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A 90-day toxicity study of L-asparagine, a food additive, in F344 rats

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ABSTRACT

L-Asparagine is an amino acid listed as an existing food additive in Japan. The present 90-day toxicity study in F344/DuCrlCrj rats was conducted for safety assessment and to determine a no observed adverse effect level (NOAEL) of L-asparagine. Groups of 10 males and 10 females were given the material at dose levels of 0%, 1.25%, 2.5% or 5% in diet for 90 days. During the experiment, there were no remarkable changes in general conditions and no deaths occurred in any group. Final body weights of male 5% and 1.25% groups were significantly decreased. There were also significant increases in relative organ weights of the brain, kidney and testis in 5% males. On serological examination, GLU, PL, K and ALT were increased significantly in 5% females, and GLU was increased significantly and CRN was decreased significantly in the female 1.25% group. However, histopathological examination did not reveal any significant variation in development of lesions among the groups. Changes in body and organ weights, as well as other parameters, were concluded to be due to treatment with 5% L-asparagine. The NOAEL was determined to be 2.5% in the diet (males, 1.65 g/kg body weight/day; females, 1.73 g/kg body weight/day).

1. Introduction

Asparagine is an amino acid named after isolation from asparagus. It has two optical isomeric forms, L and D, and although only L-asparagine is used as a food additive for seasoning and as an antioxidant nutritional supplement. It is metabolically related to aspartic acid (Benuck et al., 1970). Aspartic acid itself has low solubility, and monosodium aspartate is created from aspartic acid to make a water soluble sodium salt which can be absorbed by the sodium dependent active transport system in the intestinal mucosa (Bers and Christensen, 1990). Sodium is necessary for transport of some amino acids in the body and monosodium aspartate has already been examined in a 2-year study in F344 rats. Hyperplasias of the renal papillae and the urinary bladder were observed in 5% monosodium L-aspartate treated male and female groups, but not in the controls (Kitahori et al., 1996). However, carcinogenic potential was not concluded.

L- Or D-asparagine is generally considered a non-essential amino acid in mammals, but some studies have indicated that a dietary source may be required for maximal growth of infants (Crosby and Cline, 1973; Breuer et al., 1966). When compared with a 20% casein diet and L- or D-asparagine, young rats given the asparagine at 0.4% along with casein hydrolysate had significantly increased growth after 8 days. However, 0.8% resulted in significantly decreased growth (Breuer et al., 1966). L-Asparagine is listed as an existing food additive, developed by the Standards and Evaluation Division, Department of Food Safety, Ministry of Health, Labour and Welfare in Japan. And, from the informations of Japan Food Additives Association, it was also approved as a food additive in United States, Republic of Korea, Taiwan, Philippines and so on. However, to our knowledge, there have been no studies of the safety of asparagine supplements and evidence is lacking to determine safe levels of L-asparagine intake as a supplement for humans. The present 90-day toxicity study was therefore conducted in F344 rats to determine the no observed adverse effect level (NOAEL).

2. Materials and methods

2.1. Experimental animals

Five-week old male and female F344/DuCrlCrj rats were purchased from Charles River Japan (Atsugi, Japan) and maintained in Kagawa University Animal

Abbreviations: RBC, red blood cell count; Hb, hemoglobin concentration; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; (Kitahori et al., 1996)WBC, white blood cell count; TP, total protein; A/G, albumin/globulin ratio; ALB, albumin; BIL, total bilirubin; TC, total cholesterol; GLU, glucose; PL, phospholipid; TG, triglyceride; BUN, blood urea nitrogen; CRN, creatinine; AS, asparagines transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; Ca, Calcium; P, inorganic phosphate; Na, sodium; K, potassium; Cl, chloride; ANOVA, analysis of variance; NOAEL, no observed adverse effect level.

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Facility according to the Institutional Rules for Animal Experimentation. The Animal Care and Use Committee for Kagawa University approved the protocol of this experiment. The animals were housed five per a cage, and given free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (60 ± 10%), lighting (12 h light/dark cycle) and temperature (24 ± 2 °C). The rats were acclimated for 2 weeks prior to start of the experiment and randomly assigned to four groups, each comprising 10 males and 10 females.

2.2. Test material

L-Asparagine was supplied by the Institute of Life Sciences, Ajinomoto Co. Inc. (Kawasaki, Japan) with specification data as follows: molecular weight ($C_4H_8N_2O_3$), 132.12 gmol; isoelectric point, 5.41. It was mixed into synthetic diet (AIN-93G, Oriental Yeast Co., Ltd, Tokyo, Japan) containing minerals and vitamins and cornstarch substituted for L-asparagine as the basal diet. The dose levels of L-asparagine were set at 5%, 2.5%, 1.25% and 0% for both sexes. Details of the dietary ingredients are shown in Table 1. Each test diet was admixed by the Oriental Yeast Co., Ltd (Tokyo, Japan). Completed powdered diets were analyzed for L-asparagine content by the Institute of Life Sciences, Ajinomoto Co. Inc. and the average concentrations of L-asparagine were 5.18% (5%), 2.64% (2.5%), 1.24% (1.25%) and 0% (0%).

2.3. Experimental protocol

This study was conducted in line with the OECD Test Guideline 408 for 'Repeated dose 90-day oral toxicity study in rodents'. Rats were observed daily for clinical signs and general conditions. Body weights and food intakes were measured once a week. On day 90, after 16 h starvation, all animals were weighed and then sacrificed by exsanguination after collection of blood samples from the abdominal aorta under deep anesthesia. The collected blood samples were processed for hematological and serum biochemical examinations. ETDA-2K was used as a anticoagulant for the blood samples and serum was collected using a centrifugal separator at 300 rpm for 15 min. A complete autopsy was then performed. Macroscopic pathological findings were noted during autopsy and weights of the brain, lungs, heart, spleen, liver, adrenals, kidneys, testes, salivary glands and ovaries were determined and values relative to the body weights were calculated. In addition to the above-mentioned organs, organs and tissues including the esophagus, stomach, duodenum, jejunum, ileum (including Peyer's patches), colon, cecum, rectum, pancreas, urinary bladder, trachea, thyroid gland, tongue, skeletal muscle, ischiatic nerve, spinal cord, aorta, nasal cavity, eyeballs, Harderian glands, Zymbal's glands, skin, mammary gland, mesenteric and mandibular lymph nodes, bone marrow in femur and sternum, epididymis, prostate, seminal vesicles, uterus and vagina were fixed in 10% buffered formalin solution. Testes were fixed in 2% glutaraldehyde, 15% formalin and 3% acetic acid (GFA) solution overnight before being transferred to 10% buffered formalin solution. The pituitary, prostate, seminal vesicle and uterus were weighed at the time of processing. Paraffin sections of all tissues from the 5% treated group and control groups were stained with hematoxylin and eosin for histopathological assessment. In hematological examinations, the red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT) and white blood cell count (WBC) were determined using routine laboratory procedures by SRL Co. (Tokyo, Japan). Serum biochemical data for TP(g/dL), A/G, ALB(g/dL), BIL(mg/dL), TC(mg/dL), GLU(mg/dL), PL(mg/dL), TG(mg/dL), BUN(mg/dL), CRN(mg/dL), Ca(mg/dL), P(mg/dL), Na(mEq/L), Cl(mEq/L), K(mEq/L), AST(IU/L), ALT(IU/L), ALP(IU/L) and γ-GTP (IU/L) were also generated by SRL Co. using routine laboratory procedures.

Table 1
Dietary ingredients

Ingredient	Content (g/kg diet)
β-Corn starch[a]	629.486−*X*
Casein (vitamin free)[b]	200.000
Soybean oil (no additives)[c]	70.000
Fiber source (cellulose)[d]	50.000
Mineral Mix (AIN-93G)[e]	35.000
Vitamin Mix (AIN-93G)[f]	10.000
L-Cystine[g]	3.000
Choline bitartrate (41.1% choline)[h]	2.500
tert-butylhydroquinone (TBHQ)[i]	0.014
L-Asparagine[j]	*X*

Basal diet: $X = 0$.
1.25% L-asparagine additive diet: $X = 12.5$.
2.50% L-asparagine additive diet: $X = 25$.
5.00% L-asparagine additive diet: $X = 50$.
[a] Corn starch W, Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan.
[b] Oriental Yeast Co., Ltd., Tokyo, Japan.
[c] J-oil Mills. Inc., Tokyo, japan.
[d] Advantec Toyo Kaisya, Ltd., Tokyo, Japan (cellulose powder D 40-100 mesh).
[e] Oriental Yeast Co., Ltd., Tokyo, Japan.
[f] Oriental Yeast Co., Ltd., Tokyo, Japan.
[g] Ajinomoto Co., Inc., Tokyo, Japan.
[h] Cat No. 036-09645, Wako Pure Chemical Industries, Ltd., Osaka, Japan.
[i] Cat No. 027-07212, Wako Pure Chemical Industries, Ltd., Osaka, Japan.
[j] Ajinomoto Co., Inc., Tokyo, Japan.

2.4. Statistical analysis

Analysis of the data for body and organ weights, hematology and serum biochemistry was performed using the following general procedures as appropriate. Dunnett's multiple comparison ($P < 0.05$) was applied. If significant heterogeneity of variance was detected, the Student's *t*-test for comparing treatment and control groups was employed. The Spearman's rank correlation test was applied to examine dose–response relationships. The Fisher's exact probability test was used for histopathological data to analyze increases in incidence between treated and control rats. Incidences of differing severities of histopathological findings were also compared using the one-sided Mann–Whitney U-test.

3. Results

3.1. General observations

There were no remarkable changes in general conditions and no deaths occurred in any group during the experiment. Body weight curves for male and female F344 rats during the treatment period are shown in Fig. 1. The body weights of male rats treated 5%, 2.5%, 1.25% and 0% L-asparagine at onset and end of the experiment were 83 ± 4 (mean ± SD), 83 ± 3, 83 ± 2 and 83 ± 3 g, and 271 ± 14, 276 ± 11, 273 ± 15 and 290 ± 8 g, respectively. These of females were 74 ± 2, 74 ± 2, 74 ± 2 and 74 ± 3 g, and 162 ± 4, 158 ± 8, 161 ± 8 and 160 ± 8 g, respectively. Slight decrease of body weights was observed in L-asparagine treated groups of males at autopsy, but there were no significant differences in final body weights in either male or female groups. Data for food consumption and intake of L-asparagine are shown in Table 2. There were also no significant differences among the groups in food consumption, average daily intake per rat being about 12 g and 8 g for males and females, respectively. Average daily intakes of L-asparagine per kg body weight of 1.25%, 2.5% and 5% treated males were 753, 1537 and 3242 mg, respectively and those of 1.25%, 2.5% and 5% treated females were 857, 1708 and 3466 mg. Intake of L-asparagine was in proportion with the concentration in the diet.

3.2. Hematological and serum biochemical data

For the hematological and serum biochemistry studies, examined numbers of rats were 9 for 5% treated males and females, since collection of blood samples was not made for one rat of each of sex. No significant differences were noted in hematological data for F344 rats given L-asparagine. Serum biochemistry findings are summarized in Table 3. ALP was significantly decreased by 9.4% in 2.5% treated males but there was no dose–response relationship. In 5% treated females, GLU, PL, TG, K and ALT were significantly increased to 123%, 125%, 316%, 117% and 121%, respectively. In 2.5% treated females, TG was significantly increased to 300%. In 1.25% treated females, GLU and TG were also significantly increased to 113% and 246%, respectively, and CRN was significantly decreased to 11%. The other serum biochemical parameters were not affected by the treatment.

3.3. Macroscopic findings

On dissection, there were no characteristic findings in treated groups though some rats exhibited extra lobulation in the liver or cysts of the ovary.