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Original Article

Twenty-eight-day oral toxicity study of L-hydroxyproline in rats with 14-day post-treatment observation period

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ABSTRACT — Repeated-dose oral toxicity of L-hydroxyproline (Hyp) was assessed in male and female SD rats by gavage for 28 days at dose level of 40, 200, 1,000 or 4,000 mg/kg/day. The reversibility of treatment-related changes was also examined by providing 14-day recovery period in the control group and 4,000 mg/kg group. In the results, reduced body weight gain with decreased food consumption was noted in males at 4,000 mg/kg/day. The histopathological evaluation revealed increased incidences of focal dilatation of the renal tubules with narrowing of the tubular cell liner and focal interstitial fibroplasia in the kidney of males at 1,000 and 4,000 mg/kg and females at 4,000 mg/kg. The lack of kidney specific serum chemical or urinalysis findings supported that the renal changes were very mild. Based on the above results, it was concluded that the no observed adverse effect level (NOAEL) of Hyp was 200 mg/kg/day.

Key words: L-hydroxyproline, Repeated-dose Oral Toxicity, Rats

INTRODUCTION

L-Hydroxyproline (Hyp) is an amino acid that exists specifically in collagen, accounting for about 10% to 15% of the amino acids composing collagen (Nemethy and Scheraga, 1986; Rodwell, 2000). The physiological function of Hyp is to stabilize the triple-helix structure of collagen to protect against digestion by proteolytic enzymes (Burjanadze and Veis, 1997; Nemethy and Scheraga, 1986). It has been reported that prolyl-hydroxyproline, a food-derived collagen peptide in human blood, might stimulate the growth of fibroblasts in the skin (Shigemura *et al*., 2009). In mice, dietary Hyp was shown to enter into the bloodstream, followed by an increase in skin water content (in-house data at Kyowa Hakko Bio Co., Ltd.). It was revealed that oral administration of Hyp to rats resulted in increased soluble collagen, probably a newly synthesized collagen (Green and Lowther, 1959), content of the skin and that the serum concentration of collagen peptides was correlated with the skin content of soluble collagen, and these results suggested that orally ingested Hyp augmented collagen metabolism (Aoki *et al*., 2012). From these, it is expected that orally ingested Hyp may be effective in skin maintenance. Previously we estimated the oral LD_{50} of Hyp in rats; more than 16 g/kg (B.W.) (in-house data at Kyowa Hakko Bio Co. Ltd.), but long-term influence was unknown. In this study, we examined the repeated-dose oral toxicity of Hyp in rats.

MATERIALS AND METHODS

This study was carried out from May 18 to July 7, 2004. It was conducted at Pharmaceutical Control and Development Laboratory Co., Ltd. Budapest, Hungary in compliance with Good Laboratory Practice Regulations (US-FDA, National GLP of Hungary, and OECD), and in accordance with "Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Addi-

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tives Used in Food: Guideline for a short-term continuous exposure oral toxicity studies" (US-FDA, Bureau of Food 1982) and "OECD guidelines for the testing of chemicals, Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, 27th July 1995). The present study was also conducted in compliance with the Hungarian Act 1998: XXVIII, and Governmental Regulation 243/1998 "Rules of animal experimentation" modified by Governmental Regulation 103/2002, regulating animal protection.

Test substance

The test substance Hyp (Lot number 0240081) was supplied from KYOWA HAKKO KOGYO CO., LTD. (Tokyo, Japan). The dose formulations were prepared daily by dissolving in distilled water at concentrations of 4, 20, 100 and 200 mg/mL.

Animals

Male and female Sprague-Dawley rats (Crl:CD BR, Charles River Hungary Ltd., Budapest, Hungary) were obtained at 4 to 5 weeks of age. The animals were quarantined and acclimated for at least 7 days and then assigned to each group randomly using a computer and used to the study. The animals were reared in an animal room which maintained the temperature at 19°C to 25°C, relative humidity at 30% to 70% and 12-hr lighting per day. The animals were housed in type II makrolone (polycarbonate) cages (17.5 × 22.5 × 37.5 cm) with bedding (2 animals per cage after group allocation). The animals were allowed free access to standardized rat and mouse diet S8106-S011 ssniff SM R/M-Z+H (ssniff Spezialdiäten GmbH, Germany) and tap water via water bottles, except for the overnight fasting period to blood sampling and necropsy.

Examinations and observations

The animals were randomized into 5 main groups consisting of 10 males and 10 females (10/sex/group) and 2 recovery groups consisting of 6 males and 6 females (6/sex/group for the control and high dose groups), and assigned to the control group (distilled water) or Hyp group (40, 200, 1,000 and 4,000 mg/kg). Each dosing solution was administered to rats once a day by oral gavage for 28 days. The highest dose of Hyp was set to about 100-fold of human daily dose (approx. 40 mg/kg if calculated on 70 kg body weight of adult). The animals were observed for mortality and any abnormal clinical signs twice per day. In addition, detailed clinical observations were conducted once before the first treatment and weekly thereafter, and the signs to be observed included changes in skin, fur, eyes and visible mucous membrane; occurrence of secretions or excretions and autonomic activity (e.g., lacrimation, piloerection, diarrhea, pupil size and unusual respiratory pattern). Furthermore, potential changes in gait, posture and response to handling as well as the presence of somnolence, trembling, clonic or tonic movements, stereotypy or bizarre behavior were recorded. The body weights, food consumption and water consumption were recorded weekly. The body weight gain was also calculated. Sensory reactivity to auditory, visual and proprioceptive stimuli, assessment of grip strength and motor activity were evaluated on all animals in Week 4 of treatment according to methods described by Irwin (Irwin, 1968). In order to examine the reversibility of the test substance-related changes, observations were continued another 2 weeks in the vehicle control group and high dose group.

Urinalysis

Examination was conducted once in Week 4 of treatment. The 3-hr urine samples were collected prior to treatment following oral administration, and examined for appearance, volume, specific gravity (refractometry), pH, protein, glucose, bilirubin, urobilinogen, ketone and occult blood using a test strip (Re-urin 9, Renal, Budapest, Hungary).

Hematology

At the time of terminal necropsy, blood samples were taken from the retro-orbital sinus under diethyl-ether anesthesia into tubes containing EDTA 2K, the following parameters for hematology were determined using an analyzer (Beckman Coulter Ac.T diff, Indiana, USA): erythrocyte count (RBC), leukocyte count (WBC), hemoglobin content (Hb), hematocrit (Ht), platelet count (PLT), lymphocytes (LY), neutrophils (Ne), monocytes (Mo), eosinophils (Eo) and basophils (Ba). May-Grünwald and Giemsa-stained smears were prepared and observed using a light microscope to determine differential leukocyte ratio. Prothrombin time (PT) was also determined using an analyzer (Coagulometer, Benhk Elektronik GmbH & Co. KG, Norderstedt, Germany) on the plasma obtained by centrifuging blood samples treated with 3.8 w/v% sodium citrate.

Blood chemistry

At the same time as hematology, the following parameters for clinical chemistry were determined using an analyzer (FP-901 analyzer, Labsystems Oy, Helsinki, Finland) on the sera obtained by centrifuging whole blood: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total

cholesterol (T-CHO), total protein (TP), albumin (ALB), glucose (GLU), urea nitrogen (BUN), chloride (Cl), total calcium (Ca) and creatinine (CRE), sodium (Na), potassium (K) (IL 943 flame Photometer, GMI Inc., Minnesota, USA).

Pathology

After collecting blood samples, the animals were anesthetized by diethyl-ether and exsanguinated, and subjected to a full detailed necropsy. The following organs were weighed (absolute weight, paired organs were weighed together) and organ weight per 100 g body weight (relative weight) was calculated: liver, heart, kidneys, spleen, brain, testes, epididymides, thymus and adrenals. Then, the following organs/tissues were fixed with 8% neural buffed formaldehyde solution (however, the right testis and right epididymis were fixed with Bouin's solution): liver, kidneys, adrenals, testis (left), spleen, brain, spinal cord, thymus, heart, mesenteric lymph node, submandibular lymph node, sciatic nerve with skeletal muscle, stomach, duodenum with pancreas, lungs with mainstem bronchi, trachea, esophagus, thyroids (including parathyroids), epididymis (left), prostate, uterus, ovaries, large intestine, urinary bladder, and all gross lesions. Then all organs listed above from all animals in the main and recovery groups in the vehicle control group and high dose group were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. Because the incidence of animals with focal fibrous degeneration in the kidney was increased in males in the high dose group, histopathological examination of the kidney was extended to the lower 3 dose levels for both sexes. In addition, PAS staining was performed on the liver, heart and kidney sections. Furthermore, cryostat sections of the liver, heart and kidney were made and stained with Fat Red. Bone marrow smears were also prepared, but they were not examined as no hematological alterations were noted.

Statistical analysis

Groups of main and recovery were evaluated separately. For the numerical data, homogeneity of variance was analyzed by the Bartlett's test. Homogeneous data were assessed by one-way analysis of variance (ANOVA). If the ANOVA detected significant differences ($p < 0.05$), the Tukey test was applied to compare the test article groups versus vehicle control group (significance levels: 0.05 and 0.01). Heterogeneous data were assessed by the Kruskal-Wallis nonparametric one-way analysis. If significant differences were found among the groups, Kolmogorov-Smirnov test was applied (significance levels: 0.05 and 0.01). Non parametric data were analyzed in the same procedures as the heterogeneous data. The above analyses were performed with STATISTICA Version 5.5 (Edition 99, Statsoft Inc., Tulsa, OK, USA). Data were presented as mean ± SD.

RESULTS

Clinical observations, body weights, food consumption and ophthalmology

No deaths occurred and there were no test article-related findings in the clinical observations or detailed clinical observations. The body weight changes are shown in Fig. 1. In males at 4,000 mg/kg, lower mean body weights became apparent after one week of treatment. The difference in body weight increased progressively during the course of study, reached the statistical significance after 3 weeks and resulted markedly lower body weight was noted at the end of the treatment (−11% as compared with that of the control group). On the other hand, there was no difference in the body weights throughout the study among the female groups treated with Hyp up to 4,000 mg/kg/day in comparison to the respective control group at any time point. The food consumption was lower in males at 4,000 mg/kg during the last week of treatment (Fig. 2). There were no treatment-related changes in the water consumption. No treatment related-changes were noted in the assessment of sensory reactivity, grip strength and motor activity.

Urinalysis

There were no treatment-related changes in the urinalysis (Table 1).

Hematology

The hematological data are shown in Table 2. There were no treatment-related changes. Although statistically significant differences were recorded in Ht in males at 4,000 mg/kg and RBC in females at 40 mg/kg, they had no biological significance as the values remained within the physiological limits.

Blood chemistry

The clinical chemistry data are shown in Table 3. There were no treatment-related changes. Although statistically significant decreases were recorded in Na in males at 200 mg/kg or more and females at 4,000 mg/kg, Ca in males at 200 and 1,000 mg/kg and females at 4,000 mg/kg, and in AST in males at 1,000 and 4,000 mg/kg and females at 4,000 mg/kg, they had no biological significance as the values remained within the physiologi-

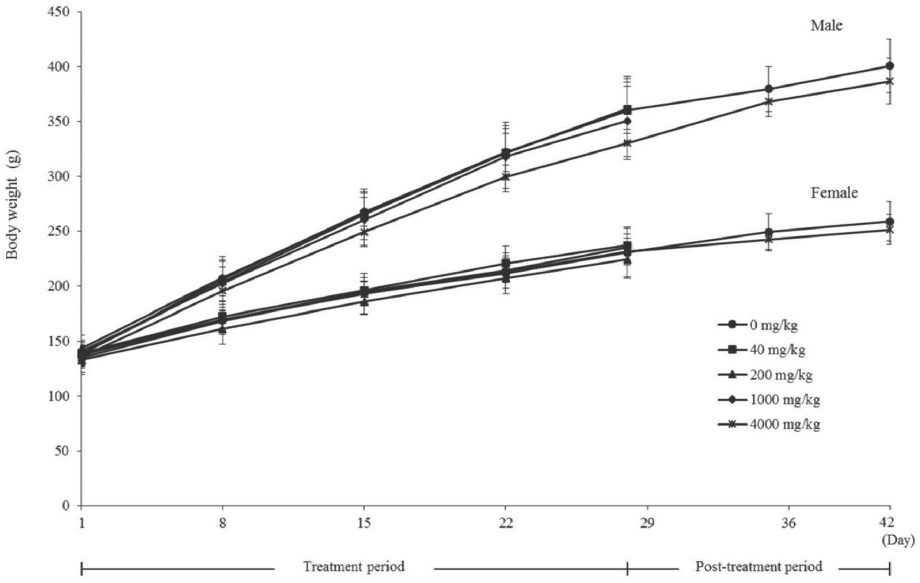

Fig. 1. Body weight changes in male and female rats treated orally with L-hydroxyproline for 4 weeks followed by a 2-week post treatment period. Values are the mean ± standard deviations of 16 (treatment period: 0 and 4000 mg/kg groups), 10 (treatment period: other groups), or 6 (post-treatment period) animals.

Table 1. Urinalysis in male and female rats treated with L-hydroxyproline for 28 days.
− Treatment period −

Dose level (mg/kg)	0	40	200	1000	4000
Male					
No. of examined	6	6	6	6	6
Appearance: normal [a)]	6	6	6	6	6
Protein: negative [a)]	6	6	6	6	6
Glucose: normal [a)](< 50 mmol/L)	6	6	6	6	6
Bilirubin: negative [a)]	6	6	6	6	6
Urobilinogen: normal [a)](< 35 μmol/L)	6	6	6	6	6
Ketone: negative [a)]	6	6	6	6	6
Blood: negative [a)]	6	6	6	6	6
Volume (0-1 hr) [b)] (mL)	2.60 ± 0.551	1.68 ± 0.799	2.63 ± 1.16	2.37 ± 0.829	2.63 ± 0.876
Volume (0-3 hr) [b)] (mL)	3.28 ± 1.09	2.48 ± 1.33	3.23 ± 1.15	3.10 ± 0.967	3.30 ± 0.748
PH [b)]	7.33 ± 0.516	7.33 ± 0.516	7.50 ± 0.548	7.33 ± 0.516	7.33 ± 0.516
Specific gravity [b)] (g/mL)	1.0161 ± 0.006	1.0166 ± 0.003	1.0178 ± 0.009	1.0157 ± 0.002	1.0155 ± 0.003
Female					
No. of examined	6	6	6	6	6
Appearance: normal [a)]	6	6	6	6	6
Protein: negative [a)]	6	6	6	6	6
Glucose: normal [a)](< 50 mmol/L)	6	6	6	6	6
Bilirubin: negative [a)]	6	6	6	6	6
Urobilinogen: normal [a)](< 35 μmol/L)	6	6	6	6	6
Ketone: negative [a)]	6	6	6	6	6
Blood: negative [a)]	6	6	6	6	6
Volume (0-1 hr) [b)] (mL)	1.55 ± 0.622	1.80 ± 0.502	1.52 ± 0.422	1.80 ± 0.923	2.25 ± 0.985
Volume (0-3 hr) [b)] (mL)	2.10 ± 0.743	2.30 ± 0.660	2.28 ± 0.293	2.58 ± 1.40	3.05 ± 1.40
PH [b)]	7.17 ± 0.753	7.17 ± 0.408	6.83 ± 0.753	7.17 ± 0.753	7.17 ± 0.408
Specific gravity [b)] (g/mL)	1.0144 ± 0.004	1.0149 ± 0.005	1.0136 ± 0.002	1.0145 ± 0.004	1.0135 ± 0.004

[a)]: Numbers in the table indicate number of animals with respective findings.[b)]: Values in the table indicate group mean ± SD.

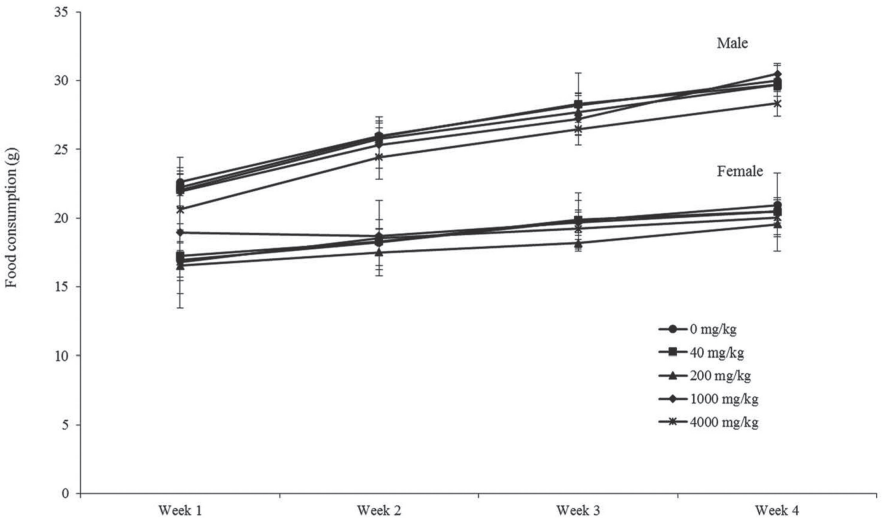

Fig. 2. Food consumption in male and female rats treated orally with L-hydroxyproline for 4 weeks. Values are the mean ± standard deviations of 16 (0 and 4000 mg/kg groups) or 10 (other groups) animals.

Table 2. Hematology in male and female rats treated with L-hydroxyproline for 28 days.

Period	Treatment					Post-treatment	
Dose level (mg/kg)	0	40	200	1000	4000	0	4000
Male							
No. of examined	10	10	10	10	10	6	6
RBC (10^{12}/L)	7.43 ± 0.49	7.66 ± 0.22	7.37 ± 0.49	7.43 ± 0.34	7.19 ± 0.42	7.92 ± 0.48	7.72 ± 0.49
WBC (10^{9}/L)	15.3 ± 2.0	13.0 ± 3.4	15.7 ± 2.8	13.5 ± 1.6	15.7 ± 3.0	14.7 ± 3.7	16.4 ± 3.4
Hb (g/L)	147 ± 8	149 ± 6	146 ± 6	147 ± 9	139 ± 6	146 ± 9	146 ± 8
Ht (%)	47.6 ± 2.6	48.0 ± 1.7	46.7 ± 1.8	47.2 ± 2.3	44.8* ± 1.7	47.1 ± 2.8	46.4 ± 2.3
Platelet (10^{9}/L)	1184 ± 110	1237 ± 126	1233 ± 128	1209 ± 134	1238 ± 172	1123 ± 60	1109 ± 145
Lymphocyte ratio (%)	87.1 ± 2.6	87.6 ± 1.4	87.7 ± 1.7	87.7 ± 1.9	88.5 ± 1.4	88.7 ± 1.2	88.8 ± 1.0
Neutrophil ratio (%)	10.7 ± 2.3	10.6 ± 1.1	10.5 ± 1.7	10.4 ± 1.6	9.6 ± 1.4	9.9 ± 1.1	9.4 ± 0.9
Eosinophil ratio (%)	0.45 ± 0.28	0.35 ± 0.34	0.45 ± 0.50	0.50 ± 0.41	0.45 ± 0.28	0.67 ± 0.26	0.50 ± 0.45
Monocyte ratio (%)	1.80 ± 0.42	1.50 ± 0.53	1.40 ± 0.39	1.50 ± 0.47	1.45 ± 0.37	0.75 ± 0.52	1.33 ± 0.41
Basophil ratio (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PT (sec.)	17.2 ± 1.37	16.4 ± 0.70	16.7 ± 1.30	17.4 ± 1.33	16.0 ± 0.95	17.6 ± 1.14	16.9 ± 1.42
Female							
No. of examined	10	10	10	10	10	6	6
RBC (10^{12}/L)	7.47 ± 0.32	7.00* ± 0.32	7.2 ± 0.47	7.45 ± 0.37	7.24 ± 0.30	7.65 ± 0.42	7.50 ± 0.28
WBC (10^{9}/L)	13.1 ± 3.4	11.4 ± 2.5	12.2 ± 3.9	11.2 ± 1.9	13.4 ± 2.5	13.4 ± 1.3	12.7 ± 2.0
Hb (g/L)	146 ± 5	141 ± 7	144 ± 6	148 ± 6	144 ± 7	141 ± 5	143 ± 6
Ht (%)	45.7 ± 1.6	44.3 ± 2.3	44.6 ± 2.0	46.4 ± 2.0	45.5 ± 2.1	44.8 ± 1.7	45.5 ± 1.9
Platelet (10^{9}/L)	1249 ± 140	1230 ± 84	1238 ± 202	1241 ± 177	1374 ± 118	1053 ± 57	1092 ± 117
Lymphocyte ratio (%)	88.3 ± 0.9	86.8 ± 2.1	88.5 ± 1.4	88.6 ± 1.2	88.4 ± 1.5	87.8 ± 1.4	88.7 ± 0.9
Neutrophil ratio (%)	9.3 ± 1.0	10.7 ± 1.9	9.7 ± 1.6	9.5 ± 0.8	9.8 ± 1.2	10.3 ± 0.9	9.7 ± 1.3
Eosinophil ratio (%)	0.75 ± 0.42	0.80 ± 0.26	0.35 ± 0.41	0.65 ± 0.41	0.50 ± 0.33	0.92 ± 0.58	0.50 ± 0.55
Monocyte ratio (%)	1.70 ± 0.42	1.70 ± 0.48	1.55 ± 0.64	1.30 ± 0.54	1.35 ± 0.58	0.92 ± 0.38	1.17 ± 0.41
Basophil ratio (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PT (sec.)	16.6 ± 0.81	16.5 ± 1.12	15.5 ± 1.02	15.8 ± 1.95	16.2 ± 1.02	14.9 ± 1.05	15.6 ± 1.27

Values in the table indicate group mean ± SD.*: $p < 0.05$ (significantly different from the control group)

Table 3. Blood chemistry in male and female rats treated with L-hydroxyproline for 28 days.

Period	Treatment					Post-treatment	
Dose level (mg/kg)	0	40	200	1000	4000	0	4000
Male							
No. of examined	10	10	10	10	10	6	6
GOT (U/L)	129 ± 19.6	128 ± 15.7	128 ± 20.0	106* ± 10.8	91.9** ± 13.7	117 ± 9.1	120 ± 32.4
GPT (U/L)	40.4 ± 4.15	41.4 ± 4.60	40.3 ± 6.03	42.2 ± 4.54	36.1 ± 6.06	40.4 ± 8.34	40.6 ± 5.37
ALP (U/L)	726 ± 147	847 ± 416	739 ± 151	662 ± 107	566 ± 64.9	608 ± 113	541 ± 79.7
T.Cho (mmol/L)	1.72 ± 0.16	1.74 ± 0.33	1.64 ± 0.15	1.83 ± 0.25	1.93 ± 0.17	1.68 ± 0.25	1.75 ± 0.17
TP (g/L)	60.2 ± 3.66	59.2 ± 3.39	58.2 ± 1.79	59.6 ± 2.76	58.8 ± 3.41	59.3 ± 1.22	58.7 ± 2.43
Albumin (g/L)	34.4 ± 1.51	35.2 ± 1.13	34.7 ± 0.94	35.0 ± 1.65	34.6 ± 1.99	34.4 ± 1.37	32.8 ± 0.52
Glucose (mmol/L)	5.22 ± 0.63	5.35 ± 0.53	5.50 ± 0.48	5.39 ± 0.66	5.42 ± 0.69	5.71 ± 0.47	5.69 ± 1.25
BUN (mmol/L)	6.64 ± 1.21	7.58 ± 0.76	6.67 ± 0.85	6.76 ± 0.62	7.33 ± 1.29	6.98 ± 0.57	6.21 ± 0.93
Na (mmol/L)	147 ± 1.04	144 ± 1.89	143** ± 2.68	142** ± 2.77	144* ± 2.05	142 ± 1.55	143 ± 1.55
K (mmol/L)	4.66 ± 0.20	4.53 ± 0.20	4.64 ± 0.28	4.42 ± 0.22	4.56 ± 0.28	4.23 ± 0.25	4.43 ± 0.20
Cl (mmol/L)	97.9 ± 3.38	95.0 ± 3.62	96.3 ± 3.85	95.1 ± 8.89	96.0 ± 4.20	102.6 ± 2.95	97.8* ± 3.01
Ca (mmol/L)	2.44 ± 0.09	2.36 ± 0.11	2.26** ± 0.14	2.29* ± 0.14	2.38 ± 0.09	2.21 ± 0.04	2.34 ± 0.12
Creatinine (mmol/L)	55.6 ± 2.49	60.7 ± 8.08	57.7 ± 5.46	55.1 ± 4.98	61.1 ± 5.50	58.6 ± 7.47	60.8 ± 10.7
Female							
No. of examined	10	10	10	10	10	6	6
GOT (U/L)	127 ± 32.2	127 ± 15.8	110 ± 21.1	112 ± 16.3	96.8* ± 13.6	130 ± 56.6	109 ± 10.0
GPT (U/L)	39.6 ± 10	39.6 ± 6.68	37.4 ± 8.82	34.0 ± 4.60	40.6 ± 24.8	49.3 ± 38.7	39.9 ± 7.44
ALP (U/L)	480 ± 326	422 ± 144	446 ± 124	470 ± 190	449 ± 213	327 ± 58.0	359 ± 126
T.Cho (mmol/L)	2.09 ± 0.35	1.94 ± 0.27	2.13 ± 0.31	2.27 ± 0.23	2.40 ± 0.38	2.25 ± 0.37	2.00 ± 0.23
TP (g/L)	67.4 ± 5.47	62.2* ± 2.10	63.1 ± 3.66	62.9 ± 3.29	63.1 ± 3.27	68.1 ± 3.92	60.9** ± 1.83
Albumin (g/L)	37.3 ± 3.35	37.1 ± 1.37	37.5 ± 2.20	37.1 ± 1.62	37.7 ± 2.16	39.4 ± 2.55	35.9* ± 1.72
Glucose (mmol/L)	5.23 ± 0.88	5.87 ± 0.64	5.41 ± 0.76	5.04 ± 0.57	5.08 ± 0.56	5.36 ± 0.40	5.51 ± 0.62
BUN (mmol/L)	8.78 ± 2.67	7.71 ± 1.54	8.52 ± 1.71	8.56 ± 3.28	7.76 ± 1.98	7.68 ± 2.21	7.45 ± 1.20
Na (mmol/L)	145 ± 2.54	144 ± 1.86	142* ± 0.81	143 ± 1.55	142* ± 1.78	141 ± 1.66	140 ± 1.67
K (mmol/L)	4.6 ± 1.35	4.23 ± 0.35	4.13 ± 0.36	4.37 ± 0.45	4.35 ± 0.36	3.88 ± 0.27	4.21* ± 0.14
Cl (mmol/L)	98.7 ± 5.87	102.4 ± 3.77	100.2 ± 3.93	98.2 ± 5.64	97.6 ± 4.99	96.5 ± 3.79	100.6 ± 6.33
Ca (mmol/L)	2.40 ± 0.16	2.34 ± 0.11	2.43 ± 0.14	2.35 ± 0.18	2.15** ± 0.17	2.39 ± 0.16	2.47 ± 0.12
Creatinine (mmol/L)	70.1 ± 14.59	63.4 ± 5.48	59.8 ± 8.97	59.6 ± 5.77	60.0 ± 5.98	61.8 ± 8.20	57.1 ± 5.88

Values in the table indicate group mean ± SD.*: $p < 0.05$, **: $p < 0.01$ (significantly different from the control group)

cal limits. The other statistically significant differences occurring sporadically had no biological significance as they were not dose-related. After the recovery period, statistically significant differences were recorded in a few parameters in males and/or females at 4,000 mg/kg; however, they were attributable to low within-group differences and judged to have no toxicological significance.

Organ weights

The organ weight data are shown in Tables 4-1 (Male) and 4-2 (Female). There was a slight increase in the absolute kidney weight in males at 4,000 mg/kg and females at 1,000 and 4,000 mg/kg, though it was not statistically significant. The relative kidney weight increased in both sexes at 1,000 and 4,000 mg/kg, though statistical significance was reached only in males at 4,000 mg/kg. A slight difference in the kidney weight remained between the male control and 4,000 mg/kg groups two weeks after cessation of treatment.

Histopathology

The histopathological findings are shown in Tables 5-1 (Treatment period) and 5-2 (Post-treatment period). Treatment-related findings were noted in the kidney in males at 1,000 and 4,000 mg/kg and females at 4,000 mg/kg. Focal dilatation of one or more renal tubules with narrowing of the tubular cell liner, focal tubular dilatation, and increased number of fibroblasts among the tubules, focal interstitial fibroplasia, were seen in 8 males and 3 females at 4,000 mg/kg. A focal interstitial fibroplasia was also seen in 5 males at 1,000 mg/kg but one of them was accompanied by focal tubular dilatation. Although

Table 4-1. Organ weights in male and female rats treated with L-hydroxyproline for 28 days.
− Male −

Period	Treatment					Post-treatment	
Dose level (mg/kg)	0	40	200	1000	4000	0	4000
No. of examined	10	10	10	10	10	6	6
Body weight (g) [a)]	344 ± 18.1	334 ± 27.0	331 ± 23.0	320 ± 37.6	301 ± 15.1	377 ± 25.6	360 ± 16.1
Liver							
abs. (g)	11.7 ± 0.999	12.0 ± 0.822	11.1 ± 0.640	11.2 ± 1.66	11.1 ± 1.19	12.1 ± 1.25	12.4 ± 2.17
rela. (g%)	3.40 ± 0.177	3.61 ± 0.213	3.37 ± 0.193	3.49 ± 0.276	3.67 ± 0.319	3.21 ± 0.200	3.44 ± 0.628
Heart							
abs. (g)	1.22 ± 0.141	1.19 ± 0.079	1.19 ± 0.082	1.18 ± 0.157	1.12 ± 0.101	1.30 ± 0.087	1.24 ± 0.075
rela. (g%)	0.353 ± 0.031	0.358 ± 0.024	0.360 ± 0.038	0.369 ± 0.016	0.373 ± 0.024	0.347 ± 0.040	0.346 ± 0.019
Kidney							
abs. (g)	2.65 ± 0.241	2.70 ± 0.196	2.65 ± 0.232	2.70 ± 0.245	3.02 ± 0.454	3.03 ± 0.158	3.13 ± 0.301
rela. (g%)	0.771 ± 0.065	0.810 ± 0.051	0.803 ± 0.065	0.847 ± 0.068	1.01** ± 0.159	0.805 ± 0.057	0.867 ± 0.051
Spleen							
abs. (g)	0.757 ± 0.073	0.728 ± 0.149	0.751 ± 0.096	0.664 ± 0.083	0.744 ± 0.094	0.785 ± 0.059	0.733 ± 0.082
rela. (g%)	0.220 ± 0.016	0.219 ± 0.042	0.227 ± 0.022	0.208 ± 0.015	0.247 ± 0.029	0.209 ± 0.020	0.204 ± 0.025
Brain							
abs. (g)	1.99 ± 0.187	1.89 ± 0.097	1.91 ± 0.075	1.93 ± 0.068	1.90 ± 0.072	1.93 ± 0.092	1.93 ± 0.100
rela. (g%)	0.579 ± 0.059	0.568 ± 0.045	0.578 ± 0.039	0.609 ± 0.057	0.633 ± 0.029	0.516 ± 0.057	0.536 ± 0.022
Testes							
abs. (g)	2.85 ± 0.391	3.11 ± 0.279	2.92 ± 0.494	2.97 ± 0.416	2.92 ± 0.426	3.00 ± 0.382	3.19 ± 0.433
rela. (g%)	0.829 ± 0.113	0.934 ± 0.078	0.888 ± 0.169	0.935 ± 0.144	0.972 ± 0.145	0.800 ± 0.118	0.886 ± 0.122
Epididymides							
abs. (g)	0.967 ± 0.124	1.00 ± 0.123	1.08 ± 0.292	0.977 ± 0.112	0.922 ± 0.128	1.37 ± 0.198	1.35 ± 0.156
rela. (g%)	0.282 ± 0.040	0.301 ± 0.041	0.328 ± 0.099	0.307 ± 0.036	0.307 ± 0.047	0.367 ± 0.071	0.375 ± 0.040
Thymus							
abs. (mg)	666 ± 116	627 ± 89.1	587 ± 102	591 ± 124	596 ± 113	486 ± 98.9	456 ± 148
rela. (mg%)	194 ± 34.7	189 ± 28.1	177 ± 30.0	185 ± 34.1	199 ± 40.2	128 ± 19.6	128 ± 43.9
Adrenals							
abs. (mg)	72.3 ± 14.0	73.5 ± 20.1	72.5 ± 12.4	68.3 ± 10.4	67.2 ± 13.1	90.5 ± 10.9	82.0 ± 14.2
rela. (mg%)	21.0 ± 3.95	22.0 ± 5.65	21.8 ± 3.02	21.6 ± 3.93	22.4 ± 4.40	24.1 ± 3.58	22.7 ± 3.41

[a)]: Body weight at necropsy after exanguination. abs.: Absolute weight, rela.: Relative weight. Values in the table indicate group mean ± SD. **: $p < 0.01$ (significantly different from the control group)

Table 4-2. Organ weights in male and female rats treated with L-hydroxyproline for 28 days.
− Female −

Period	Treatment					Post-treatment	
Dose level (mg/kg)	0	40	200	1000	4000	0	4000
No. of examined	10	10	10	10	10	6	6
Body weight (g) [a)]	207 ± 23.1	224 ± 16.2	208 ± 15.7	217 ± 10.8	210 ± 16.9	243 ± 18.3	239 ± 11.7
Liver							
abs. (g)	6.93 ± 0.683	8.29 ± 1.71	7.32 ± 0.582	7.41 ± 0.682	7.70 ± 0.982	8.17 ± 1.09	8.79 ± 0.764
rela. (g%)	3.36 ± 0.191	3.70 ± 0.625	3.53 ± 0.226	3.42 ± 0.232	3.67 ± 0.312	3.35 ± 0.223	3.70 ± 0.482
Heart							
abs. (g)	0.799 ± 0.086	0.830 ± 0.099	0.758 ± 0.065	0.826 ± 0.077	0.813 ± 0.069	0.902 ± 0.110	0.858 ± 0.092
rela. (g%)	0.387 ± 0.024	0.371 ± 0.026	0.365 ± 0.024	0.381 ± 0.030	0.388 ± 0.022	0.371 ± 0.041	0.359 ± 0.031
Kidney							
abs. (g)	1.63 ± 0.149	1.63 ± 0.120	1.65 ± 0.178	1.82 ± 0.184	1.85 ± 0.203	1.94 ± 0.278	1.95 ± 0.082
rela. (g%)	0.791 ± 0.049	0.730 ± 0.067	0.798 ± 0.094	0.842 ± 0.076	0.884 ± 0.075	0.795 ± 0.093	0.817 ± 0.048

Table 4-2. (Continued).

Period	Treatment					Post-treatment	
Dose level (mg/kg)	0	40	200	1000	4000	0	4000
No. of examined	10	10	10	10	10	6	6
Spleen							
abs. (g)	0.498 ± 0.116	0.548 ± 0.063	0.547 ± 0.119	0.520 ± 0.059	0.500 ± 0.078	0.522 ± 0.068	0.540 ± 0.052
rela. (g%)	0.239 ± 0.034	0.245 ± 0.021	0.262 ± 0.042	0.240 ± 0.025	0.238 ± 0.026	0.214 ± 0.019	0.226 ± 0.021
Brain							
abs. (g)	1.80 ± 0.073	1.77 ± 0.041	1.80 ± 0.076	1.81 ± 0.214	1.80 ± 0.080	1.80 ± 0.246	1.84 ± 0.048
rela. (g%)	0.879 ± 0.079	0.796 ± 0.066	0.869 ± 0.067	0.834 ± 0.082	0.863 ± 0.052	0.739 ± 0.093	0.771 ± 0.026
Thymus							
abs. (mg)	441 ± 120	500 ± 89.9	524 ± 82.5	428 ± 104	465 ± 98.3	418 ± 63.2	388 ± 69.2
rela. (mg%)	212 ± 45.7	224 ± 40.8	251 ± 31.1	198 ± 47.3	221 ± 39.7	171 ± 14.7	163 ± 31.7
Adrenals							
abs. (mg)	78.8 ± 15.3	77.5 ± 13.7	73.6 ± 9.87	84.9 ± 15.0	82.0 ± 13.4	109 ± 14.4	81.5** ± 8.41
rela. (mg%)	38.3 ± 7.43	34.9 ± 6.91	35.6 ± 5.45	39.2 ± 6.35	39.2 ± 6.56	44.8 ± 5.83	34.1** ± 3.35

[a)]: Body weight at necropsy after exanguination. abs.: Absolute weight, rela.: Relative weight. Values in the table indicate group mean ± SD. **: $p < 0.01$ (significantly different from the control group)

Table 5-1. Histopathological findings in male and female rats treated with L-hydroxyproline for 28 days.
−Treatment period−

Sex	Male					Female				
Dose level (mg/kg)	0	40	200	1000	4000	0	40	200	1000	4000
No. of examined	10	10	10	10	10	10	10	10	10	10
Kidney										
Focal tubular dilatation	0	0	1	1	8	1	0	0	1	3
Slight	0	0	1	1	3	1	0	0	1	2
Moderate	0	0	0	0	4	0	0	0	0	1
Marked	0	0	0	0	1	0	0	0	0	0
Focal interstitial fibroplasia	1	0	1	5	8	1	0	0	1	3
Slight	1	0	1	5	3	1	0	0	1	2
Moderate	0	0	0	0	4	0	0	0	0	1
Marked	0	0	0	0	1	0	0	0	0	0
Focal lympho-histiocytic infiltration	2	1	0	4	3	1	2	1	1	0
Slight	2	1	0	4	3	1	2	1	1	0
Mineral deposits	0	0	0	0	2	1	0	0	0	0
Slight	0	0	0	0	0	1	0	0	0	0
Moderate	0	0	0	0	1	0	0	0	0	0
Marked	0	0	0	0	1	0	0	0	0	0
Liver										
Fatty infiltration	1	−	−	−	0	8	−	−	−	7
Slight	1				0	8				7
Focal proliferation of MPS-cells	2	−	−	−	0	2	−	−	−	1
Slight	2				0	2				1
Lung										
Alveolar emphysema	1	−	−	−	1	2	−	−	−	1
Slight	1				1	2				1
Focal hemorrhage	1	−	−	−	0	0	−	−	−	0
Slight	1				0	0				0
Uterus										
Dilatation	−	−	−	−	−	3	−	−	−	1
Moderate						3				1

−: Not applicable, MPS: Mononuclear phagocyte systemNumbers in the table indicate the number of animals with respective findings.

Table 5-2. Histopathological findings in male and female rats treated with L-hydroxyproline for 28 days. −Post-treatment period−

Sex	Male		Female	
Dose level (mg/kg)	0	4000	0	4000
No. of examined	6	6	6	6
Kidney				
Focal tubular dilatation	1	3	1	0
Slight	1	2	1	0
Moderate	0	1	0	0
Focal interstitial fibroplasia	2	3	1	1
Slight	2	2	1	1
Moderate	0	1	0	0
Focal lympho-histiocytic infiltration	3	0	1	1
Slight	3	0	1	1
Liver				
Fatty infiltration	5	5	5	4
Slight	5	5	5	4
Focal proliferation of MPS-cells	1	1	1	0
Slight	1	1	1	0
Lung				
Alveolar emphysema	0	0	1	0
Slight	0	0	1	0
Focal hemorrhage	0	0	1	0
Slight	0	0	1	0
Skin				
Focal crusted inflammation	0	1	0	0
Moderate	0	1	0	0
Testis				
Decreased intensity of spermiogenesis	1	1	−	−
Slight	1	1		

−: Not applicable, MPS: Mononuclear phagocyte system.
Numbers in the table indicate the number of animals with respective findings.

focal interstitial fibroplasia was still seen in 3 males at 4,000 mg/kg after the recovery period, the incidence and severity were reduced as compared to those at the end of the treatment period, suggesting reversibility. The above renal cortical changes were graded as follows: “Slight”: unilateral and focal occurrence in approximately 1 to 5% of the renal cortex, “Moderate”: similar to the above but in bilateral change, and “Marked”: bilaterally affected larger contiguous area with intraluminar mineral deposits.

DISCUSSION

In this study, repeated-dose oral toxicity of Hyp was assessed in male and female SD rats by gavage for 28 days at the dose levels of 40, 200, 1,000 or 4,000 mg/kg/day. Reduced body weight gain with decreased food consumption was only noted in males at 4,000 mg/kg/day (Figs. 1 and 2). The histopathology revealed increased incidences of focal dilatation of the renal tubules with narrowing of the tubular cell liner and focal interstitial fibroplasia in the kidney of males at 1,000 and 4,000 mg/kg and in females at 4,000 mg/kg. The mechanism(s) of slight increase in kidney weights and slight histopathological findings such as focal dilatation of the renal tubules or interstitial fibroplasia in the kidney are unclear, but they seem to be similar to the findings seen after excessive administration of other kinds of amino acids.

Calcium oxalate is the most common constituent of urinary stones in animal model of the urolithiasis which has been established by means of feeding Hyp, an endogenous precursor of oxalate (Khan *et al*., 2007). They reported that rats receiving Hyp for 42 days showed hyperoxaluria, calcium oxalate crystalluria and nephrolithiasis. Furthermore, urinary excretion of lactate dehydrogenase, 8-isoprostane and H_2O_2 increased significantly.

The increase of urine oxalate was also observed in human study when 2,000 mg/day Hyp administrated (Akiduki *et al*., 2015). However, this elevation did not exceed the level of individuals with stones, mean oxalate excretion 40 mg/day (Knight *et al*., 2009). In another study, that 5 g and 10 g of gelatin administration to healthy subject did not influence total daily urinary oxalate excretion (Knight *et al*., 2006). So the metabolic behavior of Hyp intake in human might be similar to rat but details are unclear.

In Japan, Hyp is listed as a food additive for flavoring, and is expected to be a functional ingredient for skin health. The lack of kidney specific serum chemical or urinalysis findings supported that the renal changes in rats were very mild and reversible. However, further studies should be required to clarify the effect of oral Hyp in human urinalysis.

In conclusion, the no observed adverse effect level (NOAEL) of Hyp was 200 mg/kg/day in a 28-day oral toxicity study in rats.

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Conflict of interest---- SA, YK, FW, TK and KM are employees of KYOWA HAKKO BIO CO., LTD.

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