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Original Article

A 4-week oral toxicity study of L-alanine in rats with a recovery period of 2 weeks

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ABSTRACT — To investigate the safety of L-alanine, male and female Sprague-Dawley strain SPF rats [Crj:CD(SD)IGS] were administered L-alanine at 2,000 mg/kg/day by gavage for 4 weeks. After the end of the dosing period, reversibility was assessed following a 2-week recovery period. In the results, there were no toxicologically significant changes caused by L-alanine in general condition, body weight, food consumption, ophthalmology, hematology, blood chemistry, organ weight or at necropsy. In urinalysis, increased number of animals showing urine protein-positive or phosphate salt was observed in males and females. In addition, urine volume was significantly increased in males. In histopathological examination, squamous cell hyperplasia in the limiting ridge in the stomach was observed in males and females. These changes were reduced or no longer observed after the 2-week recovery period and thus were reversible changes. These results suggest that repeated oral administration of L-alanine at 2,000 mg/kg/day for 4 weeks is well tolerated in male and female rats.

Key words: L-Alanine, Rats, General toxicity

INTRODUCTION

Alanine (Ala) is one of the simplest nonessential amino acids and most widely found in proteins. It is the second smallest amino acid next to glycine, and is synthesized biologically within organisms from pyruvic acid, an intermediate in the glycolytic system, by transfer of an amino group from glutamic acid by alanine transaminase.

There has been a long history of Ala taken in by protein ingestion. L-Ala intake estimated based on protein intake is approximately 5.8 g/day in the Japanese population (Tanimura, 2007). Ala has also been used as a food additive for flavoring and amino acid reinforcement; DL-Ala is approved as a designated food, and L-Ala is approved as an existing additive by the Food Sanitation Law, animal feed, and animal medicinal drug. There have been toxicity studies of DL-Ala, including 26-week feed study of 0, 5, 10, and 20% DL-Ala, which report no effects on general physical condition except for lower body weight gain in the 20% DL-Ala fed group (Chow *et al.*, 1976).

In industrial processes, only DL-Ala may be obtained by chemical synthesis, while L-Ala ((2S)-2-Aminopropanoic acid) [CAS No. 56-41-7] may be obtained by hydrolysis of protein or fermentation. L-, D-, and DL- amino acids are known to differ in their functional effectiveness, as well as toxicity or deleterious effects in nutrition (Gullino *et al.*, 1956; Man and Bada, 1987). For Ala, Adkins *et al*. (1962) has reported different mortality rate and body weight gain in chicks fed 1.25% L-Ala, 1.25% D-Ala, and 2.5% DL-Ala added to protein-free, amino acid diet for 10 days. However, the general toxicity of L-Ala has not been studied.

In this study, rats were orally administered L-alanine at 2,000 mg/kg/day, the technically highest feasible dose by gavage, for 4 weeks. In addition, a 2-week recovery period was provided to examine reversibility of the toxic changes.

MATERIALS AND METHODS

Ethical considerations

The 13-week oral toxicity study was approved by

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the Animal Care and Use Committee at BoZo Research Center Inc., and was conducted in compliance with the laws or guidelines relating to animal welfare including "Law Concerning the Protection and Control of Animals" Law No. 105, 1 October 1973, Revised on 22 December 1999), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Notification No. 6 of the Prime Minister's Office, Japan, March 27, 1980), "Guidelines for Animal Experimentation" (the Japanese Association for Laboratory Animal Science, May 22, 1987) and Good Laboratory Practices (Notification no 313, Ministry of Health, Labour and Welfare, Japan, March 31, 1982) (GLP).

Chemicals and preparation of dosing solutions

L-Ala was supplied from KYOWA HAKKO BIO CO., LTD. (Tokyo, Japan). L-Ala was dissolved in sterilized water for injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) to achieve the final concentration of 100 mg/mL. Dosing solutions were prepared at least once in 7 days.

Animals, animal husbandry and group composition

Male and female Sprague-Dawley strain SPF rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) at 5 weeks of age. The animals were quarantined and acclimatized for 1 week to a pelleted diet (radiation sterilized CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water (tap water, via automatic water-supply system) *ad libitum*. They were housed individually in stainless-steel wire mesh cages in an animal room which was maintained at 23 ± 3°C, 50 ± 20% relative humidity, air ventilation of 10 to 15 times per hour and 12-hr light dark cycle. After a 1-week acclimatization period, animals were randomized into groups of 15 (10/sex/group for main group and 5/sex/group for recovery group) of each sex, and divided into the control group or L-Ala group.

Examinations and observations

Clinical observations, body weight, and food consumption

Clinical observation was done 3 times every day (predosing, immediately after and 2 hr after dosing). Body weight was measured three times in Week 1 of administration and Week 1 of recovery and thereafter twice a week, every 3 or 4 days, during the administration and recovery periods. Food consumption was measured on Day 1 of administration and thereafter twice a week, every 3 or 4 days.

Ophthalmology

Examination was done 3 times: during the quarantine/acclimatization period (all animals), in Week 4 of administration and Week 2 of recovery [5 animals of each sex per group, except the male control group where 1 animal died and thus 4 males were examined]. A mydriatic agent (Mydrine® P: Santen Pharmaceutical Co., Ltd., Osaka, Japan) was applied to dilate the pupil, and then the anterior portion, transparent body and fundus oculi were examined using an ophthalmoscope (BXα-type: NEITZ Instruments Co., Ltd., Tokyo, Japan).

Urinalysis

Examination was done twice: in Week 4 of administration and Week 2 of recovery. The following parameters were examined on 4-hr urine: pH, protein, ketone body, glucose, occult blood, bilirubin and urobilinogen (Aution Sticks-7EA test paper, ARKRAY, Inc., Kyoto, Japan), color and sediment. The following parameters were examined on 20-hr urine: osmotic pressure (AUTO & STAT OM-6030, ARKRAY, Inc., Kyoto, Japan), sodium, potassium and chloride (Automatic Electrolyte Analyzer PVA-α II, A&T Corp., Kanagawa, Japan). Water intake (24-hr) and urinary volume were also measured.

Hematology

At the time of necropsy in Week 4 of administration and Week 2 of recovery, blood samples were collected from the abdominal aorta under diethyl ether anesthesia into blood collection tubes (SB-41: Sysmex Corp., Hyogo, Japan) containing EDTA-2K. Prior to blood sample collection, the animals were deprived of food overnight. The following parameters were examined: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count and white blood cell count (WBC) (Coulter Counter T890, Beckman-Coulter Inc., Tokyo, Japan), reticulocyte ratio (Brecher method) and leukocyte differentiation (microscopic examination using May-Giemsa staining). In addition, prothrombin time (PT), activated thromboplastin time (APTT) and fibrinogen (Coagulometer ACL 100, Instrumentation Laboratory, Bedford, MA, USA) were determined on the plasma obtained by centrifuging the blood samples treated with 3.8 w/v% sodium citrate.

Blood chemistry

At the same time as hematology, the following parameters were determined on the serum: ALP, total cholesterol (T-CHO), triglyceride (TG), phospholipid (PL), total

bilirubin (T-BIL), glucose (GLU), blood urea nitrogen (BUN), creatinine (CRNN), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (P), total protein (TP), (Toshiba Biochemical Analyzer Model TBA-120FR, Toshiba Medical Systems Corp., Tokyo, Japan), A/G ratio (calculated from protein fractions) and protein fraction (Densitometer CLINISCAN SA-V, Helena Laboratories, Inc., Saitama, Japan). In addition, the following parameters were determined on the plasma obtained from blood treated with heparin: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) (Toshiba Biochemical Analyzer Model TBA-120FR, Toshiba Medical Systems Corp.).

Pathology

After collection of blood samples, the animals were exsanguinated and subjected to necropsy. The following organs were weighed: brain, pituitary, thyroids (including parathyroids), adrenals, thymus, spleen, heart, lung (including bronchus), salivary glands (submandibular plus sublingual glands), liver, kidneys, testes/ovaries, prostate/uterus and seminal vesicles. Based on the above wet weight (absolute weight) and body weight at necropsy, organ weight per 100 g body weight (relative weight) was calculated. In addition to the above organs, the following organs were fixed with phosphate buffered 10% formalin (however, eyeballs and optic nerves were fixed with fixatives containing 3% glutaraldehyde and 2.5% formalin, and testes and epidydimides were fixed with Bouin's solution and preserved in phosphate buffered 10% formalin): spinal cord, sciatic nerves, Harderian glands, submandibular lymph node, mesenteric lymph node, thoracic aorta, trachea, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, epididymidis, vagina, mammary gland, sternum (including bone marrow), femur (including bone marrow), femoral skeletal muscle and skin. All of these organs/tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) and examined histopathologically.

Statistical Analysis

For comparison of numerical data (body weight, food consumption, water intake, quantitative data of urinalysis, hematology and blood chemistry, organ weight) between groups, Tukey's test was performed for comparison between the groups (Sakuma, 1977). (levels of significance: 1 and 5%, bilaterally) .

RESULTS

Mortality and clinical signs

One male in the control group died before dosing on Day 13 of administration, but this animal had shown no remarkable changes in clinical observation. Histopathological examination of this animal showed dilatation of renal pelvis in the kidney and focal hemorrhage in the lung (Table 6), but there were no changes suggestive of the cause of death. It was judged that this animal died incidentally, since this animal was in the control group.

Clinical observations, Body weights, Food consumption and Ophthalmology

There were no test article-related changes. The results of body weight are shown in Fig. 1 and those of food consumption in Fig. 2.

Urinalysis

A tendency toward increase in the number of urine protein-positive animals was observed in males and females. A tendency toward increase in the number of animals showing phosphate in sediment was observed in males and females. Urine volume was significantly increased in males. These changes were no longer observed after the 2-week recovery period. The results are shown in Table 1.

Hematology and Blood chemistry

There were no toxicologically significant, test article-related changes in any parameter. The results of hematological examination are shown in Table 2 and those of blood chemistry examination in Table 3. α-$_2$ globulin fraction ratio was significantly high in males in the Ala administered group compared to the control. However, this change was within the normal range because no difference was observed in other protein fractions or total protein.

Organ weights

There were no toxicologically significant, test article-related changes in any organs. The results are shown in Table 4 (absolute organ weight) and Table 5 (relative organ weight).

Necropsy

There were no test article-related changes in any organs.

Histopathology

Squamous cell hyperplasia in the limiting ridge in the

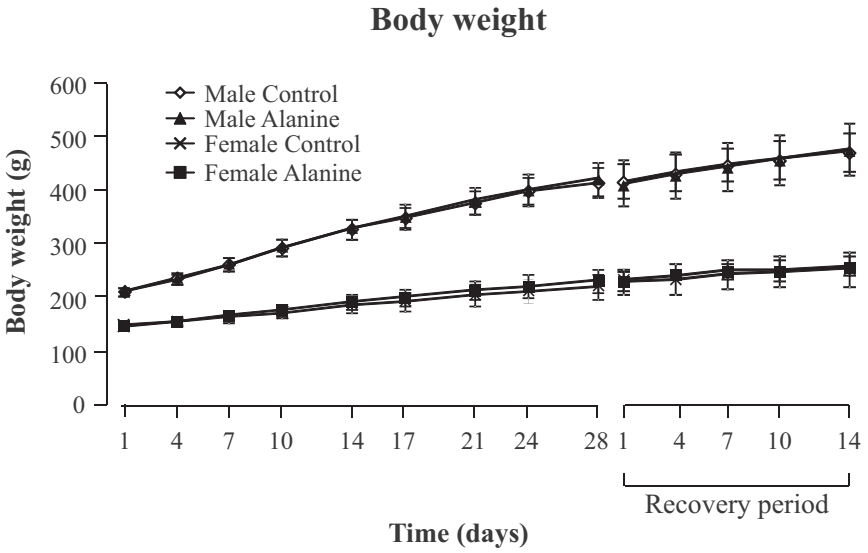

Fig. 1. Group mean body weight changes in a 4-week oral toxicity study of L-Ala in rats with a recovery period of 2 weeks.

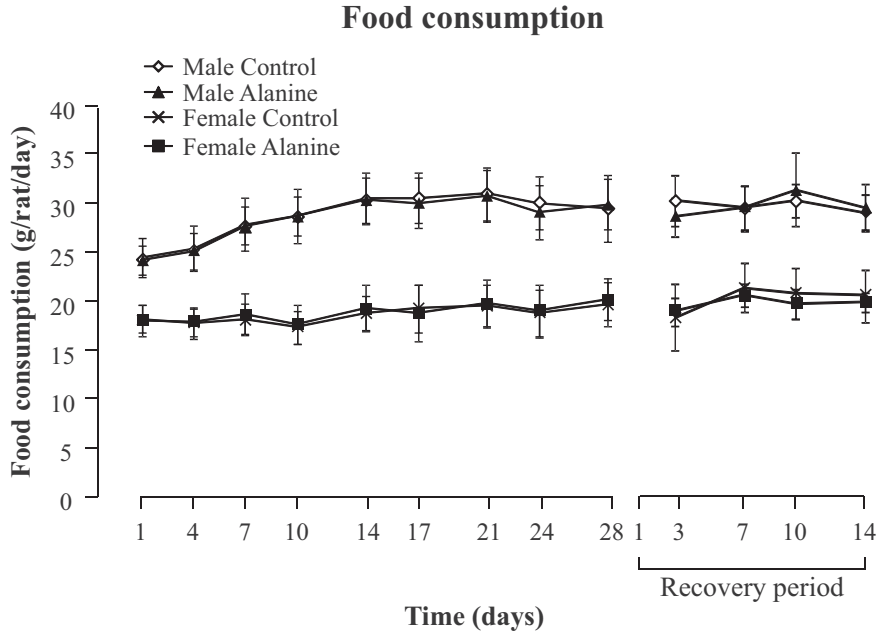

Fig. 2. Group mean food consumption in a 4-week oral toxicity study of L-Ala in rats with a recovery period of 2 weeks.

stomach was observed in males and females (Fig. 3). This change was reduced or no longer observed after the 2-week recovery period. The results are shown in Table 6.

DISCUSSION

In this study, to investigate the safety of L-Ala, male and female rats were administered L-Ala by gavage for 4 weeks. After the end of the dosing period, reversibility was assessed following a 2-week recovery period. In the results, there were no test article-related changes in clin-

Table 1. Water intake and urinalysis data.

Sex		Male				Female			
Group		Control		Alanine		Control		Alanine	
Weeks		4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)
Number of animals		10	4	10	5	10	5	10	5
pH	6.5	0	0	0	0	2	1	0	0
	7.0	0	0	0	0	1	0	3	1
	7.5	0	0	3	0	6	0	5	0
	8.0	9	0	6	0	5	0	5	1
	8.5	5	4	5	5	1	4	2	3
	9.0	0	0	1	0	0	0	0	0
Protein	-	10	0	6	0	15	3	11	3
	±	4	2	8	2	0	1	4	1
	1+	0	2	1	3	0	1	0	1
Ketone Body	-	13	2	15	5	15	4	15	5
	±	1	2	0	0	0	1	0	0
Glucose	-	14	4	15	5	15	5	15	5
Occult Blood	-	13	4	15	3	15	5	14	5
	±	0	0	0	2	0	0	1	0
	1+	0	0	0	0	0	0	0	0
	2+	1	0	0	0	0	0	0	0
Bilirubin	-	13	4	15	5	15	5	15	5
	1+	1	0	0	0	0	0	0	0
Urobilinogen	±	14	4	15	5	15	5	15	5
Color	Yellow	14	4	15	5	15	5	15	5
RBC	-	14	4	15	5	15	5	15	5
WBC	-	14	4	15	5	15	5	15	5
Ep.SEC	±	14	3	15	5	15	5	15	5
	1+	0	1	0	0	0	0	0	0
Ep.SREC	-	14	3	15	5	15	5	15	5
	±	0	1	0	0	0	0	0	0
Cast	-	14	4	15	5	15	5	15	5
Cr.PS	-	12	2	10	2	14	2	6	4
	±	2	2	5	3	1	3	8	1
	1+	0	0	0	0	0	0	1	0
Cr.CO	-	14	4	15	5	15	5	15	5
Water Intake (mL/24 hr)		38 ± 7	43 ± 3	44 ± 10	42 ± 6	31 ± 7	36 ± 9	33 ± 6	36 ± 6
Urine Volume（mL/24 hr）		13.4 ± 3.6	11.8 ± 2.7	19.7 ± 5.1##	12.4 ± 2.3	9.2 ± 4.2	11.3 ± 5.1	10.9 ± 4.8	10.9 ± 5.2
Osmolality (mOsm/kg)		1881 ± 370	1902 ± 223	1627 ± 313	1910 ± 238	2300 ± 638	1704 ± 489	2001 ± 519	1586 ± 406
Na (mmol/24 hr)		2.17 ± 0.58	1.76 ± 0.53	2.26 ± 0.41	1.99 ± 0.45	1.62 ± 0.67	1.35 ± 0.49	1.43 ± 0.52	1.36 ± 0.65
K (mmol/24 hr)		3.83 ± 1.16	3.35 ± 0.96	4.32 ± 0.54	3.63 ± 0.91	2.89 ± 1.20	2.65 ± 0.51	2.70 ± 1.12	2.48 ± 1.40
Cl (mmol/24 hr)		2.61 ± 0.74	2.22 ± 0.68	2.78 ± 0.44	2.50 ± 0.57	2.00 ± 0.83	1.71 ± 0.56	1.73 ± 0.69	1.67 ± 0.87

Protein) -:< 10, +/-:10-25, 1+:26-85 mg/dL
Ketones) -:< 5, +/-:5-7.5 mg/dL
Glucose) -:< 30 mg/dL
Occult Blood) -:< 0.03, +/-:0.03-0.05, 1+:0.06-0.15, 2+:0.16-0.75 mg/dL
Bilirubin) -:< 0.5, 1+:0.5-1.5 mg/dL
Urobilinogen) +/-:< 2.0 mg/dL
RBC: Red Blood Cells) -:Negative
WBC: White Blood Cells) -:Negative
Ep.SEC: Squamous Epithelial Cells) +/-:Slight, 1+:Mild
Ep.SREC: Small Round Epithelial Cells) -:Negative, +/-:Slight
Cast) -:Negative
Cr.PS: Crystal Phosphate Salts) -:Negative, +/-:Slight, 1+:Mild
Cr.CO: Crystal Calcium Oxalate) -:Negative
Values are mean ± S.D.
$p < 0.01$: Significant difference between control group and the Alanine administered group.

Table 2. Group means of hematological parameters.

Sex	Male				Female			
Group	Control		Alanine		Control		Alanine	
Weeks	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)
Number of animals	10	4	10	5	10	5	10	5
Red blood cell count (10^4/mL)	770 ± 41	784 ± 27	774 ± 26	788 ± 20	778 ± 30	769 ± 15	782 ± 27	782 ± 16
Hemoglobin (g/dL)	15.4 ± 0.5	15.3 ± 0.3	15.7 ± 0.5	15.6 ± 0.5	15.5 ± 0.5	15.4 ± 0.5	15.6 ± 0.5	15.5 ± 0.4
Hematocrit (%)	46 ± 2	44 ± 1	47 ± 2	44 ± 1	46 ± 2	44 ± 1	46 ± 2	45 ± 1
Mean corpuscular volume (fL)	59.4 ± 1.4	56.5 ± 1.5	60 ± 1.6	56.3 ± 1.0	58.5 ± 1.0	57.4 ± 1.0	58.2 ± 0.6	57.6 ± 1.0
Mean corpuscular hemoglobin (pg)	20.0 ± 0.6	19.5 ± 0.9	20.3 ± 0.6	19.7 ± 0.5	19.9 ± 0.3	20.0 ± 0.5	19.9 ± 0.3	19.8 ± 0.3
Mean corpuscular hemoglobin concentration (%)	33.7 ± 0.3	34.6 ± 0.8	33.8 ± 0.4	35.1 ± 0.4	33.9 ± 0.3	34.9 ± 0.5	34.2 ± 0.3	34.3 ± 0.2
Reticulocyte ratio (%)	2.0 ± 0.4	1.8 ± 0.2	2.2 ± 0.3	2.1 ± 0.1	1.7 ± 0.3	2.0 ± 0.4	1.6 ± 0.5	1.9 ± 0.4
Platelet count (10^4/mL)	105 ± 6.1	101 ± 5.5	110.7 ± 7.5	107.5 ± 5.6	107 ± 10.7	107 ± 16.9	106 ± 8.1	103.0 ± 12.4
Prothrombin time (s)	14.0 ± 1.5	13.1 ± 0.4	13.7 ± 0.7	13.0 ± 0.7	12.4 ± 0.4	12.1 ± 0.7	12.3 ± 0.3	12.5 ± 0.3
Activated partial thromboplastin time (sec)	19.3 ± 1.4	19.1 ± 1.3	19.5 ± 1.3	19.2 ± 1.2	15.1 ± 1.0	15.1 ± 0.9	15.3 ± 0.6	14.8 ± 0.7
Fibrinogen (mg/dL)	312 ± 18	296 ± 21	337 ± 46	320 ± 18	261 ± 34	241 ± 15	253 ± 25	248 ± 29
White blood cell count (10^2/mL)	95 ± 10	95 ± 17	100 ± 21	111 ± 30	78 ± 18	66 ± 15	73 ± 16	57 ± 8
Lymphocytes (%)	88.1 ± 5.0	88.9 ± 2.1	87.9 ± 3.6	88.8 ± 2.7	88.2 ± 3.8	85.4 ± 5.3	88.0 ± 6.3	82.7 ± 6.5
Stab (%)	0.1 ± 0.2	0.1 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.1 ± 0.2	0.5 ± 0.4	0.2 ± 0.2	0.4 ± 0.5
Seg. (%)	10.8 ± 4.6	9.3 ± 1.8	10.7 ± 3.1	9.7 ± 3.0	10.7 ± 3.5	13.0 ± 5.3	11 ± 5.6	15.3 ± 6.2
Eosinophils (%)	0.8 ± 0.5	1.3 ± 0.3	1.0 ± 0.6	1.0 ± 0.6	0.8 ± 0.9	0.9 ± 0.8	0.6 ± 0.8	1.5 ± 0.4
Basophils (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Monocytes (%)	0.4 ± 0.4	0.5 ± 0.0	0.3 ± 0.3	0.2 ± 0.3	0.3 ± 0.3	0.2 ± 0.3	0.4 ± 0.3	0.1 ± 0.2
Others (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Erythroblast counts(/200 leukocyte)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Values are mean ± S.D.

Table 3. Group means of blood chemical parameters.

Sex	Male				Female			
Group	Control		Alanine		Control		Alanine	
Weeks	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)
Number of animals	10	4	10	5	10	5	10	5
AST (IU/L)	69 ± 10	61 ± 4	74 ± 16	59 ± 7	65 ± 7	63 ± 7	67 ± 6	60 ± 2
ALT (IU/L)	30 ± 6	23 ± 3	31 ± 9	24 ± 3	21 ± 3	23 ± 5	24 ± 5	22 ± 2
LDH (IU/L)	62 ± 12	45 ± 10	59 ± 7	49 ± 10	45 ± 5	50 ± 9	49 ± 8	42 ± 15
ALP (IU/L)	602 ± 130	544 ± 119	639 ± 149	441 ± 35	429 ± 140	294 ± 113	403 ± 126	305 ± 47
Total cholesterol (mg/dL)	61 ± 10	62 ± 12	75 ± 21	74 ± 11	59 ± 18	65 ± 17	72 ± 15	63 ± 11
Triglyceride (mg/dL)	58 ± 28	66 ± 23	47 ± 22	66 ± 25	9 ± 4	11 ± 6	15 ± 17	10 ± 4
Phospholipid (mg/dL)	104 ± 14	106 ± 13	120 ± 25	117 ± 13	104 ± 25	117 ± 22	122 ± 25	109 ± 14
Total bilirubin (mg/dL)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
Glucose (mg/dL)	150 ± 17	165 ± 18	157 ± 20	169 ± 26	118 ± 13	127 ± 10	121 ± 16	117 ± 22
Blood urea nitrogen (mg/dL)	12 ± 1	12 ± 1	12 ± 1	13 ± 3	15 ± 3	16 ± 1	16 ± 1	18 ± 3
Creatinine (mg/dL)	0.27 ± 0.04	0.29 ± 0.03	0.27 ± 0.02	0.27 ± 0.04	0.31 ± 0.04	0.3 ± 0.02	0.28 ± 0.03	0.37 ± 0.04 #
Na (mmol/L)	141 ± 2	141 ± 1	141 ± 1	141 ± 1	140 ± 1	140 ± 1	141 ± 1	140 ± 1
K (mmol/L)	4.2 ± 0.3	3.8 ± 0.2	4.2 ± 0.1	4.3 ± 0.3 #	4.5 ± 0.3	4.2 ± 0.3	4.3 ± 0.4	3.8 ± 0.2
Chloride (mmol/L)	107 ± 2	105 ± 3	107 ± 2	106 ± 2	110 ± 2	110 ± 2	110 ± 2	111 ± 1
Calcium (mg/dL)	9.4 ± 0.3	10 ± 0.1	9.3 ± 0.2	9.9 ± 0.2	9.5 ± 0.4	9.9 ± 0.3	9.7 ± 0.4	9.8 ± 0.3
P (mg/dL)	8.0 ± 0.4	7.6 ± 0.6	8.1 ± 0.5	7.5 ± 0.6	8.2 ± 0.6	6.8 ± 0.5	8.3 ± 0.9	6.5 ± 1.1
Total protein (g/dL)	5.8 ± 0.2	5.8 ± 0.1	5.8 ± 0.2	5.9 ± 0.2	6.0 ± 0.3	6.3 ± 0.2	6.1 ± 0.2	6.1 ± 0.3
A/G ratio	0.82 ± 0.06	0.83 ± 0.04	0.81 ± 0.06	0.8 ± 0.03	0.89 ± 0.05	0.95 ± 0.05	0.88 ± 0.04	0.92 ± 0.05
Albumin (%)	45.2 ± 1.8	45.3 ± 1.2	44.5 ± 1.8	44.5 ± 0.9	47.2 ± 1.5	48.7 ± 1.4	46.8 ± 1	48 ± 1.4
Globulin(%)								
α_1-globulin (%)	22.9 ± 1.8	21.5 ± 2.3	22.6 ± 1.7	23.8 ± 0.7	20.6 ± 1.9	19.6 ± 2	20.7 ± 1.5	19.5 ± 2.3
α_2-globulin (%)	9.6 ± 0.6	10.4 ± 0.5	10.3 ± 0.7 #	10.4 ± 0.5	9.0 ± 1.2	8.5 ± 0.8	9.3 ± 0.9	8.4 ± 1.3
β-globulin (%)	16.7 ± 0.9	17.6 ± 0.7	17.4 ± 1.2	17.3 ± 0.5	16.4 ± 1.3	16.3 ± 1.6	16 ± 0.7	17 ± 0.7
γ-globulin (%)	5.7 ± 0.9	5.2 ± 0.7	5.1 ± 0.8	4.0 ± 0.8	6.9 ± 1.2	6.8 ± 1.6	7.2 ± 0.9	7 ± 0.5

Values are mean ± S.D.

: $p < 0.05$: Significant difference between the control group and the Alanine administered group.

Table 4. Group means of absolute organ weight data.

Sex	Male				Female			
Group	Control		Alanine		Control		Alanine	
Weeks	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)
Number of animals	10	4	10	5	10	5	10	5
Body weight on the day of necropsy (g)	381 ± 26	444 ± 34	390 ± 28	444 ± 48	201 ± 19	235 ± 29	214 ± 21	238 ± 20
Brain (g)	1.99 ± 0.07	2.07 ± 0.13	2.02 ± 0.12	2.05 ± 0.08	1.81 ± 0.08	1.84 ± 0.09	1.84 ± 0.08	1.82 ± 0.05
Pituitary (mg)	10.9 ± 1.9	11.7 ± 2.0	11.6 ± 1	11.2 ± 1	12.3 ± 2.8	12.6 ± 3.4	13.1 ± 2.2	11.6 ± 1.5
Thyroid gland (mg)	18.6 ± 4.3	17.7 ± 5.8	18 ± 3	15.3 ± 5.5	12.9 ± 2.9	12.8 ± 2.1	11.1 ± 1.7	10.7 ± 1.8
Salivary gland (mg)	612 ± 58	628 ± 80	628 ± 96	649 ± 81	368 ± 37	393 ± 45	399 ± 22	385 ± 40
Thymus (mg)	514 ± 95	477 ± 138	490 ± 73	484 ± 92	418 ± 96	396 ± 97	464 ± 93	342 ± 80
Heart (g)	1.32 ± 0.13	1.31 ± 0.07	1.36 ± 0.15	1.33 ± 0.19	0.74 ± 0.07	0.79 ± 0.11	0.80 ± 0.06	0.78 ± 0.07
Lung (g)	1.35 ± 0.07	1.38 ± 0.17	1.35 ± 0.11	1.38 ± 0.17	0.95 ± 0.08	0.99 ± 0.08	1 ± 0.07	1.03 ± 0.05
Liver (g)	11.5 ± 1.41	13.1 ± 1.15	11.8 ± 1.33	13.1 ± 1.74	5.98 ± 0.75	6.69 ± 1.08	6.43 ± 1.04	6.2 ± 0.36
Spleen (g)	0.68 ± 0.11	0.77 ± 0.23	0.72 ± 0.1	0.75 ± 0.09	0.43 ± 0.07	0.53 ± 0.11	0.47 ± 0.1	0.49 ± 0.07
Kidney (g)	2.89 ± 0.31	3.22 ± 0.39	2.82 ± 0.20	3.12 ± 0.35	1.65 ± 0.19	1.78 ± 0.25	1.73 ± 0.14	1.55 ± 0.19
Adrenal (mg)	62 ± 8	77 ± 21	62 ± 7	63 ± 10	62 ± 7	70 ± 5	65 ± 8	55 ± 8 #
Testis (g)	3.07 ± 0.23	3.24 ± 0.23	3.09 ± 0.23	3.05 ± 0.19	NA	NA	NA	NA
Seminal vesicles (g)	1.03 ± 0.17	1.21 ± 0.09	0.96 ± 0.13	1.17 ± 0.14	NA	NA	NA	NA
Prostate (g)	0.91 ± 0.09	1.06 ± 0.19	0.91 ± 0.15	1.1 ± 0.16	NA	NA	NA	NA
Ovary (mg)	NA	NA	NA	NA	75.1 ± 11.2	77.8 ± 5.7	76.9 ± 12	75.1 ± 11.6
Uterus (mg)	NA	NA	NA	NA	413 ± 81	489 ± 138	420 ± 130	455 ± 154

Values are mean ± S.D.
NA: Not applicable
: $p < 0.05$: Significant difference between the Control group and the Alanine administered group.

Table 5. Group means of relative organ weight data.

Sex	Male				Female			
Group	Control		Alanine		Control		Alanine	
Weeks	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)
Dose(mg/kg)	0	0	2000	2000	0	0	2000	2000
Number of animals	10	4	10	5	10	5	10	5
Brain (g/100g)	0.53 ± 0.03	0.47 ± 0.01	0.52 ± 0.03	0.46 ± 0.05	0.9 ± 0.06	0.8 ± 0.12	0.87 ± 0.09	0.77 ± 0.07
Pituitary (mg/100g)	2.9 ± 0.4	2.6 ± 0.3	3 ± 0.3	2.5 ± 0.3	6.1 ± 1.1	5.4 ± 1.8	6.1 ± 1	4.9 ± 0.7
Thyroid gland (mg/100g)	4.9 ± 1.1	3.9 ± 1.1	4.6 ± 0.8	3.5 ± 1.1	6.4 ± 1.4	5.6 ± 1.7	5.2 ± 0.8	4.5 ± 0.8
Salivary gland (mg/100g)	161 ± 14	142 ± 18	161 ± 24	147 ± 17	184 ± 20	168 ± 20	188 ± 10	161 ± 7
Thymus (mg/100g)	134 ± 20	109 ± 38	126 ± 18	111 ± 31	206 ± 41	167 ± 30	217 ± 35	144 ± 35
Heart (g/100g)	0.35 ± 0.02	0.3 ± 0.02	0.35 ± 0.04	0.3 ± 0.02	0.37 ± 0.02	0.34 ± 0.01	0.38 ± 0.02	0.33 ± 0.01
Lung (g/100g)	0.36 ± 0.02	0.31 ± 0.03	0.35 ± 0.02	0.31 ± 0.01	0.48 ± 0.03	0.42 ± 0.02	0.47 ± 0.04	0.43 ± 0.03
Liver (g/100g)	3 ± 0.18	2.95 ± 0.07	3.02 ± 0.2	2.94 ± 0.13	2.96 ± 0.16	2.84 ± 0.18	3 ± 0.2	2.61 ± 0.1
Spleen (g/100g)	0.18 ± 0.03	0.17 ± 0.04	0.18 ± 0.02	0.17 ± 0.02	0.21 ± 0.02	0.22 ± 0.03	0.22 ± 0.03	0.21 ± 0.03
Kidney (g/100g)	0.76 ± 0.04	0.73 ± 0.05	0.73 ± 0.03	0.7 ± 0.05	0.82 ± 0.05	0.76 ± 0.06	0.81 ± 0.07	0.65 ± 0.04 ##
Adrenal (mg/100g)	16 ± 2	17 ± 4	16 ± 2	14 ± 2	31 ± 5	30 ± 3	30 ± 3	23 ± 4
Testis (g/100g)	0.81 ± 0.07	0.73 ± 0.05	0.79 ± 0.07	0.69 ± 0.09	NA	NA	NA	NA
Seminal vesicles (g/100g)	0.27 ± 0.05	0.27 ± 0.04	0.25 ± 0.03	0.26 ± 0.03	NA	NA	NA	NA
Prostate (g/100g)	0.24 ± 0.03	0.24 ± 0.03	0.23 ± 0.04	0.25 ± 0.03	NA	NA	NA	NA
Ovary (mg/100g)	NA	NA	NA	NA	37.4 ± 4.8	33.3 ± 3.2	36 ± 4.8	31.4 ± 3.1
Uterus (mg/100g)	NA	NA	NA	NA	206 ± 37	213 ± 76	198 ± 64	188 ± 51

Values are mean ± S.D.
NA: Not applicable
: $p < 0.01$: Significant difference between the Control group and the Alanine administered group.

Table 6. Histopathological examination data.

Sex	Male				Female			
Group	Control		Alanine		Control		Alanine	
Weeks	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)	4	6 (Recovery)
Number of animals	10	4	10	5	10	5	10	5
Eye								
Atrophy, retinal (Total)	0	0	0	0	0	0	0	0
slight	0	0	0	0	0	0	0	0
Fold/rosette, retina (Total)	0	0	2	0	0	0	0	0
slight	0	0	1	0	0	0	0	0
mild	0	0	1	0	0	0	0	0
Harderian gland								
Cell infiltration, focal (Total)	0	0	1	0	0	0	0	0
slight	0	0	1	0	0	0	0	0
Heart								
Myocarditis, focal (Total)	0	0	1	1	0	0	0	0
slight	0	0	1	0	0	0	0	0
mild	0	0	0	1	0	0	0	0
Kidney								
Cyst (Total)	1	0	0	2	0	0	0	0
present	1	0	0	2	0	0	0	0
Basophilia, tubular (Total)	1	3	1	3	0	0	0	0
slight	1	3	1	3	0	0	0	0
Eosinophilic body, tubular cell (Total)	1	0	0	0	0	0	0	0
slight	1	0	0	0	0	0	0	0
Cell infiltration, interstitial (Total)	2	0	1	0	0	1	0	0
slight	2	0	1	0	0	1	0	0
Fibrosis, focal (Total)	1	0	0	0	0	0	0	0
slight	1	0	0	0	0	0	0	0
Dilatation, pelvic (Total)	1 (Dead)							
mild	1 (Dead)							
Liver								
Necrosis, focal (Total)	0	0	1	0	0	0	0	1
slight	0	0	0	0	0	0	0	1
mild	0	0	1	0	0	0	0	0
Microgranuloma (Total)	5	4	5	3	8	5	9	5
slight	5	4	5	3	8	5	9	5
Lung(bronchus)								
Hemorrhage, focal (Total)	2 (Including Dead)	0	4	0	0	0	0	0
slight	2 (Including Dead)	0	4	0	0	0	0	0
Foamy cell, alveolar (Total)	0	0	0	0	0	0	0	1
slight	0	0	0	0	0	0	0	1
Pancreas								
Atrophy, acinar, focal (Total)	1	0	1	0	0	1	0	1
slight	1	0	1	0	0	1	0	1
Cell infiltration, periductal (Total)	0	1	0	0	0	0	0	0
slight	0	1	0	0	0	0	0	0
Pituitary								
Cyst (Total)	1	0	2	0	0	0	0	0
present	1	0	2	0	0	0	0	0
Prostate								
Cell infiltration, interstitial (Total)	1	3	2	1	NA	NA	NA	NA
slight	1	3	2	1	NA	NA	NA	NA
Prostatitis (Total)	0	0	0	1	NA	NA	NA	NA
slight	0	0	0	1	NA	NA	NA	NA
Skeletal muscle, femoral								
Cell infiltration, focal (Total)	1	0	0	0	0	0	0	0
slight	1	0	0	0	0	0	0	0
Spleen								
Hematopoiesis, increased (Total)	0	0	0	0	0	0	0	0
mild	0	0	0	0	0	0	0	0
Stomach								
Hyperplasia, squamous, limiting ridge (Total)	0	0	6	0	0	0	2	0
slight	0	0	6	0	0	0	2	0
Urinary bladder								
Cell infiltration, mucosal (Total)	0	0	0	0	0	0	1	0
slight	0	0	0	0	0	0	1	0
Hyperplasia, mucosal, diffuse (Total)	0	0	0	0	0	0	1	0
slight	0	0	0	0	0	0	1	0

NA : Not applicable

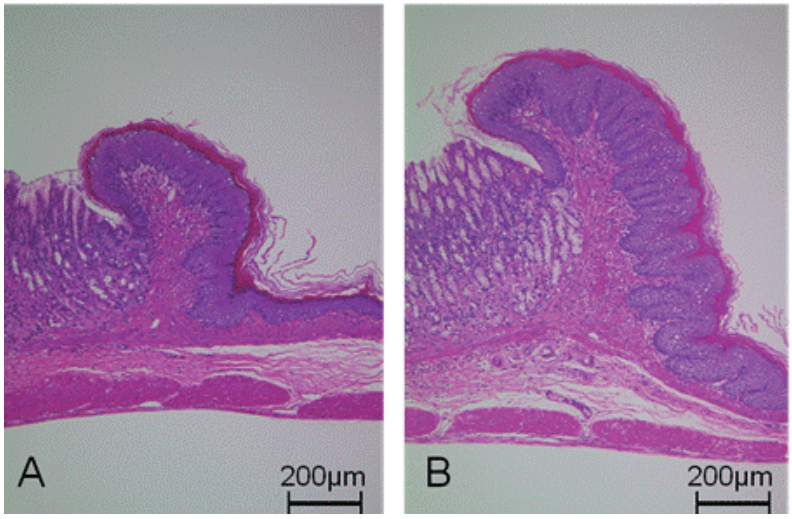

Fig. 3. Histopahological findings in the stomach. (A) No abnormality was observed in the control group. (B) Squamous cell hyperplasia in the limiting ridge was observed in the L-Ala administered group. H.E. staining.

ical signs, body weight, food consumption, ophthalmology, hematology or blood chemistry.

In urinalysis, increased number of animals showing urine protein-positive or phosphate salt was observed in males and females. In addition, urine volume was significantly increased in males. It has been reported that excessive amino acids are not accumulated in the body (Murray *et al.*, 2001; William, 2005). Considering that a relatively small amino acid (molecular weight: 89.09) was orally administered in a large quantity in this study, the changes in urinary proteins and urine volume may reflect excretion of the test article (or its metabolites) into urine. After the 2-week recovery, these changes were no longer observed. In addition, phosphate salts are generally observed in rat urine; however, there were no changes in blood chemical parameters indicative of renal function damage, no test article-related effects in the kidney or urinary bladder in the histopathology, no changes in electrolytes in blood, and this change was no longer observed after the 2-week recovery. Therefore, these changes in urinalysis were reversible, and judged to have no toxicological significance. In histopathological examination, squamous cell hyperplasia in the limiting ridge in the stomach was observed in males and females. After the 2-week recovery period, the change was reduced or no longer observed. It is known that squamous cell hyperplasia in the stomach is generally observed after oral administration of chemical substances with various stimuli and appear more commonly in the limiting ridge (Manabe *et al.*, 2000). These results suggest that administration of L-alanine in a large amount may induce effects of some sorts on the stomach. However, because the lesion was limited to the limiting ridge and that tissue is specific to rodents, the lesion was judged to have little toxicological significance.

In conclusion, administration of L-Ala was mainly associated with the changes in urinalysis that might be caused by oral administration of amino acid in a large quantity and squamous cell hyperplasia in the limiting ridge in the stomach. However, these changes were reversible, and therefore we judged them to have no toxicological significance. These results suggest that repeated oral administration of L-Ala at 2,000 mg/kg/day for 4 weeks is well tolerated in male and female rats.

Conflict of interest---- The authors declare that there is no conflict of interest.

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