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Effects of four-week feed restriction on toxicological parameters in beagle dogs

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Abstract: This study was conducted to examine any changes caused by feed restriction in dogs to contribute to safety evaluation in toxicity studies. Two male 7-month-old beagle dogs/group were fed 300 (control), 150 (50% of control), or 70 g/animal of diet daily (23% of control) for 4 weeks. Effects of feed restriction, except for clinical signs, were noted depending on the feed dosage in almost all examinations. The principal outcomes were: decreased body weight and water consumption, ECG changes (decreased heart rate and prolonged QTc), and hematopoietic and lymphopoietic suppression (decreased reticulocyte ratio or white blood cell count in hematology, decreased nucleated cell count in bone marrow, decreased erythroid parameters in myelography, and hypocellularity of bone marrow and thymic atrophy in histopathology). In addition, some changes were noted in urinalysis (decreased urine volume and sodium and potassium excretion), blood chemistry (decreased ALP and inorganic phosphorus and increased creatinine), organ weights, and gastric histopathology. These results provide important reference data for distinguishing the primary effects of test compounds from secondary effects of decreased food consumption in toxicity studies in beagle dogs.
Key words: beagle dogs, bone marrow, electrocardiography, feed restriction, toxicological parameter

Introduction

Decreased food consumption is frequently observed in toxicity studies for candidate drugs and affects the physiological conditions of animals. This often complicates the toxicological assessment of test compounds, making it difficult to distinguish findings due to the direct effects of the test article from those incurred due to the malnourished condition. Close investigation of the biological responses in feed-restricted animals is therefore important and contributes to proper toxicological assessment.

Changes in the toxicological parameters of feed-restricted animals have been reported in rats [13, 18, 20]. Levin *et al*. [13] examined overall toxicological parameters with feed restriction for 2 weeks at 75, 50 or 25% of the control diet and found a myelosuppression in bone marrow in all the feed-restricted groups, indicating that the primary response to the feed restriction was hematopoietic suppression in rats. Other examinations focusing on hematopoiesis in feed-restricted rodents showed a decreased blood cell count and hypocellularity in bone marrow [3–9, 15, 17, 19]. These rodent examinations suggested, as described by a number of review articles

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[15, 21, 22], the importance of hematopoietic evaluation in the malnourished condition. In contrast to rodents, however, few malnourished examinations have been reported in dogs, despite this being the most commonly used non-rodent species in toxicity studies. For dogs, Hill *et al*. [11] and Lawlar *et al*. [12] used the grayhound or Labrador retriever, respectively, and they did not consider any toxicological aspects except for clinical pathology. Morita *et al*. [16] used the beagle which is a common breed in toxicity studies, and only conducted electrocardiography and hematology. Their data are considered insufficient as background data for toxicity studies in dogs.

Given this lack of data, we comprehensively evaluated the biological responses, with particular attention to hematopoiesis, in feed-restricted beagle dogs, including toxicological examination items for general signs, body weight, water consumption, electrocardiography, urinalysis, hematology, blood chemistry, bone marrow examination (including nucleated cell count and myelography), organ weight, and histopathology.

Materials and Methods

Study design and conditions

Animals: Six male beagle dogs purchased from Kitayama Labes Co., Ltd (Iwakuni, Japan) were used. The dogs were 7 months old at the initiation of restricted feeding. This study was approved by the Institutional Animal Care and Use Committee and was performed in accordance with the animal welfare by laws of Drug Safety Research Laboratories, Shin Nippon Biomedical Laboratories. The animals were individually housed in stainless steel cages in a room controlled for temperature (20.4 to 23.9°C), humidity (37 to 80%), ventilation (15 times/h), and light (12 h/day: 07:00 to 19:00).

Feeding and water supply: NVE-10 (Nippon Pet Food, Co., Ltd., Tokyo, Japan) was provided to each animal daily between 14:30 and 16:00. On the days before blood sampling (for hematology and blood chemistry and blood glucose level measurement) and gross pathology, any remaining food was removed from each animal at approximately 17:00. Water was available *ad libitum* from an automatic water supply system or bottle for measurement of water consumption.

Grouping: The three groups established for feed restriction were 300 g/animal/day (standard feed dosage in toxicity studies), 150 g/animal/day (50% of control), and 70 g/animal/day (23% of control), with 2 animals allocated per group. Animals were identified by number, as follows: No. 1 and 2 for 300 g/animal/day (control group), No. 3 and 4 for 150 g/animal/day, and No. 5 and 6 for 70 g/animal/day.

Feed restriction period: A four-week period of feed restriction was determined, which is the standard dosing period for subacute toxicity studies.

Examination items

The examination days were defined as follows. The day of initiation of feed restriction was defined as day 1. The day before day 1 was defined as day –1.

Clinical signs: Animals were observed in the morning and afternoon for clinical signs including external appearance, behavior, respiration, position, urinary and fecal condition, response to stimulation, and mortality.

Body weight: Body weights were measured on days –21, –7, –1, 4, 7, 11, 14, 18, 21, 25, and 28.

Food consumption: The quantity supplied and remaining for each animal was weighed daily using an electronic balance, and daily food consumption calculated.

Water consumption: Water consumption was measured on days –18, –11, 6, 12, 19, and 26 using a graduated cylinder, and the difference between the volume of water supplied and remaining was calculated.

Electrocardiography: Electrocardiogram examinations were conducted on days –15, 1, 8, 14, 21, and 28. Data from standard leads (I, II, and III) were recorded continuously for 24 h, and analysis was performed at 10:00 and 18:00 on each examination day and at 10:00 on the following day using JET transmitters (JET-3ETA, Data Sciences International Inc., St. Paul, MN, USA), JET receivers (JET-RCV, Data Sciences International Inc.), and JET systems (Ponemah Physiology Platform Plus, version 4.9, Data Sciences International Inc.). Heart rate, PR interval, QRS duration, QT interval, and QTc (Matsunaga's correction) from five consecutive waves of lead II at each time point as well as the mean values were calculated.

Urinalysis: Urinalysis was conducted on days –18, –11, 6, 12, 19, and 26. Six-hour urine (excreted from 9:00 to 15:00) and 18-h urine (excreted from 15:00 to 9:00 on the following day) were collected under ice-cooled conditions. The volume, pH, ketone bodies, bilirubin, occult blood, urobilinogen, protein, and glucose were measured or observed for 6- and 18-h urine, color and sediment were observed for 6-h urine, and specific

gravity, sodium, potassium, chloride, creatinine, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase (G-GTP), N-acetyl-beta-D-glucosaminidase (NAG), and osmolality were measured for 18-h urine.

Hematology: Blood was derived from the external jugular vein on days –17, –10, 7, 13, 20, and 27 to measure the erythrocyte count (RBC), leukocyte count (WBC), hematocrit value, hemoglobin concentration, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio, eosinophil count, basophil count, monocyte count, lymphocyte count, neutrophil count, large unstained cell (LUC) count, prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen.

Blood chemistry: Blood was derived from the external jugular vein on days –17, –10, 7, 13, 20, and 27 and left at room temperature for 20 to 60 min. Serum was obtained by centrifugation at room temperature at 1,710 × *g* for 15 min to measure aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), ALP, G-GTP, amylase, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, total cholesterol, free cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, phospholipid, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorus (IP), calcium (Ca), sodium (Na), potassium (K), chloride (Cl), total bile acid, globulin, and albumin/globulin ratio (A/G). In addition to blood chemistry, circadian changes in blood glucose and total protein were measured on days –7, –4, 5, 11, 18, and 25 with the following procedure. Approximately 0.5 ml of blood was derived from the external jugular vein with a syringe containing heparin sodium at 10:00 and 18:00 on the day of examination and at 10:00 the following day. Blood was immediately cooled on ice and centrifuged at 4°C at 1,710 × g for 15 min. Plasma was collected (at approximately 10:00 and 18:00 on the examination day and at 10:00 on the following day: total 3 points) to measure glucose and protein.

Gross pathology: The animals were weighed, anesthetized by an intravenous injection of sodium pentobarbital (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) solution (64.8 mg/ml, 0.5 ml/kg) into the cephalic vein of the forearm, and euthanized by exsanguination. External appearance, and internal organs and tissues were examined macroscopically.

Bone marrow examination: Bone marrow fluid was collected from the sternum at gross pathology. The bone marrow nucleated cell count was measured, and myelography were conducted.

• Bone marrow nucleated cell count: Bone marrow fluid was diluted and stained with Türk solution. The number of nucleated bone mallow cells was counted using an automatic F-820 cell counter (Sysmex Corporation, Kobe, Japan).

• Myelography: Bone marrow smears were prepared and stained with May-Grünwald and Giemsa stains. Myelograms of the smears were examined microscopically.

Organ weight and histopathology: Measurement of organ weight and histopathologic examination were performed for the heart, thymus, spleen, lungs, submandibular glands, liver, pancreas, kidneys, pituitary, thyroid glands, adrenals, testes, prostate, and brain. In addition to these organs, histopathology was performed for the aorta, sternum, sternal bone marrow, femur, femoral bone marrow, submandibular lymph nodes, mesenteric lymph nodes, Peyer's patches (ileum), trachea, bronchus, tongue, sublingual glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, gallbladder, urinary bladder, parathyroid glands, epididymides, optic nerves, spinal cord, sciatic nerves, eyeballs, lacrimal glands, femoral skeletal muscle, skin, and mammary glands. The eyeballs and optic nerves were fixed in a mixture of 3% glutaraldehyde and 2.5% formalin, and the testes were fixed in Bouin's solution. Other organs were fixed in 10% neutral buffered formalin. The sternum and femur were decalcified with Kalkitox (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical analysis: Statistical analysis was not conducted.

Results

Clinical signs

No abnormal clinical signs were observed in any animals.

Body weight

The time courses of body weight are shown in Fig. 1. The body weights in the 150 g/animal and 70 g/animal groups decreased gradually during the 4-week feed restriction period. The degrees of change depended on the feed dosage; that is, 150 g/animal and 70 g/animal groups showed decreases of approximately 14 and 25%, respec-

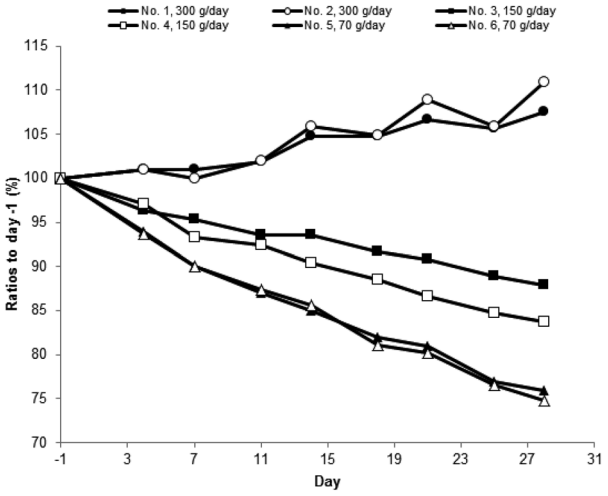

Fig. 1. Time course of body weight for each individual.

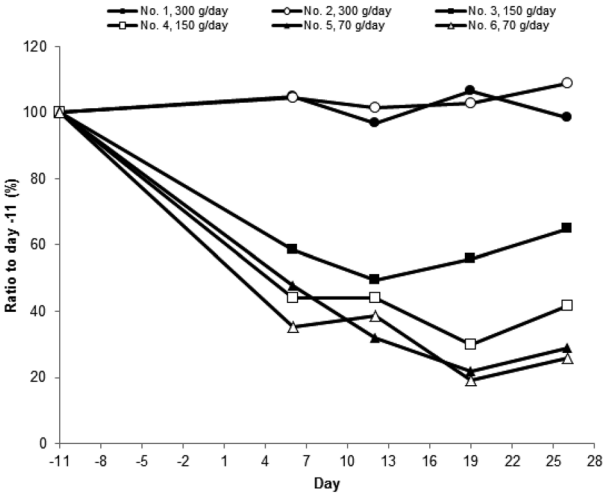

Fig. 2. Time course of water consumption for each individual.

tively, from day –1 to 28.

Food consumption

All animals consumed the entire amount of provided food during the examination period.

Water consumption

The time courses of water consumption are shown in Fig. 2. Water consumption decreased depending on the feed dosage in all animals in the 150 and 70 g/day groups. The amount of water consumption in the 150 and 70 g/day groups were approximately half and one-fourth of those on day -11, respectively, on all examination days during the feed restriction period.

Electrocardiography

The time courses of the mean values for electrocardiographic parameters in each group are shown in Fig. 3, and the individual values for the parameters at all examination points are listed in Supplementary Table 1. Decreased heart rate and prolonged PR interval were noted depending on the feed dosage in the 150 and 70 g/day groups. Since tendencies towards prolongation of QTc were observed in the 150 and 70 g/day groups (Fig. 3D), individual values were evaluated in detail using criteria based on the variation during the acclimation period (Supplementary Table 1). Although prolongation beyond the criteria based on the variation during the acclimation period was were noted transiently in only one animal in the 150 g/day group, both animals in the 70 g/day group exhibited a tendency toward increased levels at some examination points. The time courses of animals with noticeable tendencies in each group are shown in Fig. 3E. Tendencies towards prolongation of the QRS duration were also noted in the 150 g/day group; however, the degree of changes was very slight. The above changes tended to continue during the feed restriction period.

Urinalysis

A notable change depending on the feed dosage was observed in urine volume. The time courses of the mean value for daily total urine volume in each group are shown in Fig. 4A. The urine volume in the feed-restricted groups was consistently lower than that in the 300 mg/day group. The ratios to that on day -18 ranged from 20 to 60% in both groups during the feed restriction period. Excretion of sodium and potassium in urine changed in a manner similar to the urine volume (Figs. 4B and 4C). No notable change was observed in other parameters in any groups. The individual values of parameters at all examination points are listed in Supplementary Table 2, except for parameters in which no changes were noted at any examination points.

Hematology

The mean values of parameters with notable changes caused by feed restriction are shown in Table 1. Decreases in reticulocyte ratio and WBC, including the neutrophil, eosinophil, basophil, monocyte, and lymphocyte count, were noted depending on the feed dosage from day 7 up to the end of the feed restriction period.

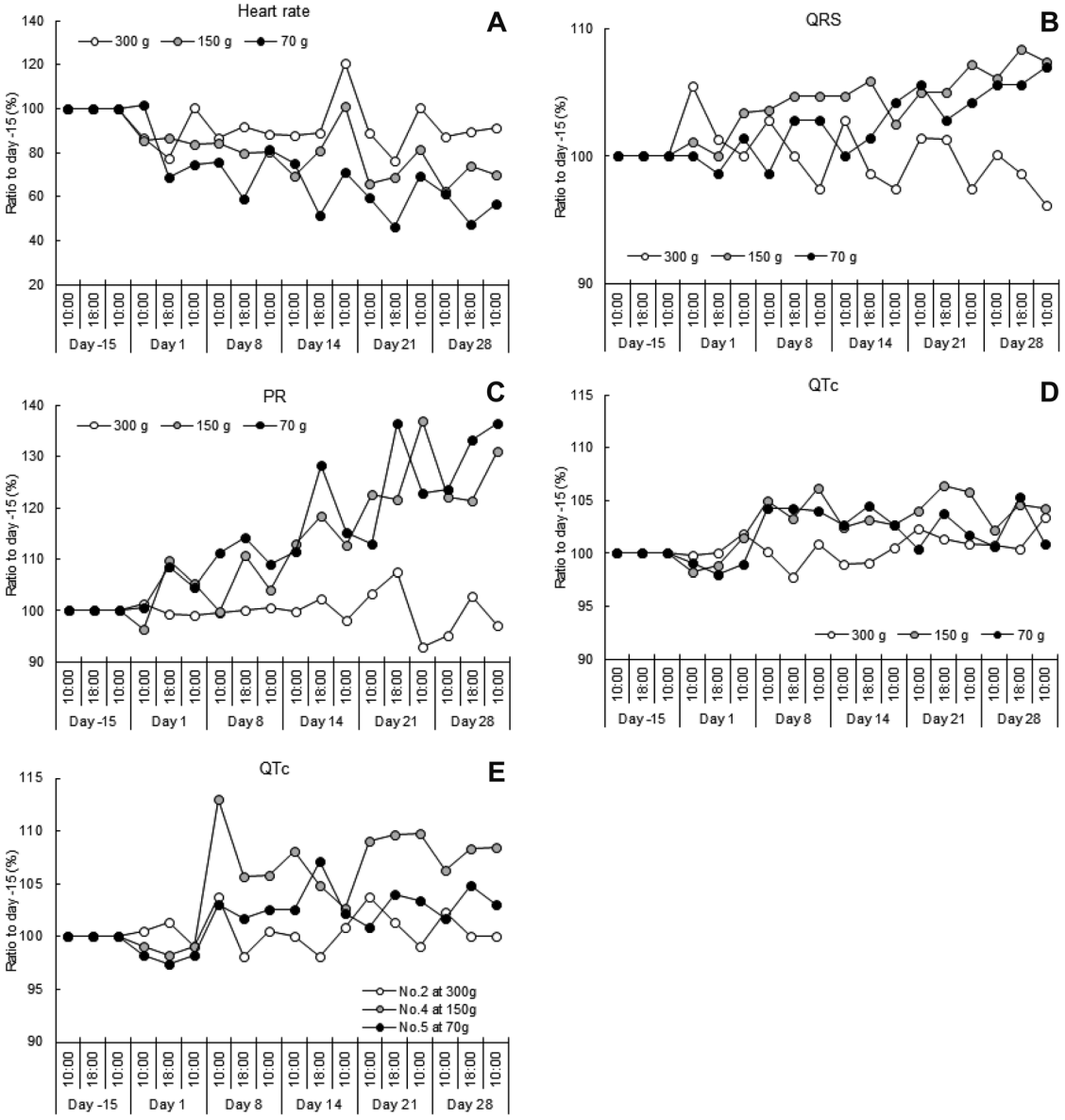

Fig. 3. Time course of each parameter in electrocardiography. A: Time course of the mean values of heart rate in each group. B: Time course of the mean values of QRS in each group. C: Time course of the mean values of PR interval in each group. D: Time course of the mean values of QTc in each group. E: Time course of the individual values in each group.

The time course of the changes in reticulocyte ratio and WBC are shown in Fig. 5. The individual values of parameters at all examination points are listed in Supplementary Table 3, except for parameters in which no changes were noted at any examination points.

Blood chemistry

Both feed-restricted groups exhibited a decrease or tendency to decrease in serum ALP, amylase, phospholipid, inorganic phosphorus, glucose, and calcium and an increase or tendency to increase in ALAT, creatinine, G-GTP, chloride, BUN, and total bile acid. Only the 70 g/day group exhibited an increase in bilirubin. These data are shown in Table 2. Almost all these parameters changed only at day 27 or at random days during the feed restriction period (Supplementary Table 4); however, creatinine constantly increased, and ALP and inorganic phosphorus constantly decreased during the feed restriction period (Fig. 6), although the magnitudes of change were not necessarily large. Regarding examina-

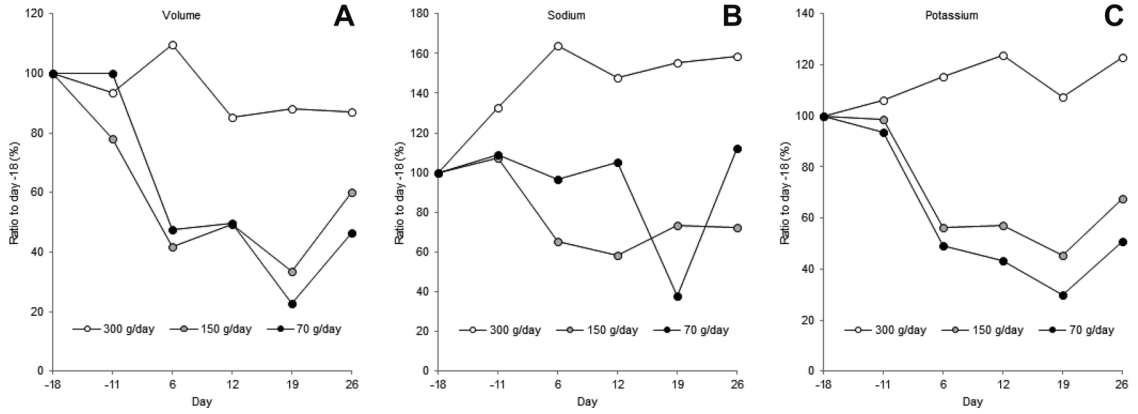

Fig. 4. Time courses of urinary volume, sodium excretion, and potassium excretion. A: Time course of the mean values of urinary volume in each group. B: Time course of the mean values of sodium excretion in each group. C: Time course of the mean values of potassium excretion in each group.

Table 1. Individual and mean hematologic parameter values at days -10 and 27

Parameters	Day	300 g/day			150 g/day			70 g/day		
		No. 1	No. 2	Mean	No. 3	No. 4	Mean	No. 5	No. 6	Mean
Reticulocyte ratio	-10	0.7	0.5	0.6	0.9	0.5	0.70	0.7	0.6	0.65
(%)	27	0.4	0.6	0.5	0.5	↓0.1	0.30	↓0.1	↓0.1	0.10
WBC	-10	9.80	10.15	9.98	11.90	10.78	11.34	8.64	9.41	9.03
(10^3/mm^3)	27	8.53	8.85	8.69	9.65	↓6.45	8.05	↓5.08	↓4.35	4.72
Neutrophil count	-10	4.13	5.72	4.93	5.8	4.3	5.05	3.04	3.92	3.48
(10^3/mm^3)	27	3.64	4.95	4.30	5.47	(↓) 2.92	4.20	(↓) 1.86	↓2.25	2.06
Eosinophil count	-10	0.77	0.39	0.58	1.21	1.96	1.59	0.47	1.02	0.75
(10^3/mm^3)	27	0.52	0.30	0.41	↓0.72	↓1.00	0.86	↓0.41	↓0.19	0.30
Basophil count	-10	0.10	0.07	0.09	0.12	0.05	0.09	0.11	0.06	0.09
(10^3/mm^3)	27	0.06	0.05	0.06	0.06	(↓) 0.04	0.05	↓0.03	↓0.02	0.03
Monocyte count	-10	0.72	0.6	0.66	0.53	0.71	0.62	0.49	0.36	0.43
(10^3/mm^3)	27	0.63	0.58	0.61	(↓) 0.39	(↓) 0.49	0.44	↓0.18	↓0.23	0.21
Lymphocyte count	-10	4.05	3.34	3.70	4.20	3.75	3.98	4.50	4.02	4.26
(10^3/mm^3)	27	3.67	2.95	3.31	3.00	↓2.00	2.50	↓2.58	↓1.64	2.11

Simbols: ↑, increase; ↓, decrease; (↑), tended to increase; (↓), tended to decrease (evaluated only for individual data). Criteria were calculated based on the range of variance (%) in the acclimation period or control group obtained for each animal, and the highest individual variation (%) was adopted as the criterion after rounding (except for reticulocyte ratio, basopil count, and lymphocyte count). Range of variance in WBC, eosinophil count, monocyte count, and neutrophil count: (lowest value) / (highest value) × 100. Individual criteria were calculated using the following equation:WBC, eosinophil, monocyte, and neutrophil counts: [value on day −17 or −10]* × [range of variance (%)]. *Criteria <100%: from the highest value on day −17 or −10. Criteria >100%: from the lowest value on day −17 or −10. Reticulocyte ratio (<0.3%), basophil count (<0.04 10^3/mm^3), lymphocyte count (<2.95 10^3/mm^3).

tion of circadian changes in glucose and total protein, no apparent circadian changes were noted in any groups, and no abnormalities were observed in any animals (Supplementary Table 5). The individual values of parameters at all examination points are listed in Supplementary Table 4, except for parameters in which no changes were noted at any examination points.

Bone marrow examination

Notable results from the bone marrow examination are shown in Table 3. The nucleated cell count decreased in the feed-restricted groups depending on the feed dosage. The feed restricted-groups also exhibited decreased ratio of erythroblasts at the proerythroblast, basophilic erythroblast, polychromatic erythroblast, and mitotic erythroblast stages of the erythroblastic series and of total erythropoietic cells (ECs), and increased ratio of granulocytes at the myeloblast, eosinophilic metamyelocyte, segmented neutrophil, and eosinophil stages of granulocytic series and of total granulopoietic cells

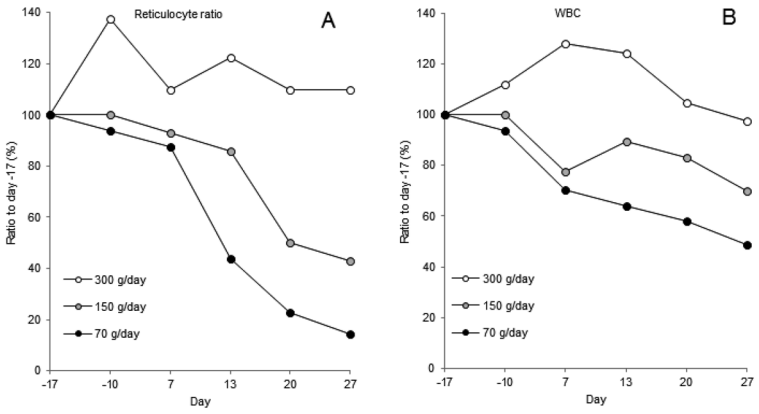

Fig. 5. Time courses of reticulocyte ratio and WBC. A: Time course of the mean values of the reticulocyte ratio in each group. B: Time course of the mean values of the WBC in each group.

(GCs) and monocytes. These changes led to an increased ratio of total GCs to total ECs.

Gross pathology

The thymus was found to be small in 1 animal in the 150 g/day group and both animals in the 70 g/day group. One animal in 70 g/day also exhibited a white focus of the mucosa in the stomach.

Organ weight

All organ weight data are shown in Table 4. Organ weight changes were evaluated by comparing the feed-restricted group values with the control group values or control background data with 300 g/day feeding. All animals in the feed-restricted groups exhibited a decrease or tendency to decrease in the absolute and relative thymus weights and absolute liver and kidney weights and an increase in relative brain and lung weights. One animal in the 150 g/day group and both animals in the 70 g/day group exhibited decrease in absolute and/or relative heart weight. One animal in each of the 150 g/day and 70 g/day groups also exhibited a decrease in absolute spleen weight. Only the 70 g/day group exhibited an increase in relative pituitary weight and absolute and relative adrenal weight and a decrease in absolute and relative testis weights and absolute prostate weight.

Histopathology

No abnormal changes were noted in the 300 g/day group. In the 150 g/day group, hypocellularity and an increase in the mature granulocyte ratio in the sternal and femoral bone marrow (Figs. 7B and 7E), as well as atrophy of the thymus and testicular seminiferous tubules, were observed in 1 animal. In the 70 g/day group, an increase in the mature granulocyte ratio in the sternal and femoral bone marrow and atrophy of the thymus were observed in both animals. Hypocellularity in the sternal and femoral bone marrow (Figs. 7C and 7F) and atrophy of the seminiferous tubules were observed in 1 animal, and regeneration was observed in the mucosa in the stomach body corresponding to a gross lesion (white focus in the mucosa) in another animal (Fig. 8). In the two animals with hypocellularity in the bone marrow, no necrotic picture was observed in the bone marrow (Fig. 7). An atrophic change was noted in the seminiferous tubules in the 70 g/day group (Supplementary Fig. 1). Atrophic seminiferous tubules with a reduced number of germinal cells were randomly distributed in testicular cross sections. No degenerative or necrotic cells were observed in the atrophic tubules, and many other seminiferous tubules looked intact. The magnitude of the testicular change was the same as that in the 150 g/day group, and a similar change was occasionally noted in the control background data.

Discussion

We examined the toxicological parameters of beagle dogs with a 4 weeks of feed restriction at 150 or 70 g/animal/day versus a standard diet of 300 g/animal/day. All animals ate all the supplied feed, indicating that the experimental condition for evaluation of the feed-re-

Table 2. Individual and mean blood chemical parameter values at days -10 and 27

Parameters	Day	300 g/day			150 g/day			70 g/day		
		No. 1	No. 2	Mean	No. 3	No. 4	Mean	No. 5	No. 6	Mean
ALAT	-10	30	34	32	32	38	35	53	44	49
(IU/l)	27	28	38	33	32	(↑) 52	42	↑139	30	85
ALP	-10	404	351	378	438	523	481	271	394	333
(IU/l)	27	321	289	305	(↓) 296	(↓) 294	295	↓161	↓231	196
G-GTP	-10	3	2	3	2	3	3	1	3	2
(IU/l)	27	5	5	5	(↑) 6	(↑) 7	7	↑8	↑10	9
Amylase	-10	649	624	637	1,440	666	1,053	839	1018	929
(IU/l)	27	704	643	674	(↓) 1,323	↓511	917	(↓) 751	↓707	729
Total bilirubin	-10	0.07	0.03	0.05	0.05	0.04	0.05	0.05	0.05	0.05
(mg/dl)	27	0.06	0.02	0.04	0.06	0.04	0.05	↑0.10	0.06	0.08
Direct bilirubin	-10	0.03	0.02	0.03	0.03	0.02	0.03	0.02	0.02	0.02
(mg/dl)	27	0.02	0.01	0.02	0.02	0.02	0.02	(↑) 0.04	0.03	0.04
Indirect bilirubin	-10	0.04	0.01	0.03	0.02	0.02	0.02	0.03	0.03	0.03
(mg/dl)	27	0.04	0.01	0.03	0.04	0.02	0.03	↑ 0.06	0.03	0.05
Total protein	-10	5.0	5.2	5.1	5.0	5.0	5.0	5.5	4.9	5.2
(g/dl)	27	5.1	5.1	5.1	5.1	↓4.5	4.8	5.2	5.0	5.1
Albumin	-10	3.0	3.1	3.1	3.0	3.1	3.1	3.2	3.1	3.2
(g/dl)	27	3.0	3.0	3.0	3.0	↓2.8	2.9	3.3	3.1	3.2
Globulin	-10	2.0	2.1	2.1	2.0	1.9	2.0	2.3	1.8	2.1
(g/dl)	27	2.1	2.1	2.1	2.1	(↓) 1.7	1.9	1.9	1.9	1.9
Phospholipid	-10	322	291	307	278	256	267	363	293	328
(mg/dl)	27	311	264	288	(↓) 256	↓215	236	↓335	(↓) 272	304
Glucose	-10	108	102	105	96	105	101	105	103	104
(mg/dl)	27	105	97	101	97	↓91	94	99	↓87	93
BUN	-10	12.0	12.9	12.5	13.9	10.5	12.2	11.8	13.6	12.7
(mg/dl)	27	13.5	12.8	13.2	10.6	↑16.5	13.6	↑16.1	↑17.8	17.0
Creatinine	-10	0.57	0.59	0.58	0.62	0.63	0.63	0.7	0.66	0.68
(mg/dl)	27	0.67	0.59	0.63	↑0.75	(↑) 0.70	0.73	↑0.96	(↑) 0.73	0.85
IP	-10	7.13	6.62	6.88	6.56	6.18	6.37	5.77	7.17	6.47
(mg/dl)	27	7.04	6.05	6.55	↓5.70	↓4.96	5.33	↓5.13	↓5.50	5.32
Ca	-10	10.4	10.9	10.7	10.4	10.5	10.5	10.8	10.5	10.7
(mg/dl)	27	10.4	10.6	10.5	10.3	↓9.8	10.1	10.3	↓9.8	10.1
Cl	-10	112	114	113	115	117	116	112	114	113
(mEq/l)	27	114	115	115	(↑) 117	↑120	119	(↑) 116	↑118	117
Total bile acid	-10	2.5	2.1	2.3	BLOQ	2.3	1.2	2.1	2.1	2.1
(μmol/l)	27	BLOQ	BLOQ	0.0	BLOQ	↑6.7	3.4	BLOQ	↑10.0	5.0

Symbols:↑, increase; ↓, decrease; (↑), tended to increase; (↓), tended to decrease (evaluated only for individual data). Criteria were calculated based on the range of variance (%) in the acclimation period or control group obtained for each animal, and the highest individual variation (%) adopted as the criterion after rounding (except for ALP, G-GTP, total bilirubin, indirect bilirubin, albumin, globulin, Ca, Cl, and total bile acid).Range of variance in amylase, total protein, total protein, phospholipid, glucose, and IP: (lowest value) / (highest value) × 100. Range of variance in ALAT, A/G, BUN, and creatinine: (highest value) / (lowest value) × 100. Individual criteria were calculated using the following equation: [value on day −17 or −10] $^{*}\times$ [range of variance (%)]. *Criteria <100%: from the highest value on day −17 or −10. Criteria >100%: from the lowest value on day −17 or −10. ALP (<271 IU/l), G-GTP (>7 IU/l), total bilirubin (>0.08 mg/dl), indirect bilirubin (>0.05 mg/dl), albumin (<2.9 g/dl), globulin (−0.3 g/dl: from the lowest value on day −17 or −10), Ca (−0.4 mg/dl: from the lowest value on day −17 or −10), Cl (>117 mEq/l), total bile acid (+0.4 μmol/l: from the highest value on day −17 or −10). BLOQ: below the limit of quantification.

stricted effects were established. Decreases in body weight and water consumption that corresponded to the levels of feed restriction were noted.

The most notable finding in the present study was the effect on the hematopoietic system. Changes in a number of parameters were noted in hematology and bone marrow examinations, especially in the erythroid ratio, which was decreased. The decreased erythroid ratio was reflected in histopathology as an increase in the mature granulocyte ratio in the sternal and femoral bone marrow. In addition, a decrease in the numbers of nucleated bone marrow cells was noted. One animal in each feed-restricted group exhibited hypocellularity of the bone marrow in histopathology. With regard to effects on leukocytes, decreases in the numbers of neutrophils, eosinophils, basophils, and monocytes were noted in

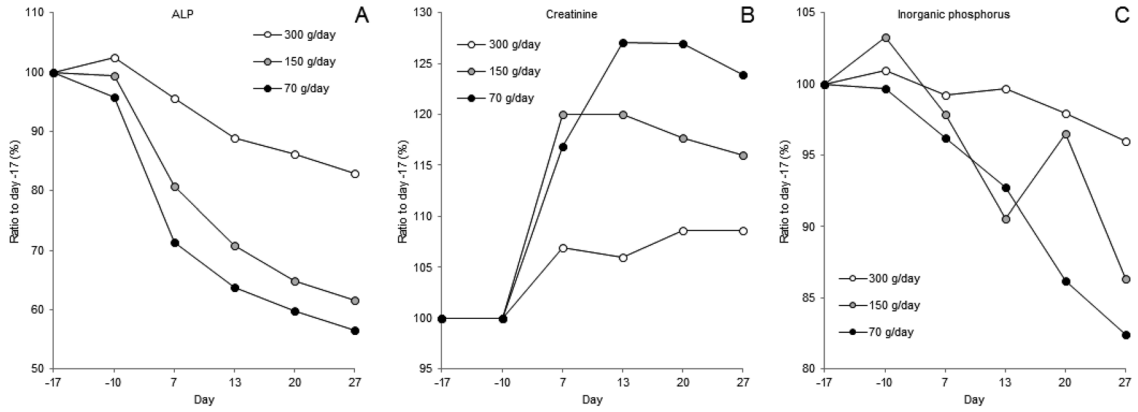

Fig. 6. Time courses of ALP, creatinine, and inorganic phosphorus in serum. A: Time course of the mean values of ALP in each group. B: Time course of the mean values of creatinine in each group. C: Time course of the mean values of inorganic phosphorus in each group.

Table 3. Individual and mean parameter values in bone marrow examination

Parameters	300 g/day			150 g/day			70 g/day		
	No. 1	No. 2	Mean	No. 3	No. 4	Mean	No. 5	No. 6	Mean
Nucleated cell count (10^4/mm^3)	126	150	138	152	↓65	109	↓82	↓86	84
Myelogram (%)									
Proerythroblasts	0.4	0.4	0.4	↓0.2	↓0.0	0.1	0.4	↓0.0	0.2
Basophilic erythroblasts	2.4	1.7	2.1	1.6	↓0.2	0.9	1.6	↓0.6	1.1
Polychromatic erythroblasts	38.6	38.9	38.8	↓26.3	↓14.6	20.5	↓13.7	↓13.0	13.4
Mitotic erythroblasts	0.8	1.0	0.9	↓0.0	↓0.4	0.2	↓0.0	↓0.6	0.3
Total erythropoietic cells (ECs)	42.4	42.2	42.3	↓28.1	↓15.2	21.7	↓15.9	↓14.4	15.2
Myeloblasts	0.0	0.0	0.0	0.0	0.0	0.0	↑0.2	0.0	0.1
Eosinophilic metamyelocytes	0.8	0.4	0.6	↑1.3	↑3.6	2.5	↑1.2	0.6	0.9
Segmented neutrophils	7.2	3.5	5.4	↑10.3	↑10.2	10.3	↑15.3	↑12.4	13.9
Eosinophils	1.6	0.8	1.2	↑2.5	↑10.6	6.6	↑4.0	↑2.1	3.1
Total granulopoietic cells (GCs)	44.4	49.4	46.9	↑62.9	↑70.9	66.9	↑70.3	↑57.4	63.9
Monocytes	0.6	0.2	0.4	↑0.9	↑0.8	0.9	↑1.0	↑1.2	1.1
Total GCs/Total ECs	1.05	1.17	1.11	↑2.24	↑4.66	3.45	↑4.42	↑3.99	4.21

Symbols: ↑, increase; ↓, decrease (evaluated only for individual data).

these groups. These results suggest that feed restriction affected all types of hematopoietic cells, particularly erythroid cells. Effects were also observed in the lymphoid system accompanied by thymic atrophy and a decreased lymphocyte count. Myelosuppression and thymic atrophy have been reported in feed-restricted rats by Levin *et al.* [13]. Morita *et al.* [18] reported decreased leukocytic parameters in feed-restricted dogs. These findings indicate that the effects on the hematopoietic and lymphocytic systems are reproducible and common events across different species.

Other changes noted in the feed-restricted groups were a decreased urinary volume and urinary potassium excretion, which were considered to be associated with decreased water consumption. Regarding cardiovascular events, feed-restricted animals exhibited a decreased heart rate. Some animals exhibited a prolongation of the PR interval and tendency towards prolongation of QRS that were associated with decreased heart rate. Bradycardia might be related to possible depression of cardiac function in response to malnutrition [1, 2]. Morita *et al*. [16] also reported a decreased heart rate in feed-restricted beagle dogs and considered the cause to be a decreased circulating blood volume. The decreased water consumption of the feed-restricted dogs in our examination is also consistent with the cause suggested by Morita *et al*. Regarding other cardiac parameters, slight prolongation of the mean QTc in the feed-restricted groups was noted during the feed restriction period. In individual analysis, 1 animal in each feed-restricted

Table 4. Individual and mean organ weights in each animal

Organ (Uppervalue, Absolute weight; lower value, relative weight to body weght)	300 g/day			150 g/day			70 g/day		
	No. 1	No. 2	Mean	No. 3	No. 4	Mean	No. 5	No. 6	Mean
Pituitary (mg)	62	73	68	64	58	61	64	61	63
(mg/kg)	5.5	6.8	6.2	6.7	6.6	6.7	↑8.5	↑7.4	8.0
Thyroid gland (bilateral weight, g)	0.98	0.96	0.97	1.02	0.67	0.85	0.70	0.90	0.80
(g/kg)	0.087	0.089	0.088	0.107	0.076	0.092	0.093	0.110	0.102
Adrenal (bilateral weight, g)	0.9	0.88	0.89	0.89	0.76	0.83	0.82	↑1.16	0.99
(g/kg)	0.080	0.081	0.081	0.094	0.086	0.090	↑0.109	↑0.141	0.125
Testis (bilateral weight, g)	15.0	18.4	16.7	14.1	14.9	14.5	↓11.2	↓9.6	10.4
(g/kg)	1.33	1.70	1.52	1.48	1.69	1.59	1.49	↓1.17	1.33
Pancreas (g)	21.8	30.4	26.1	20.4	21.5	21.0	16.7	17.4	17.1
(g/kg)	1.93	2.81	2.37	2.15	2.44	2.30	2.23	2.12	2.18
Thymus (g)	16.7	19.0	17.9	↓11.7	↓3.2	7.5	↓2.2	↓2.2	2.2
(g/kg)	1.48	1.76	1.62	↓1.23	↓0.36	0.80	↓0.29	↓0.27	0.28
Submandibular gland (bilateral weight, g)	9.6	10.2	9.9	9.7	9.1	9.4	8.9	7.6	8.3
(g/kg)	0.85	0.94	0.90	1.02	1.03	1.03	1.19	0.93	1.06
Spleen (g)	26.7	24.1	25.4	30.5	↓15.9	23.2	↓15.4	20.3	17.9
(g/kg)	2.36	2.23	2.30	3.21	1.81	2.51	2.05	2.48	2.27
Brain (g)	79.8	79.9	79.9	82.4	87.5	85.0	75.8	83.1	79.5
(g/kg)	7.06	7.4	7.23	(↑) 8.67	↑9.94	9.31	↑10.11	↑10.13	10.12
Heart (g)	72.3	77.0	74.7	74.6	↓68.1	71.4	↓68.4	↓63.6	66.0
(g/kg)	6.40	7.13	6.77	7.85	7.74	7.80	↑9.12	7.76	8.44
Lung (g)	78.7	75.9	77.3	80.3	79.7	80.0	↓63.2	↓70.6	66.9
(g/kg)	6.96	7.03	7.00	↑8.45	↑9.06	8.76	↑8.43	↑8.61	8.52
Liver (g)	279.6	276.9	278.3	↓221.4	↓207.4	214.4	↓170.4	↓188.5	179.5
(g/kg)	24.74	25.64	25.19	23.31	23.57	23.44	22.72	22.99	22.86
Kidney (bilateral weight, g)	44.0	43.7	43.9	(↓) 39.7	(↓) 39.3	39.5	↓31.8	↓33.7	32.8
(g/kg)	3.89	4.05	3.97	4.18	4.47	4.33	4.24	4.11	4.18
Prostate (g)	3.5	2.6	3.1	3.1	2.7	2.9	2.6	↓1.8	2.2
(g/kg)	0.31	0.24	0.28	0.33	0.31	0.32	0.35	0.22	0.29

Symbols: ↑, increase; ↓, decrease; (↑), tended to increase; (↓), tended to decrease (evaluated only for individual data).

group exhibited apparent QTc prolongation. Although the mechanism of QTc prolongation in the feed-restricted dogs was unclear, this finding suggested that animals with decreased food consumption might exhibit QTc prolongation. To our knowledge, this is the first report of QTc prolongation in feed-restricted dogs.

The slight increases in serum creatinine and BUN levels in all and some feed-restricted animals, respectively, were considered to be due to a decreased glomerular filtration rate (GFR) associated with a decreased renal blood flow and circulating blood volume. Taking into consideration the small fluctuation ranges of the serum creatinine and BUN levels, the inhibitory effect on GFR caused by feed restriction was considered to be not so significant. While the serum inorganic phosphate (IP) concentration is expected to increase when GFR decreases, all feed-restricted animals exhibited a decrease in the serum IP concentration in our examination. The reduction in phosphorus intake associated with feed restriction is therefore considered to lead to a subsequent decrease in serum IP concentration. Another potential cause of the increase in BUN might be catabolism of protein, which is supported by animal No. 4 which showed a decrease in serum total protein and albumin levels with the largest increase in BUN, and in animal No.5 which showed an increase in serum ALAT levels, which is a parameter of protein catabolism.

Changes in organ weight were noted in the pituitary, adrenal, spleen, brain, heart, lung, liver, kidney, and prostate without accompanying histopathological changes, except for decreased weights of the thymus and testis, reflecting atrophic changes in histopathology. Trieb *et al*. [23] described that body weight (x) and absolute organ weight (y) have an equality of $y=ax^b$, where a is the integration constant and b is the ratio of the specific growth rates for the organ and species. When b equals 1, this indicates an isometric increase with constant proportionality for body weight and organ weight. When b is greater than 1, this indicates a positive allometric increase in organ weight compared with body weight.

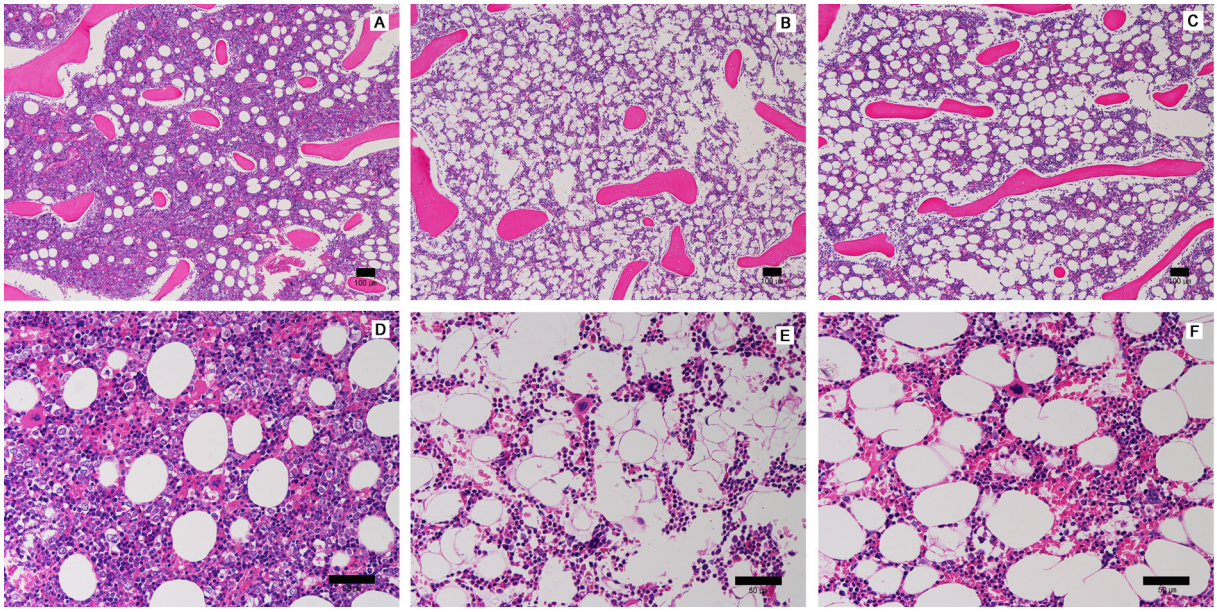

Fig. 7. Histological section of the sternal bone marrow. Scale bars: A to C, 100 μm; D to F, 50 μm. A: 300 g/day. B: 150 g/day. C: 70 g/day. D: 300 g/day, magnified image. E: 150 g/day, magnified image. F: 70 g/day, magnified image. Hypocellularities are observed in B and C, and hypocellularity and an increase in the mature granulocyte ratio are observed in E and F.

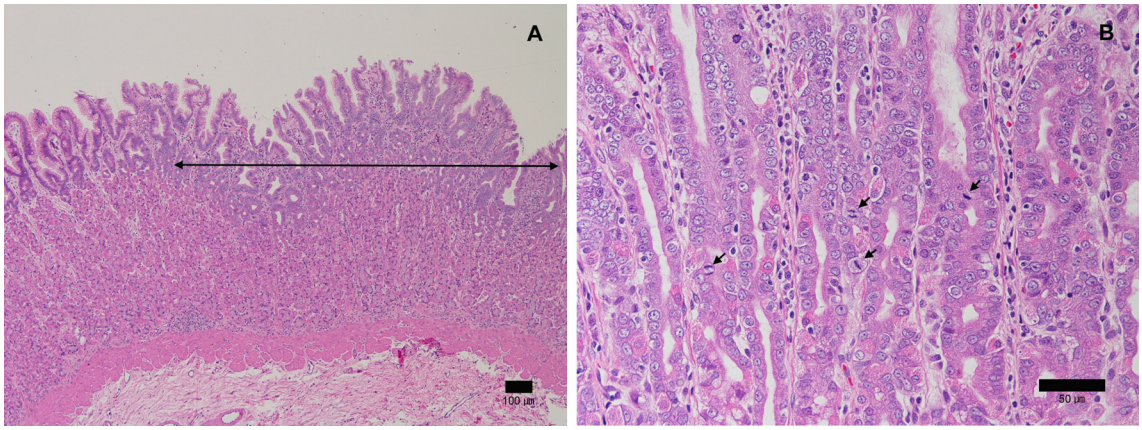

Fig. 8. Histological section of the stomach at 70 g/day. Scale bars: A, 100 μm; B, 50 μm. A: A regenerative mucosa can be seen in the right half area of the picture. B: Regenerative mucosa, magnified image. The cytoplasm of the epithelium shows basophilia. Mitotic figures can be seen (arrows).

Negative allometry or slower organ growth results if *b* is less than 1 [23]. The organs that decreased in weight in the present study had relatively large values for *b* [14]. That is, the weights of the organs with a large rate of increase associated with body weight gain decreased along with body weight. These data indicate the importance of carefully assessing organ weight in toxicity studies referring to *b* for weight change secondary to body weight change or as a direct effect of the test articles. In some organs, the weights of relative organs increased, indicating that the weight changes of these organs did not follow the rapid decrease in body weight.

In histopathology, atrophic changes in the bone marrow and thymus and regeneration of the gastric mucosa were noted. Regeneration of the gastric mucosa is considered to be a repair process for mucosal injury, which might be induced by stress through corticosteroid release [13]. Similar findings were also observed in rats by Levin *et al*. [13] and considered a common response in different species. Testicular changes, such as degeneration of

the seminiferous epithelium or reduced number of the spermatids, were reported as the effects of feed restriction in rats [13]. Atrophy of the seminiferous tubules was also observed in the present study; a similar change is occasionally noted as a spontaneous change [10] probably due to endocrinological imbalance. The precise mechanism of testicular atrophy caused by feed restriction is unclear.

In summary, the principal responses in feed-restricted dogs with decreased body weight and water consumption were suppressed hematopoiesis and lymphopoiesis, which are common in feed-restricted rats. The stomach mucosa and cardiovascular system were also targets: the dogs showed a decreased heart rate accompanied by prolongation of the PR interval and QTc. These results provide important information for assessing responses of beagle dogs with decreased food consumption in toxicity studies.

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References

1. Abel, R.M., Grimes, J.B., Alonso, D., Alonso, M., and Gay, W.A. Jr. 1979. Adverse hemodynamic and ultrastructural changes in dog hearts subjected to protein-calorie malnutrition. *Am. Heart J.* 97: 733–744. [Medline] [CrossRef]
2. Alden, P.B., Madoff, R.D., Stahl, T.J., Lakatua, D.J., Ring, W.S., and Cerra, F.B. 1987. Left ventricular function in malnutrition. *Am. J. Physiol.* 253: H380–H387. [Medline]
3. Alt, H.L. 1938. The relation of growth and nutrition to the reticulocyte level in the young rat. *J. Nutr.* 16: 597–602.
4. Aschkenasy, A. 1957. On the pathogenesis of anemias and leukopenias induced by dietary protein deficiency. *Am. J. Clin. Nutr.* 5: 14–25. [Medline]
5. Bethard, W.F., Wissler, R.W., Thompson, J.S., Schroeder, M.A., and Robson, M.J. 1958. The effect of acute protein deprivation upon erythropoiesis in rats. *Blood* 13: 216–225. [Medline]
6. Borelli, P., Mariano, M., and Borojevic, R. 1995. Protein malnutrition: Effect on myeloid cell production and mobilization into inflammatory reactions in mice. *Nutr. Res.* 15: 1477–1485. [CrossRef]
7. Brown, J.W. 1954. A quantitative study of cellular changes occurring in bone marrow following protein deficiency in the rat. *Anat. Rec.* 120: 515–532. [Medline] [CrossRef]
8. Fried, W., Barone, S.J., Anagnostou, A., and Anagnostou, A. 1978. Effect of protein deprivation on hematopoietic stem cells and on peripheral blood counts. *J. Lab. Clin. Med.* 92: 303–310. [Medline]
9. Fruhman, G.J. and Gordon, A.S. 1955. Influence of starvation upon the formed elements of blood and bone marrow of the rat. *Anat. Rec.* 122: 492.
10. Goedken, M.J., Kerlin, R.L., and Morton, D. 2008. Spontaneous and age-related testicular findings in beagle dogs. *Toxicol. Pathol.* 36: 465–471. [Medline] [CrossRef]
11. Hill, R.C., Lewis, D.D., Randell, S.C., Scott, K.C., Omori, M., Sundstrom, D.A., Jones, G.L., Speakman, J.R., and Butterwick, R.F. 2005. Effect of mild restriction of food intake on the speed of racing Greyhounds. *Am. J. Vet. Res.* 66: 1065–1070. [Medline] [CrossRef]
12. Lawler, D.F., Ballam, J.M., Meadows, R., Larson, B.T., Li, Q., Stowe, H.D., and Kealy, R.D. 2007. Influence of lifetime food restriction on physiological variables in Labrador retriever dogs. *Exp. Gerontol.* 42: 204–214. [Medline] [CrossRef]
13. Levin, S., Semler, D., and Ruben, Z. 1993. Effects of two weeks of feed restriction on some common toxicologic parameters in Sprague-Dawley rats. *Toxicol. Pathol.* 21: 1–14. [Medline] [CrossRef]
14. Lützen, L., Trieb, G., and Pappritz, G. 1976. Allometric analysis of organ weights: II. Beagle dogs. *Toxicol. Appl. Pharmacol.* 35: 543–551. [Medline] [CrossRef]
15. Meierhenry, E.F. 1990. Literature review – The effects of inanition on rat bone marrow. *Toxicol. Pathol.* 18: 707–708.
16. Morita, J., Izumi, S., Sunouchi, S., Tsutsumi, S., Ohno, R., Arima, K., and Sato, Y. 2012. Effect of body weight loss on electrocardiography and blood parameters in dogs under reduced feeding conditions. *J. Toxicol. Sci.* 37:(Supplement I): S250.
17. Ogawa, Y., Matsumoto, K., Kamata, E., Ikeda, Y., and Kaneko, T. 1985. Effect of feed restriction on the peripheral blood and bone marrow cell counts of Wistar rats. *Jikken Dobutsu* 34: 407–416. [Medline]
18. Oishi, S., Oishi, H., and Hiraga, K. 1979. The effect of food restriction for 4 weeks on common toxicity parameters in male rats. *Toxicol. Appl. Pharmacol.* 47: 15–22. [Medline] [CrossRef]
19. Reissmann, K.R., Dietrich, M.R., and Kennedy, M.J. 1964. Protein metabolism and erytropoiesis. II. Erythropietin formation and erythroid responsiveness in protein-deprived rats. *Blood* 23: 146–153. [Medline]
20. Seki, M., Yamaguchi, K., Marumo, H., and Imai, K. 1997. Effects of food restriction on reproductive and toxicological parameters in rats—in search of suitable feeding regimen in long-term tests. *J. Toxicol. Sci.* 22: 427–437. [Medline] [CrossRef]
21. Travlos, G.S. 2006a. Normal structure, function, and histology of the bone marrow. *Toxicol. Pathol.* 34: 548–565. [Medline] [CrossRef]
22. Travlos, G.S. 2006b. Histopathology of bone marrow. *Toxicol. Pathol.* 34: 566–598. [Medline] [CrossRef]
23. Trieb, G., Pappritz, G., and Lützen, L. 1976. Allometric analysis of organ weights. I. Rats. *Toxicol. Appl. Pharmacol.* 35: 531–542. [Medline] [CrossRef]