic to various organs.

Kojic acid is a fermentation product of *Aspergillus oryzae* and is structurally related to maltol. The structures of maltol and kojic acid are shown in Fig. 1. Kojic acid is used as a food additive in Japan, for example, to impede the browning in shrimp and crab, as well as its usage worldwide in cosmetics. It also forms Al-complexes.¹²⁾ When Al was injected with kojic acid, hepatic Al concentration was higher than that after Al-maltol administration.¹¹⁾

The Al complexes of maltol and kojic acid are formed *via* dissociated hydroxyl

Fig. 1. Ligand Structures

group and adjacent carbonyl group. The stability constants for 1:1, 1:2, and 1:3 complexes of Al with maltol and kojic acid are 8.25, 15.81, 22.48 and 7.24, 13.72, 19.26 (25°C, ionic strength 0.6), respectively.¹²⁾ Thus, Al-maltol and Al-kojic acid systems have comparable stability constants. When Al is mixed with maltol and kojic acid in a 1:4 molar ratio at neutral pH region, Al·L₃ complexes (L=ligand) are mainly formed.¹²⁾

In this study, the hepatotoxicity of Al administered with maltol was compared to that of Al with kojic acid. As indices of hepatic injury, plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and several hepatic essential element levels were investigated. When significant changes were observed in the hepatic element levels, the compounds (fractions) responsible for the changes were also studied using a high performance liquid chromatograph (HPLC) connected directly to an inductively coupled plasma-atomic emission spectrometer (ICP) (hereafter abbreviated as HPLC-ICP).

Materials and Methods

Solution for Injection — Aluminum chloride hexahydrate and maltol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Kojic acid was obtained from Riedel-de Haën AG (Seelze, Germany). Aluminum chloride was mixed with maltol at 1:2 and 1:4 molar ratios or with kojic acid at a 1:4 molar ratio, and the solutions were adjusted to pH 7 with sodium hydroxide. The white precipitates were not formed, which suggested the formation of Al-ligand

complexes. The solution of maltol alone (at a concentration corresponding to that of a 1:4 molar ratio) was also neutralized. pH of the aluminum chloride and saline solutions was not adjusted.

Animal Experiments — Male mice of the ICR strain (mean body weight $\pm$ S.D.; 33.8 ± 1.7 g, Charles River Japan, Atsugi) were maintained in an environmentally controlled facility on a 12 h light/dark cycle. Animals were injected once intravenously *via* the tail vein with Al only (abbreviated as group ALO), Al and maltol at 1:2 (AM2) and 1:4 (AM4) molar ratios, or Al and kojic acid at a 1:4 molar ratio (AK4) at a dose of 0.25 mmol Al/5 ml/kg (5 mice/group). Mice were also injected with maltol only (MAL) or saline (SAL) at the same volume (5 mice/group). The mice were hydrated (50 ml/kg) at 22 h after the injection and urine was collected for 2 h as reported.¹³⁾ Blood was collected by cardiac puncture under ether anesthesia 24 h after the administration, and then the liver was excised.

Preparation of the Liver Supernatant Fraction for HPLC-ICP Study — A 0.5-g sample of the liver from each mouse was pooled for each group and homogenized in 4 vol. of 0.1 M Tris-HCl buffer (pH 7.4, 0.25 M glucose) under a nitrogen atmosphere to prevent the oxidation of substances containing thiol (SH).¹⁴⁾ The homogenates were centrifuged at $100000 \times g$ for 60 min at 4°C, and the supernatant fraction was obtained.

Element Determination — A portion of the liver (about 0.3 g) obtained from each mouse and a 0.2 ml-aliquot of the liver homogenate and the supernatant fraction prepared as above were digested with an acid mixture (HNO₃: HClO₄ = 5:1 (v/v), acids of metal analysis grade). After digestion, the solutions were diluted with distilled water. Element concentrations were determined using a vacuum-ultraviolet ICP (Thermo-Jarrell Ash ICAP-61, Waltham, MA, U.S.A.).

HPLC-ICP Study — The liver supernatant fraction (100 μ l) was applied to an HPLC (LC-6A, Shimadzu, Kyoto) equipped with a gel-filtration column (TSKgel G3000SW, 7.5 mm i.d. $\times$ 60 cm, with a guard column, 7.5 mm i.d. $\times$

7.5 cm, Tosoh, Tokyo). The fraction was eluted with 10 mM Tris-HCl buffer solution (pH 8.0) containing 0.1% NaN_3 at a flow rate of 1 ml/min. The eluate flowed continuously into the nebulizer tube of the ICP machine, and the atomic emission intensities of various elements were monitored for 3 s and stored every 3 s for 45 min using a personal computer. HPLC-ICP chromatograms were obtained as described.¹⁵⁾ A 0.1-ml aliquot of the urine sample from each mouse was pooled for each group and filtered (0.45 μm , Millipore, Bedford, MA, U.S.A.). The mixed urine sample was also examined by the HPLC-ICP.

Plasma Enzyme Activity — The activities of AST and ALT in plasma were determined colorimetrically, based on the method of Karmen,¹⁶⁾ using commercial kits (Wako).

Statistics — The actual data or logarithmic conversions were analyzed by a one-way analysis of variance followed by Duncan's test or the Cochran-Cox test. A value of $p < 0.05$ was considered significant.

Results

Figure 2 shows the Al concentration in the liver 24 h after the injection. Al could not be determined in the sample solution of SAL or MAL due to its low level. AM4 and AK4 showed significantly lower Al levels than ALO and AM2. There was not a significant difference between AM4 and AK4.

Figure 3 shows the plasma activities of AST and ALT for the six groups. AM4 exhibited significantly higher values of AST and ALT than SAL (control), indicating hepatic injury in AM4, while AK4 did not show any significant augmentation.

Fig. 2. Hepatic Al Concentration after Administration of Al-Ligand Mixtures

SAL, saline; MAL, maltol only; ALO, aluminum only; AM2, Al + maltol (1:2); AM4, Al + maltol (1:4); AK4, Al + kojic acid (1:4). Each value is the mean $\pm$ S.D. of five mice. ND, not determined. Values with a different superscript are significantly different at $p < 0.05$.

Fig. 3. Plasma Enzyme Activities after Injection of Al-Ligand Mixtures

SAL, saline; MAL, maltol only; ALO, aluminum only; AM2, Al + maltol (1:2); AM4, Al + maltol (1:4); AK4, Al + kojic acid (1:4). Each value is the mean $\pm$ S.D. of five mice. *Significantly different from the control (SAL) at $p < 0.05$.

TABLE I. Hepatic Element Concentrations ($\mu\text{g/g}$)^{a)}

	Ca	Fe	K	Mg	Na	P	S
SAL	32.1 $\pm$ 1.7	74.4 $\pm$ 32.4	2985 $\pm$ 106	210 $\pm$ 3	534 $\pm$ 42	3402 $\pm$ 55	2490 $\pm$ 70
MAL	30.4 $\pm$ 3.5	83.4 $\pm$ 21.4	3049 $\pm$ 155	221 $\pm$ 8	538 $\pm$ 54	3522 $\pm$ 140	2509 $\pm$ 53
ALO	37.5 $\pm$ 7.3	68.9 $\pm$ 22.7	3121 $\pm$ 48	222 $\pm$ 6	535 $\pm$ 39	3463 $\pm$ 80	2548 $\pm$ 162
AM2	32.7 $\pm$ 7.3	87.6 $\pm$ 33.4	3070 $\pm$ 126	208 $\pm$ 12	481 $\pm$ 33	3234 $\pm$ 121	2364 $\pm$ 111
AM4	32.9 $\pm$ 3.0	113.9 $\pm$ 21.4	3210 $\pm$ 120	206 $\pm$ 11	478 $\pm$ 58	2792 $\pm$ 143*	2203 $\pm$ 107*
AK4	31.4 $\pm$ 2.5	78.4 $\pm$ 39.6	3016 $\pm$ 202	214 $\pm$ 14	543 $\pm$ 27	3441 $\pm$ 241	2418 $\pm$ 117

a) Each value is the mean $\pm$ S.D. of five mice. *Significantly different from control (SAL) at $p < 0.05$.

Fig. 4. HPLC-ICP Chromatograms of the Liver Supernatant Fraction in SAL (Left) and AM4 (Right)

A 100- μ l aliquot of the liver supernatant fraction was applied to an HPLC equipped with a gel-filtration column, and eluted with 10 mM Tris-HCl buffer (pH 8.0). The eluate was continuously introduced into the nebulizer tube of an ICP, and the atomic emission intensities of the respective elements were continuously monitored. The numerical values following element symbols show the relative amplification factors for the ordinate (atomic emission intensity).

Table I gives several essential element concentrations in the liver. The levels of phosphorus (P) and sulfur (S) were significantly decreased in AM4 compared with SAL. However, no significant differences were observed in AK4.

Figure 4 shows HPLC-ICP chromatograms of the liver supernatant fraction of SAL and AM4. In the chromatogram for the S level, the peaks at 21.6 and 25.7 min were ascribed to reduced glutathione (GSH) and taurine, respectively,¹⁷⁾ by chromatography with the standard solutions. The peak height of taurine at 25.7 min (taurine) was markedly reduced in AM4, while that of GSH slightly increased. Since about 70% of S in the homogenate was recovered into the supernatant fraction, the changes observed in the supernatant fraction were believed to well reflect those in the total tissue (homogenate).

In the chromatogram for the P level, the height of the main peak was slightly reduced in AM4 (Fig. 4). Since only 30% of P in the liver homogenate was recovered into the supernatant fraction, a more profound change might take place in the sediment fraction.

Table II gives the peak areas for taurine and GSH along with their relative peak areas

to total S in the HPLC-ICP chromatograms (S level) for the six groups. The peak area for total S (data not shown) was the smallest in AM4 in accord with the hepatic S content (Table I). The peak area for taurine and its relative area to total S in AM4 were markedly lower than those in other groups. Thus, the decrease in the hepatic S concentration was ascribed to decrease of the hepatic taurine content. The peak area for GSH and the relative area to total S were the largest in AM4.

Figure 5 shows HPLC-ICP chromatograms of urine sample from SAL and AM4. The peaks at 19.9, 22.3, and 25.9 min were ascribed to inorganic sulfate, isethionate, and taurine, respectively, by chromatography

TABLE II. Peak Areas of Taurine and GSH in Liver Supernatant Fraction (Arbitrary Units)

	Taurine	Taurine/S	GSH	GSH/S
SAL	63.0	0.302	19.7	0.094
MAL	49.1	0.259	17.7	0.093
ALO	62.9	0.300	17.0	0.081
AM2	50.0	0.244	15.5	0.076
AM4	29.7	0.164	23.0	0.127
AK4	49.1	0.258	14.5	0.076

Fig. 5. HPLC-ICP Chromatograms of the Urine Samples in SAL (Upper) and AM4 (Lower)

See the legend to Fig. 4.

TABLE III. Peak Areas of Taurine in Urine (Arbitrary Units)

	Taurine/S	Taurine/SO ₄ ²⁻
SAL	0.362	0.89
MAL	0.363	0.86
ALO	0.418	1.06
AM2	0.481	1.28
AM4	0.569	2.49
AK4	0.395	1.06

with the standard solutions. The peak heights of taurine and isethionate (metabolite of taurine) increased in AM4. Namely, the urinary excretion of taurine was markedly increased in AM4, in accord with the decrease in the hepatic taurine content.

Table III gives the relative peak areas of taurine to total S and inorganic sulfate in the HPLC-ICP chromatograms (S level) of urine samples for the six groups. The increase in urinary taurine was most profound in AM4.

Discussion

Al complexes in which six coordination sites of Al are occupied by the coordinating atoms of ligands cannot interact with tissues in the body. Therefore, the Al complexes in the circulation are excreted into urine. Among the four groups given Al (ALO, AM2, AM4, and AK4), AM4 and AK4 showed lower hepatic Al levels than the other two (Fig. 2). These findings suggested that the Al complex-

es formed in AM4 and AK4 (Al·L₃ complexes) did not accumulate highly in the liver and were excreted into the urine. This means that Al chelation is a very important factor in the biochemical and toxicological behaviors of Al.^{18,19)}

Nevertheless, hepatic injuries were suggested in AM4 by the augmented AST and ALT activities, indicating that the Al-maltol complex, especially tris(maltolato)aluminum (III) (Al·L₃), may be a hepatotoxin. The toxicity of tris(maltolato)aluminum (III) may be, in part, associated with its neutrality, because the analogous tris(kojato)aluminum (III) formed in AK4, in which a dissociation of hydroxymethylene group may occur, did not cause hepatic injury.

Hepatic injury was accompanied by a decrease in the hepatic taurine content, when analyzed by HPLC-ICP (Fig. 4 and Table II). In mammalian species, high levels of taurine are found in the heart,²⁰⁾ liver,²¹⁾ and brain.²²⁾ Therefore, a deficiency of taurine produces abnormalities in cerebellar development.²³⁾ Furthermore, taurine is found in nerves at a high concentration,²⁴⁾ so it may be a neurotransmitter, although this is a matter of debate.²⁵⁾ Nevertheless, taurine is a very important substance in neurons.

Maltol enhances the brain toxicity⁷⁾ and, as shown here, the liver toxicity of Al. In the liver, Al itself did not decrease the hepatic taurine content, whereas the Al-maltol complex induced a decrease under our experimental conditions. On the other hand, Al itself causes a decrease in the taurine level of the frontoparietal and posterior parietal cortex of the brain *in vivo*,²⁶⁾ and stimulates a release of endogenous taurine from cultured cerebral cortical astrocytes *in vitro*.²⁷⁾ Consequently, the high brain toxicity of Al-maltol complex might be associated with an accelerated decrease or release of taurine in the brain, although the mechanism is far different from that in the liver. The Al-maltol complex may be a useful model²⁸⁾ with which to study the

adverse effects of Al.

The hepatic GSH content showed a tendency to increase slightly in AM4 (Table II). Although the finding is based only on the mean, the tendency was also reconfirmed after two injections performed as a supplementary experiment. No augmentation was observed, however, and there was actually a slight decrease in ALO. Jeffery *et al.*²⁹⁾ have also reported that the liver GSH content does not change even after intraperitoneal injections of aluminum chloride at a dose of 40 mg Al/kg for 3 d into rats. Therefore, the increased GSH level observed in this study may also suggest the toxicity of the Al-maltol complex, though the mechanism is not clear.

In connection with the decrease in hepatic taurine content, the urinary excretion of taur-

ine increased in AM4 (Fig. 5 and Table III). Taurine plays an important role in the conjugation of bile acids in liver. The increased urinary excretion of taurine in AM4 may therefore reflect the fact that hepatic taurine was not used effectively for the conjugation with bile acids due to the hepatic injury.

In summary, the administration of Al with maltol at a 1:4 molar ratio caused hepatic injury and decreased the hepatic S concentration mainly due to a decrease in the taurine content. At the same time, an increase in urinary taurine was also observed. Al administered with kojic acid at the same molar ratio as in maltol did not show any toxicity. These findings may suggest that tris (maltolato)aluminum(III) is a specific hepatotoxin.

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