

Original Article

A 4-week oral gavage toxicity study of L-citrulline in rats

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(Received August 22, 2017; Accepted August 31, 2017)

ABSTRACT — The safety of L-citrulline was investigated in male and female Sprague-Dawley rats by oral gavage administration for 4 weeks at a dose level of 2,000 mg/kg/day. Animals were sacrificed following the administration period. In the results, there were no toxicologically significant changes in general condition, clinical observations, body weight, food consumption, ophthalmology, urinalysis, hematology, blood chemistry, organ weights, or necropsy. In histopathological evaluation, squamous cell hyperplasia in the limiting ridge in the stomach was observed in some males and females in the test group. However, the lesion was limited and that tissue is specific to rodents. Thus it was considered to be less toxicologically significant. Our results indicate that repeated oral administration with L-citrulline under the present experimental conditions is well tolerated in male and female rats.

Key words: L-Citrulline, Rats, General toxicity

INTRODUCTION

L-Citrulline is a water soluble α -amino acid that is abundant in watermelon (*Citrullus vulgaris*) from which it was first isolated in 1930's. It has the formula $H_2NC(O)NH(CH_2)_3CH(NH_2)CO_2H$ [CAS No. 372-75-8], and is a key intermediate in the urea cycle in the liver by which mammals excrete ammonia (Murry and Robert, 1988).

In mammals, L-citrulline is a common molecule to restore nitrogen balance and has a unique metabolism. As a key regulator of endogenous L-citrulline production, the small intestine plays an important role in L-citrulline supply to the body (Moinard and Cynober, 2007). In addition, orally supplemented L-citrulline is converted to L-argininosuccinate by argininosuccinate synthase and subsequently to L-arginine by argininosuccinate lyase, mainly in the kidney (Moinard *et al.*, 2008; Romero *et al.*, 2006). Based on these metabolic properties, L-citrulline strongly contributes to elevation of plasma and tissue levels of L-arginine as a major precursor of L-arginine.

Recent studies have demonstrated that L-citrulline administration exerts beneficial effects on the cardiovascular system by supporting enhanced nitric oxide (NO) production through the L-citrulline to L-arginine recycling

pathway involving modulation of the vascular endothelium as well as blood circulation (Morita *et al.*, 2013; Morita *et al.*, 2015). It was also suggested that L-citrulline could play a pivotal role in the control of nitrogen homeostasis (Moinard and Cynober, 2007). Thus, L-citrulline is widely used as a food supplement for vascular function and exercise performance in sport nutrition in Japan and USA. For healthy humans, clinically relevant or any other side effects were not observed in a daily dose of 6 g up to 4 weeks, or a single dose of 15 g in human clinical studies (Figuroa *et al.*, 2009; Moinard *et al.*, 2008). However, the available knowledge for the general toxicity of L-citrulline is limited so far.

As a part of the safety evaluation of L-citrulline, we assessed its toxicity in rats by oral administration for 4 weeks at a dose level of 2,000 mg/kg/day, which is generally regarded as the maximum feasible dose by oral gavage administration in repeated dose toxicity studies.

MATERIALS AND METHODS

Ethical considerations

This study was approved by the Institutional 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 24 June 2011), “Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain” (Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006) and “Guidelines for Proper Conduct of Animal Experiments” (Science Council of Japan, June 1, 2006).

Chemicals and preparation of dosing solutions

L-Citrulline (Lot. 11004) was supplied from KYOWA HAKKO BIO CO., LTD. (Tokyo, Japan). L-Citrulline was dissolved in sterilized water for injection (Japanese Pharmacopoeia, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) to achieve a final concentration of 200 mg/mL. Dosing solutions were prepared at least once in 7 days.

Animals, animal husbandry and group composition

Male and female Sprague-Dawley (CrI:CD (SD)) SPF rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) at 5 weeks of age. The animals were quarantined and acclimated for 1 week and pelleted diet (radiation sterilized CR-LPF (Lot. 110809, 110915), Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water (tap water, via automatic water-supply system) were supplied *ad libitum*. The animals were housed individually in stainless-steel wire mesh cages in an animal room which was maintained at $23 \pm 3^\circ\text{C}$, relative humidity at $50 \pm 20\%$, air ventilation of 12 to 17 times per hour and 12-hr light dark cycle. After a 1-week quarantine/acclimation period, the animals were randomized into 2 groups consisting of 6 males and 6 females (6/sex/group), and assigned to the control group (sterilized water) or L-citrulline group (2,000 mg/kg/day). Each dosing solution was administered to rats once a day by oral gavage for 4 weeks.

Examinations and observations

Clinical observations, body weight and food consumption

Clinical observations were conducted 3 times every day (pre-dosing, immediately after and 1 to 3 hr after dosing). Body weights were measured three times in Week 1 of administration and thereafter twice a week, every 3 or 4 days, during the administration period. Food consumption was measured on Day 1 of administration and thereafter twice a week, every 3 or 4 days.

Ophthalmology

Examination was conducted twice: during the quarantine/acclimation period (all animals delivered) and in Week 4 of administration (all animals in each test group). 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 (Omega 200, HEINE Optotechnik GmbH & Co. KG, Germany).

Urinalysis

Examination was conducted once in Week 4 of administration. The following parameters were examined on 4-hr urine samples collected under fasting but with free access to water: pH, protein, ketone body, glucose, occult blood, bilirubin and urobilinogen (CLINITEK 500, Siemens Healthcare Diagnostics Inc., Illinois, USA), color and urinary sediment. The following parameters were examined on 20-hr urine samples collected with free access to the feed and water: osmotic pressure (Automatic Osmotic Pressure Analyzer AUTO & STAT OM-6030 (Arkray Inc., Kyoto, Japan), sodium, potassium and chloride (Chemistry Automatic Analyzer TBA-120FR, Toshiba Medical Systems Corp., Tokyo, Japan). Water intake (24-hr) and urine volume were also measured.

Hematology

At the time of necropsy in Week 4 of administration, blood samples were collected from the abdominal aorta under isoflurane 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, white blood cell count (WBC), reticulocyte ratio and leukocyte differentiation (ADVIA 120 Hematology System, Siemens Healthcare Diagnostics Inc., Illinois, USA). In addition, prothrombin time (PT), activated thromboplastin time (APTT) and fibrinogen (Coagulometer ACL Elite Pro, Instrumentation Laboratory, MA, USA) were determined on 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 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) and total protein (TP) (Clinical chemistry automated analyzer TBA-120FR, Toshiba Medical Systems Corp., Tokyo, Japan), A/G ratio (calculated from protein fractions) and protein fractions (agarose gel electrophoresis, QuickScan densitometer, Helena Laboratories Corp., TX, USA). In addition, the following parameters were determined on plasma obtained from blood treated with heparin: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) (Clinical chemistry automated analyzer TBA-120FR, Toshiba Medical Systems Corp., Tokyo, Japan).

Pathology

After collection of blood samples, the animals were exsanguinated via the abdominal artery 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 in phosphate buffered 10% formalin (however, the eyeballs and optic nerves were fixed in fixatives containing 3% glutaraldehyde and 2.5% formalin, and the testes and epididimides were fixed in Bouin's solution and then 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, skin, nasal cavity and Zymbal gland. 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 and organ weights) between groups, Tukey's test was applied (Tukey, 1949). (levels of significance: 1 and 5%, two-tailed). Data were presented as means \pm S.D.

RESULTS

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

The results are shown in Table 1. There were no test article-related changes in any parameter.

Hematology

The results are shown in Table 2. There were no toxicologically significant, test article-related changes in

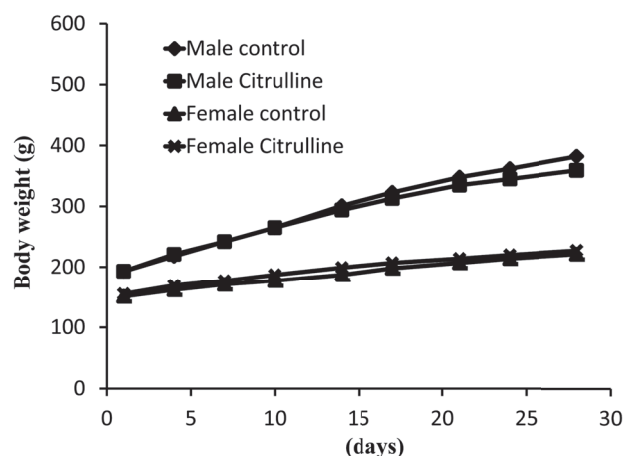


Fig. 1. Group mean body weight changes in a 4-week oral toxicity study of L-citrulline in rats.

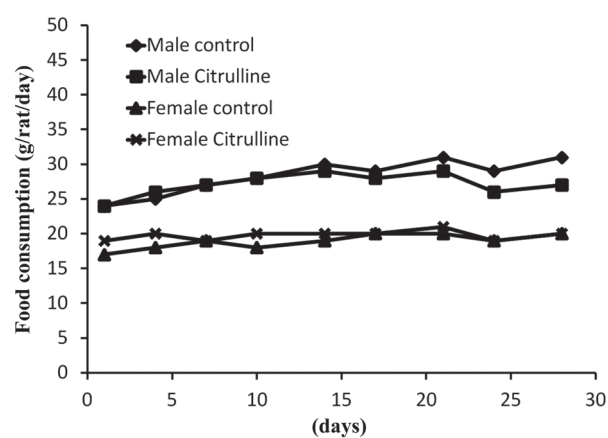


Fig. 2. Group mean food consumption in a 4-week oral toxicity study of L-citrulline in rats.

Table 1. Water intake and urinalysis data.

	Sex	Male		Female	
		Control	Citrulline	Control	Citrulline
	Group				
	Weeks	4	4	4	4
	Dose (mg/kg)	0	2000	0	2000
	Number of animals	6	6	6	6
pH	5.0	0	0	0	0
	5.5	0	0	0	0
	6.0	0	0	0	0
	6.5	0	0	0	0
	7.0	0	0	0	1
	7.5	0	0	0	0
	8.0	0	0	0	0
	8.5	4	3	5	4
	9.0	2	3	1	1
Protein	-	2	2	6	4
	±	4	3	0	2
	1+	0	1	0	0
Ketone Body	-	2	1	6	5
	±	2	3	0	1
	1+	2	2	0	0
Glucose	-	6	6	6	6
Occult Blood	-	4	5	6	6
	±	2	0	0	0
	1+	0	0	0	0
	2+	0	1	0	0
Bilirubin	-	6	6	6	6
	1+	0	0	0	0
Urobilinogen	±	6	6	6	6
Color	Yellow	6	6	6	6
RBC	-	6	5	6	6
	±	0	1	0	0
WBC	-	6	6	6	6
	±	0	0	0	0
Ep.SEC	±	6	6	6	6
	1+	0	0	0	0
Ep.SREC	-	6	6	6	6
	±	0	0	0	0
Cast	-	6	6	6	6
Cr.PS	-	5	1	6	1
	±	1	5	0	5
	1+	0	0	0	0
Cr.CO	-	6	6	6	6
Water Intake	(mL/24 hr)	36	32	33	27
Urine Volume	(mL/24 hr)	13.2	11.4	10.2	6.8
Osmolality	(mOsm/kg)	1719	2039	1647	2057
Na	(mmol/24 hr)	1.9	1.7	1.4	1.0
K	(mmol/24 hr)	4.7	3.9	3.2	2.1
Cl	(mmol/24 hr)	2.9	2.4	2.1	1.4

Protein) -:Negative, +/-:15, 1+:30 mg/dL, Ketone Body) -:Negative, +/-:5, 1+:15 mg/dL, Glucose) -:Negative, Occult Blood) -:Negative, +/-:0.015, 1+:0.062, 2+:0.135 mg/dL, Bilirubin) -:Negative, 1+:0.8 mg/dL, Urobilinogen), +/-:0.1-1.0 Ehrlich U/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.

General toxicity study of L-citrulline in rats

Table 2. Group means of hematological parameters.

Sex	Male		Female	
	Control	Citrulline	Control	Citrulline
Group				
Weeks	4	4	4	4
Dose (mg/kg)	0	2000	0	2000
Number of animals	6	6	6	6
Red blood cell count ($10^4/\mu\text{L}$)	844 \pm 47	844 \pm 24	813 \pm 36	846 \pm 22
Hemoglobin (g/dL)	15.7 \pm 0.7	16.3 \pm 0.6	15.4 \pm 0.3	16.4 \pm 0.4#
Hematocrit (%)	45.5 \pm 2.0	46 \pm 1.8	43.2 \pm 1.0	45.4 \pm 1.0
Mean corpuscular volume (fL)	53.9 \pm 1.6	54.8 \pm 1.7	53.2 \pm 1.8	53.7 \pm 1.9
Mean corpuscular hemoglobin (pg)	18.7 \pm 0.7	19.4 \pm 0.6	19.0 \pm 0.6	19.4 \pm 0.6
Mean corpuscular hemoglobin concentration (g/dL)	34.6 \pm 0.4	35.4 \pm 0.2#	35.7 \pm 0.3	36.0 \pm 0.5
Reticulocyte ratio (%)	2.6 \pm 0.4	2.3 \pm 0.4	2.3 \pm 0.2	1.6 \pm 0.3#
Platelet count ($10^4/\mu\text{L}$)	111.0 \pm 16.3	122.0 \pm 13.9	121.3 \pm 7.4	119.8 \pm 7.0
Prothrombin time (s)	14.7 \pm 2.2	14.4 \pm 1.7	13.0 \pm 0.6	12.8 \pm 0.9
Activated partial thromboplastin time (s)	21.3 \pm 3.0	21.7 \pm 2.8	18.5 \pm 1.7	16.0 \pm 2.5
Fibrinogen (mg/dL)	300 \pm 20	278 \pm 22	232 \pm 8	213 \pm 17
White blood cell count ($10^2/\mu\text{L}$)	93.6 \pm 17.1	97.6 \pm 26.0	64.6 \pm 11.2	67.0 \pm 16.6
Lymphocytes (%)	80.3 \pm 4.3	83.0 \pm 2.4	79.3 \pm 5.8	81.3 \pm 4.7
NEUT (%)	15.3 \pm 3.8	12.9 \pm 2.0	16.1 \pm 5.2	14.0 \pm 4.6
Eosinophils (%)	1.1 \pm 0.5	0.9 \pm 0.4	1.3 \pm 0.6	1.3 \pm 0.8
Basophils (%)	0.3 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.1	0.2 \pm 0.1
Monocytes (%)	2.1 \pm 0.3	2.1 \pm 0.4	2.1 \pm 0.8	2.3 \pm 0.8
LUC (%)	0.9 \pm 0.3	0.9 \pm 0.3	0.9 \pm 0.3	1.0 \pm 0.3
Lymphocytes ($10^2/\text{mL}$)	75.3 \pm 15.2	80.9 \pm 21.1	51.3 \pm 10.0	54.8 \pm 15.5
NEUT ($10^2/\text{mL}$)	14.3 \pm 3.9	12.6 \pm 4.2	10.3 \pm 3.5	9.2 \pm 3.4
Eosinophils ($10^2/\text{mL}$)	1.0 \pm 0.5	0.9 \pm 0.3	0.9 \pm 0.4	0.8 \pm 0.3
Basophils ($10^2/\text{mL}$)	0.3 \pm 0.1	0.3 \pm 0.2	0.2 \pm 0.1	0.2 \pm 0.1
Monocytes ($10^2/\text{mL}$)	2.0 \pm 0.5	2.1 \pm 0.8	1.4 \pm 0.7	1.5 \pm 0.4
LUC ($10^2/\text{mL}$)	0.8 \pm 0.3	0.9 \pm 0.5	0.6 \pm 0.2	0.7 \pm 0.3

Values are mean \pm S.D.

: $p < 0.05$: Significant difference between the Control group and the Citrulline administered group.

any parameter. Although a significantly high value in mean corpuscular hemoglobin concentration (MCHC) in males, a significantly high value in hemoglobin (HGB) in females and a significantly low value in reticulocyte ratio in females were recorded in the test article group, they were judged to have no toxicological significance since they were fluctuations within the historical control range (MCHC: $n=201$, mean; 35.8 g/dL, 32.7 to 39.4 g/dL, ± 2 S.D.; 33.0 to 38.6 g/dL, hemoglobin: $n=191$, mean; 15.5 g/dL, 13.1 to 17.7 g/dL, ± 2 S.D.; 13.9 to 17.0 g/dL, reticulocyte ratio : $n=191$, mean; 2.2 %, 1.0 to 3.6 %, ± 2 S.D.; 1.2 to 3.2%).

Blood chemistry

The results are shown in Table 3. There were no toxicologically significant changes in any parameter. Although significantly low values in sodium and chloride were recorded in males in the test article dose group, they

were judged to have no toxicological significance since they were minimal changes.

Organ weights

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

Necropsy

There were no test article-related changes in any organ/tissue.

Histopathology

The results are shown in Table 6 and Fig. 3. Squamous cell hyperplasia in the limiting ridge in the stomach was observed in 3/6 males and 1/6 females in the test article group. Other changes were considered to be inciden-

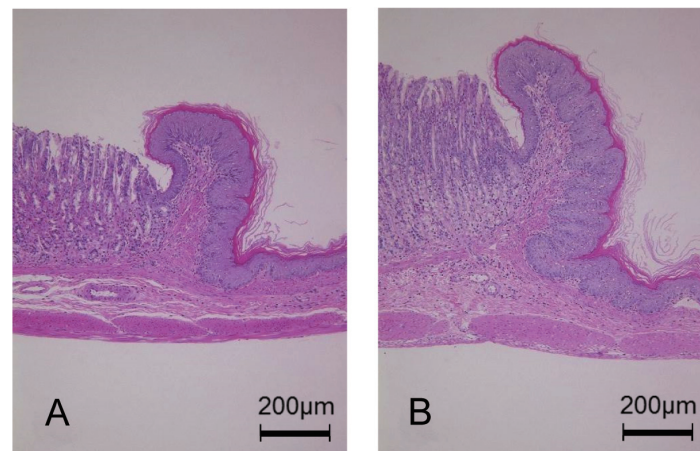


Fig. 3. Histopathological findings in the stomach. (A) No abnormality was observed in the control group. (B) Squamous cell hyperplasia in the limiting ridge was observed in the L-citrulline administered group. H.E. staining.

Table 3. Group means of blood chemical parameters.

Sex	Male		Female	
	Control	Citrulline	Control	Citrulline
Group				
Weeks	4	4	4	4
Dose (mg/kg)	0	2000	0	2000
Number of animals	6	6	6	6
AST (IU/L)	59 ± 5	67 ± 7	59 ± 5	59 ± 9
ALT (IU/L)	27 ± 3	30 ± 5	20 ± 3	20 ± 3
LDH (IU/L)	44 ± 6	52 ± 12	38 ± 5	47 ± 10
ALP (IU/L)	662 ± 115	588 ± 101	411 ± 75	316 ± 34
r-GTP(IU/L)	1 ± 1	0 ± 1	1 ± 1	0 ± 1
Total cholesterol (mg/dL)	71 ± 9	66 ± 9	72 ± 17	67 ± 15
Triglyceride (mg/dL)	47 ± 16	30 ± 13	18 ± 8	14 ± 3
Phospholipid (mg/dL)	114 ± 7	103 ± 11	133 ± 30	120 ± 21
Total bilirubin (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Glucose (mg/dL)	121 ± 4	123 ± 7	121 ± 11	114 ± 11
Blood urea nitrogen (mg/dL)	12 ± 2	14 ± 2	15 ± 2	17 ± 2
Creatinine (mg/dL)	0.21 ± 0.03	0.21 ± 0.02	0.26 ± 0.02	17 ± 0.02
Na (mmol/L)	143 ± 1	141 ± 1###	142 ± 1	142 ± 1
K (mmol/L)	4.4 ± 0.4	4.4 ± 0.1	4.3 ± 0.3	4.3 ± 0.2
Chloride (mmol/L)	106 ± 1	104 ± 1##	108 ± 1	106 ± 1
Calcium (mg/dL)	10.2 ± 0.3	9.9 ± 0.4	10.0 ± 0.2	9.9 ± 0.3
P (mg/dL)	8.0 ± 0.7	8.0 ± 0.8	6.7 ± 0.8	6.9 ± 0.8
Total protein (g/dL)	5.9 ± 0.2	5.8 ± 0.2	6.0 ± 0.2	5.9 ± 0.3
Albumin (g/dL)	3.2 ± 0.1	3.2 ± 0.1	3.5 ± 0.2	3.4 ± 0.1
A/G ratio	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Albumin (%)	53.5 ± 1.6	54.2 ± 2.3	56.8 ± 2.9	56.9 ± 1.7
Globulin(%)				
α ₁ -globulin (%)	16.6 ± 2.7	15.7 ± 2.0	14.0 ± 1.1	14.0 ± 2.6
α ₂ -globulin (%)	11.8 ± 1.6	12.2 ± 1.0	11.5 ± 1.1	12.0 ± 0.9
β-globulin (%)	13.0 ± 1.1	12.1 ± 0.8	11.4 ± 0.6	11.0 ± 1.4
γ-globulin (%)	5.1 ± 1.1	5.9 ± 1.9	6.3 ± 2.2	6.1 ± 2.9

Values are mean ± S.D.

: p < 0.01, ### : p < 0.001 : Significant difference between the Control group and the Citrulline administered group.

General toxicity study of L-citrulline in rats

Table 4. Group means of absolute organ weight data.

	Sex	Male		Female	
		Control	Citrulline	Control	Citrulline
Group					
Weeks		4	4	4	4
Dose (mg/kg)		0	2000	0	2000
Number of animals		6	6	6	6
Body weight on the day of necropsy (g)		352 ± 22	333 ± 20	206 ± 17	212 ± 12
Brain (g)		2.00 ± 0.05	1.96 ± 0.10	1.83 ± 0.08	1.90 ± 0.06
Pituitary (mg)		11.3 ± 1.7	10.0 ± 0.7	12.4 ± 1.8	11.5 ± 1.6
Thyroid gland (mg)		16.9 ± 2.2	17.8 ± 2.6	14.8 ± 2.4	15.2 ± 3.8
Salivary gland (mg)		560 ± 44	562 ± 39	415 ± 27	394 ± 21
Thymus (mg)		508 ± 108	530 ± 84	455 ± 154	458 ± 65
Heart (g)		1.16 ± 0.05	1.14 ± 0.04	0.77 ± 0.08	0.81 ± 0.08
Lung (g)		1.26 ± 0.08	1.21 ± 0.05	0.94 ± 0.08	0.99 ± 0.05
Liver (g)		9.97 ± 0.81	8.84 ± 0.91	5.79 ± 0.56	5.91 ± 0.49
Spleen (g)		0.71 ± 0.08	0.61 ± 0.09	0.40 ± 0.05	0.48 ± 0.05
Kidney (g)		2.55 ± 0.12	2.67 ± 0.20	1.55 ± 0.14	1.71 ± 0.09
Adrenal (mg)		56 ± 7	59 ± 9	58 ± 7	60 ± 5
Testis (g)		3.22 ± 0.23	3.06 ± 0.17	NA	NA
Prostate (g)		0.83 ± 0.11	0.80 ± 0.07	NA	NA
Seminal vesicles (g)		0.87 ± 0.13	0.86 ± 0.14	NA	NA
Ovary (mg)		NA	NA	82.4 ± 11.3	86.9 ± 20.6
Uterus (mg)		NA	NA	494 ± 91	403 ± 107

Values are mean ± S.D.

NA: Not applicable

No significant difference between the Control group and the Citrulline administered group.

Table 5. Group means of relative organ weight data.

	Sex	Male		Female	
		Control	Citrulline	Control	Citrulline
Group					
Weeks		4	4	4	4
Dose (mg/kg)		0	2000	0	2000
Number of animals		6	6	6	6
Brain (g/100g)		0.57 ± 0.05	0.59 ± 0.05	0.89 ± 0.07	0.90 ± 0.06
Pituitary (mg/100g)		3.2 ± 0.4	3.0 ± 0.1	6.1 ± 1.0	5.4 ± 0.4
Thyroid gland (mg/100g)		4.8 ± 0.7	5.4 ± 1.0	7.2 ± 1.4	7.2 ± 2.2
Salivary gland (mg/100g)		159 ± 10	170 ± 21	202 ± 9	186 ± 12
Thymus (mg/100g)		144 ± 25	160 ± 26	220 ± 65	217 ± 35
Heart (g/100g)		0.33 ± 0.02	0.34 ± 0.01	0.38 ± 0.02	0.38 ± 0.04
Lung (g/100g)		0.36 ± 0.02	0.37 ± 0.03	0.46 ± 0.03	0.47 ± 0.03
Liver (g/100g)		2.83 ± 0.08	2.65 ± 0.13	2.82 ± 0.16	2.78 ± 0.15
Spleen (g/100g)		0.20 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	0.23 ± 0.02
Kidney (g/100g)		0.73 ± 0.05	0.80 ± 0.03	0.75 ± 0.05	0.81 ± 0.03
Adrenal (mg/100g)		16 ± 2	18 ± 2	28 ± 3	28 ± 1
Testis (g/100g)		0.92 ± 0.08	0.92 ± 0.08	NA	NA
Seminal vesicles (g/100g)		0.24 ± 0.04	0.24 ± 0.02	NA	NA
Prostate (g/100g)		0.25 ± 0.05	0.26 ± 0.06	NA	NA
Ovary (mg/100g)		NA	NA	40.0 ± 2.9	40.9 ± 9.5
Uterus (mg/100g)		NA	NA	241 ± 40	189 ± 45

Values are mean ± S.D.

NA: Not applicable

No significant difference between the Control group and the Citrulline administered group.

Table 6. Histopathological examination data.

	Sex	Male		Female	
		Control	Citrulline	Control	Citrulline
	Group				
	Weeks	4	4	4	4
	Dose (mg/kg)	0	2000	0	2000
	Number of animals	6	6	6	6
Eye					
Dysplasia, retinal (Total)		1	1	0	0
minimal		1	1	0	0
Heart					
Myocarditis, focal (Total)		0	1	0	0
minimal		0	1	0	0
Intestine, cecum					
Cell infiltration, mucosal (Total)		0	0	1	0
minimal		0	0	1	0
Kidney					
Dilatation, tubular, cystic (Total)		0	1	1	0
minimal		0	1	1	0
Regeneration, tubular (Total)		0	1	2	0
minimal		0	1	2	0
Urinary cast, hyaline (Total)		1	0	0	0
minimal		1	0	0	0
Liver					
Microgranuloma (Total)		1	2	2	3
minimal		1	2	2	3
Nasal cavity					
Cell infiltration (Total)		1	0	0	0
minimal		1	0	0	0
Prostate					
Cell infiltration (Total)		1	3	NA	NA
minimal		1	3	NA	NA
Spleen					
Hematopoiesis,extramedullary (Total)		4	4	0	1
minimal		4	4	0	1
Stomach					
Squamous cell hyperplasia, limiting ridge (Total)		0	3	0	1
minimal		0	3	0	1
Testis					
Atrophy,seminiferous tubular (Total)		1	0	NA	NA
minimal		1	0	NA	NA

NA : Not applicable

tal based on the incidence of their occurrences and their pathological nature.

DISCUSSION

L-Citrulline is non-essential α -amino acid that is linked to L-arginine metabolism, urea cycle and nitrogen homeostasis. To date, we have already reported some clinical utilities of the supplemented L-citrulline, focusing on the prevention of vascular dysfunction and the functional reg-

ulation of arterial stiffness (Ochiai *et al.*, 2012; Morita *et al.*, 2013; Morita *et al.*, 2015). Recently, L-citrulline has also been used as a popular supplement among athletes, because L-citrulline exerts some physiological effects related to exercise performance in humans (Suzuki *et al.*, 2016).

For the safety of L-citrulline, a previous clinical study showed that acute supplementation of L-citrulline is well tolerated without side effects at high dose (i.e. 15 g), and the increased plasma concentrations of L-citrulline return

General toxicity study of L-citrulline in rats

to baseline values within 5-8 hr post intake, which suggests that L-citrulline has no accumulative property (Moinard *et al.*, 2008). However, little is known about the general toxicity of L-citrulline.

In this study, to investigate the safety of L-citrulline, male and female rats were administered L-citrulline by gavage for 4 weeks. In the results, there were no test article-related changes in clinical signs, body weight, food consumption, ophthalmology, urinalysis, hematology, blood chemistry or at necropsy.

In histopathological examination, squamous cell hyperplasia in the limiting ridge in the stomach was observed in males and females in the test article group. It is known that squamous cell hyperplasia in the stomach is generally observed after oral administration of chemicals with irritating properties and occurs commonly in the limiting ridge (Manabe, 2000). However, it was judged to have no toxicological significance since there were no histopathological abnormalities in the esophagus or forestomach composed of stratified squamous epithelium as well as the limiting ridge, and the limiting ridge is a structure specific to rodents (Ito, 1994).

Based on the above results, it was concluded that L-citrulline does not induce any toxic changes in rats by repeated oral administration for 4 weeks.

Conflict of interest---- M, Morita, K, Morishita and A, Kamimura are employees of KYOWA HAKKO BIO CO., LTD.. This work was supported by a grant from KYOWA HAKKO BIO CO., LTD.

ACKNOWLEDGMENT

The Authors would like to thank Mr. Pete Aughton, B.Sc, D.A.B.T., Dip.R.C.Path., ITR Laboratories Canada Inc., for proofreading.

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