

Original

Importance of Starting Age for Myelotoxicity Study in Dietary Restricted Rats

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Abstract: The aim of this study was to prove our hypothesis that adult rats with lowering of body weight gain, rats at 12 weeks of age as an example, are suitable for evaluation of myelotoxicity. Age-related differences between young rats (6-week-old study) and adult rats (12-week-old study) were analyzed in hematological examination values. The data of the young rats were reprinted from our previous report (Miyata et al., 2009) since our hypothesis was verified by comparison with that previous report. Several experimental groups were defined for the 12-week-old study as well as for the 6-week-old study; these included 5-fluorouracil (5-FU) treated groups receiving 12, 15 and 18 mg/kg/day (FU12, FU15 and FU18), pair-feeding groups (R12, R15 and R18 receiving the same amount of food as in the FU12, FU15 and FU18 groups, respectively) and a nontreated control group. Numerous hematologic and bone marrow parameters in the 5-FU treated groups were comparable to those in the corresponding pair-feeding groups in both age studies. Generally, the influences of undernutrition were more apparent in the young rats than in the adult rats. Histopathological examinations showed a decrease in hematopoiesis in the bone marrow in the 5-FU treated and pair-feeding groups. No apparent differences were observed in the decreased hematopoiesis between the 5-FU treated and pair-feeding groups in the 6-week-old study, but a difference between these groups was noted in the 12-week-old study; decreased hematopoiesis was more frequently noted in the 5-FU treated groups. These facts suggest that adult rats are more suitable than young rats for evaluation of 5-FU-induced myelotoxicity. (*J Toxicol Pathol* 2009; 22: 153–166)

Key words: myelotoxicity, age-related differences, dietary restriction, 5-fluorouracil, pair-feeding, rat

Introduction

A short-term repeated dose toxicity study conducted for 14 days is often used to predict the appropriate doses of test articles in subsequent long-term studies or to search for target organs of toxicity. The rat is a widely used animal model for safety assessments of such things as pharmaceuticals. Dosing is generally initiated in animals that are approximately 6 weeks of age. Rats of this age are easy to obtain and economical. Various administration periods, such as those used in subacute, chronic and carcinogenicity studies, are necessary to assess the safety of a new drug. In carcinogenicity studies, it is recommended that dosing of the rodents should begin as soon as possible after weaning and acclimatization and preferably before the

animal reaches 6 weeks of age¹. Generally, the age of the animals used in the short-term repeated dose toxicity study is similar to that of the animals used in carcinogenicity studies. The weight of a male rat increases throughout much of its life. In particular, rapid weight gain occurs up until 10 weeks after birth². Young rats at this stage of growth are often used in the short-term repeated dose toxicity study, and suppression of body weight gain and decreased food consumption are often recognized in drug administration groups in this toxicity study. Consequently, whether hematology, bone marrow cytology or histopathological changes are caused directly by the drug or indirectly by suppression of body weight gain and decreased food consumption can be difficult to evaluate.

We previously reported that many of the influences of dietary restriction on hematological examination values were comparable to those caused by 5-fluorouracil (5-FU) administration in young rats (6 weeks of age at start of the experiment)³. In fact, undernutrition during the rapid growth stage is thought to cause changes, such as hemocytopenia, that are nearly equal to those induced by immunotoxicants. Many reports concerning hematological changes in dietary

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restricted rats⁴⁻⁹ or nontreated rats with age-related physiological data¹⁰⁻¹³ have been published. However, few reports have examined age-related hematological changes in dietary restricted rats. In this study, we set out to prove our hypothesis that adult rats with lowering of body weight gain, rats at 12 weeks of age as an example, are suitable for evaluation of myelotoxicity. Our hypothesis was verified by comparison with our previous report³.

The anti-cancer drug 5-FU belongs to a category of chemotherapy agents called antimetabolites and functions as a pyrimidine analog^{14,15}; the myelotoxic effect of 5-FU on bone marrow is a serious adverse effect associated with its clinical use¹⁶. We selected 5-FU as a positive control, since this agent is known to cause blood toxicity and bone marrow depression¹⁷⁻²⁰.

In this study, 5-FU treated and pair-feeding groups (animals were given the same amount of food as the average amount of food consumption in the 5-FU treated groups) were used for the experimental design. We compared the hematological, bone marrow cytological and histopathological results of the 5-FU treated and pair-feeding groups after a 14-day administration period and after a 7-day recovery period. Thereafter, age-related differences between the young rats (6 weeks of age at start of the experiment) and adult rats (12 weeks of age at start of the experiment) were analyzed. The data of the young rats used for the age-related differences were reprinted from our previous report³. Here, we discuss the age-related differences in hematological and/or myelotoxic effects under decreased food consumption based on examinations of general toxicity studies. This study provides useful indices for evaluating myelotoxicity when suppression of body weight gain and decreased food consumption are noted in toxicity studies. Our hypothesis that adult rats may be suitable for evaluation of myelotoxicity was verified by comparison of age-related differences.

Materials and Methods

Chemicals and dose selection rationale

The 5-FU was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and was dissolved in water for injection for dosing. All other chemicals and reagents were purchased from commercial sources.

In a previous 14-day dose-finding study involving oral administration of 10 and 30 mg/kg/day of 5-FU in 6-week-old rats, all the animals given 30 mg/kg died on Days 10 to 13; meanwhile, no abnormalities in body weight, hematology, bone marrow cytology and so on were observed in the group dosed with 10 mg/kg. In an additional 5-day dose-finding study involving oral administration of 20 and 25 mg/kg/day of 5-FU in 5- or 6-week-old rats, body weight, food consumption and the marrow nucleated cell count were remarkably decreased in the 25 mg/kg dosing group and slightly decreased in the 20 mg/kg dosing group. Therefore, dosages of 12, 15 and 18 mg/kg/day were chosen for use in this study.

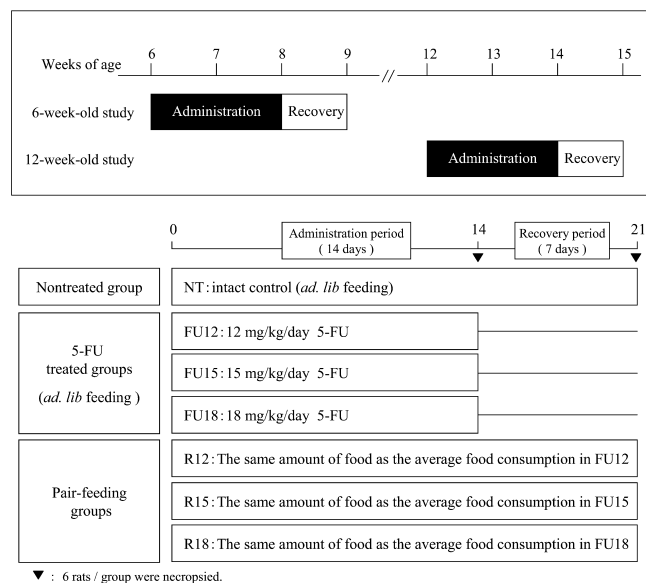


Fig. 1. Experimental design.

Animals and housing conditions

A total of 84 male Crl:CD(SD) rats were purchased from Charles River Japan Inc. (Tsukuba, Ibaraki, Japan). The animals were housed individually in stainless steel cages (W:225 mm × D:350 mm × H:200 mm) with an artificial lighting cycle of 12 hours (7:15 to 19:15), a temperature of $23 \pm 3^\circ\text{C}$, a relative humidity of $50 \pm 20\%$ and a ventilation cycle of 10 to 20 times/hour. Before group assignment, all the animals were allowed free access to a standard laboratory animal chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water. After group assignment, the R12, R15 and R18 groups described below were given restricted diets. At the start of dosing / pair feeding, the animals were 12 weeks of age.

All the animals were treated in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Taisho Pharmaceutical Co., Ltd.

Study groups

The experimental design is shown in Fig. 1. The animals were divided into the following 7 groups: NT, FU12, FU15, FU18, R12, R15 and R18. The animals used were as follows: 12 rats / group; 6 rats per group were euthanized at the end of the administration and recovery periods, respectively. The animals in the NT group were nontreated and were allowed free access to a standard laboratory animal chow; this group was used as the control group. The animals in the FU12, FU15 and FU18 groups were orally treated with 5-FU for 14 consecutive days at doses of 12, 15 and 18 mg/kg, respectively. The dose volume was set at 10 mL/kg body weight and was calculated based on the most recent body weight. The animals in the R12, R15 and R18 groups were not given 5-FU, but were given amounts of food equal to those consumed by the rats in

the FU12, FU15 and FU18 groups, respectively. If all the food was not consumed in any of the R12, R15 or R18 groups, the next portion of food was supplied without removing the food that had not been consumed. The next day of initial administration of 5-FU was set as a start date of pair-feeding. After an administration period of 14 days in the 5-FU treated groups, the recovery period was set at 7 days, which is considered appropriate to examine the reversibility of changes, and administration was withdrawn during this period.

Examinations and methods

The starting day of 5-FU administration or pair-feeding was designated as Day 0 in this study. Body weight and food consumption were measured every day during the administration and recovery periods in each animal. The ratio of the amount of limited food was calculated using the following formula: $fu / nt - 100$, where fu represents the total amount of food consumption in each 5-FU group $\times 100$ and nt represents the total amount of food consumption in the NT group. In the 5-FU treated groups, abnormal eating behavior (spilled food) was observed from Day 4 to Day 17 in many rats. Therefore, food consumption in the 5-FU treated groups was corrected using the amount of spilled food. The following examinations were performed in all the animals (excluding those that died unexpectedly) at the end of the administration period (6 rats/group) or at the end of the recovery period (6 rats/group), respectively. The animals were fasted for at least 16 hours prior to necropsy, and blood samples were collected via the abdominal aorta under ether anesthesia into dipotassium ethylenediaminetetraacetic acid (EDTA-2K) treated tubes for the hematological examination. The complete blood count (CBC) and differential white blood cell count were measured using a hematology analyzer Technicon H-1E (Bayer Medical Ltd., Tarrytown, NY, USA). The percentage of reticulocytes was measured using a flow cytometer (EPICS-XL; Beckman Coulter Inc., Fullerton, CA, USA) with Coriophosphine-O stain. After blood sampling, all the animals were euthanized by exsanguination. For the marrow cytological evaluation, the right side femur was obtained and used. The bone marrow nucleated cell count was measured using the above-mentioned hematology analyzer. The differential cell count was determined by counting 500 cells in bone marrow smears stained with May-Grünwald and Giemsa. Then, the absolute numbers of each type of marrow cell (myeloid, erythroid, lymphoid and other cells) were calculated using the data for the marrow cell number and marrow differential counts. The spleen and thymus were weighed, and the ratios of these organ weights to the body weight (relative weights) were calculated based on the final body weight. For the histopathological evaluation, the left side femur (bone marrow), liver, spleen, kidney, thymus, adrenal, stomach, duodenum, ileum and colon were fixed in 10% neutral buffered formalin. The femur was decalcified by the Plank-Rychlo method. After fixation, hematoxylin and eosin

(H&E) stained specimens were prepared and subjected to microscopic observation.

Statistical analysis

Significant differences between the NT and 5-FU treated groups or between the NT and pair-feeding groups were analyzed using the following procedure. The homogeneity of the variance among the groups was first tested using a Bartlett's test. When a homogenous variance was noted, all the groups were compared using a one-way analysis of variance. When a heterogeneous variance was noted, the Kruskal-Wallis test was subsequently performed. Finally, the Dunnett's test (if homogeneous) or Dunnett's-type multiple comparison test (if heterogeneous) was used if a significant difference was noted between the groups.

Significant differences between the 5-FU treated and pair-feeding groups (i.e., FU12 vs. R12, FU15 vs. R15, or FU18 vs. R18) were analyzed using the following procedure. The homogeneity of the variance among the groups was first tested using the F-test, and then the Student's *t*-test (if homogeneous) or Aspin-Welch's *t*-test (if heterogeneous) was performed.

The Bartlett's test, one-way analysis of variance, Kruskal-Wallis test and F-test were conducted using a significance level of 5% (two-tailed except for the F-test, which was one-tailed), while the other tests were conducted using significance levels of 1% and 5% (two-tailed). A statistical analysis of clinical observation, necropsy and histopathology results was not performed.

Analysis of age-related differences

The degree of variability (relative standard deviation: RSD) of the main inspection values in the NT group were below about 20%. The variation of each group from the NT group (relative error: RE) was calculated in this study (hereinafter referred to as 12-week-old study). RE was similarly calculated for the numerical data of our previous report³ (hereinafter referred to as 6-week-old study), and when the difference in the subtracted RE value of the 6-week-old study from the 12-week-old study was 20% or more, a significant change was assumed.

Results

Mortality and clinical signs

One rat in each of the FU12 and FU15 groups died on Day 13. A subcapsular hemorrhagic cyst in the kidney and a marked subungual hemorrhage of the forelimb were seen in the animals that died in the FU12 and FU15 groups, respectively. Alopecia was seen in one rat in each of the FU12 and FU15 groups, and loose stools were seen in a few rats in the FU18 group. Animals that died unexpectedly were not included in the assessments of hematology, bone marrow cytology and organ weight performed at the end of the administration and/or recovery periods.

No abnormalities were seen in the pair-feeding groups.

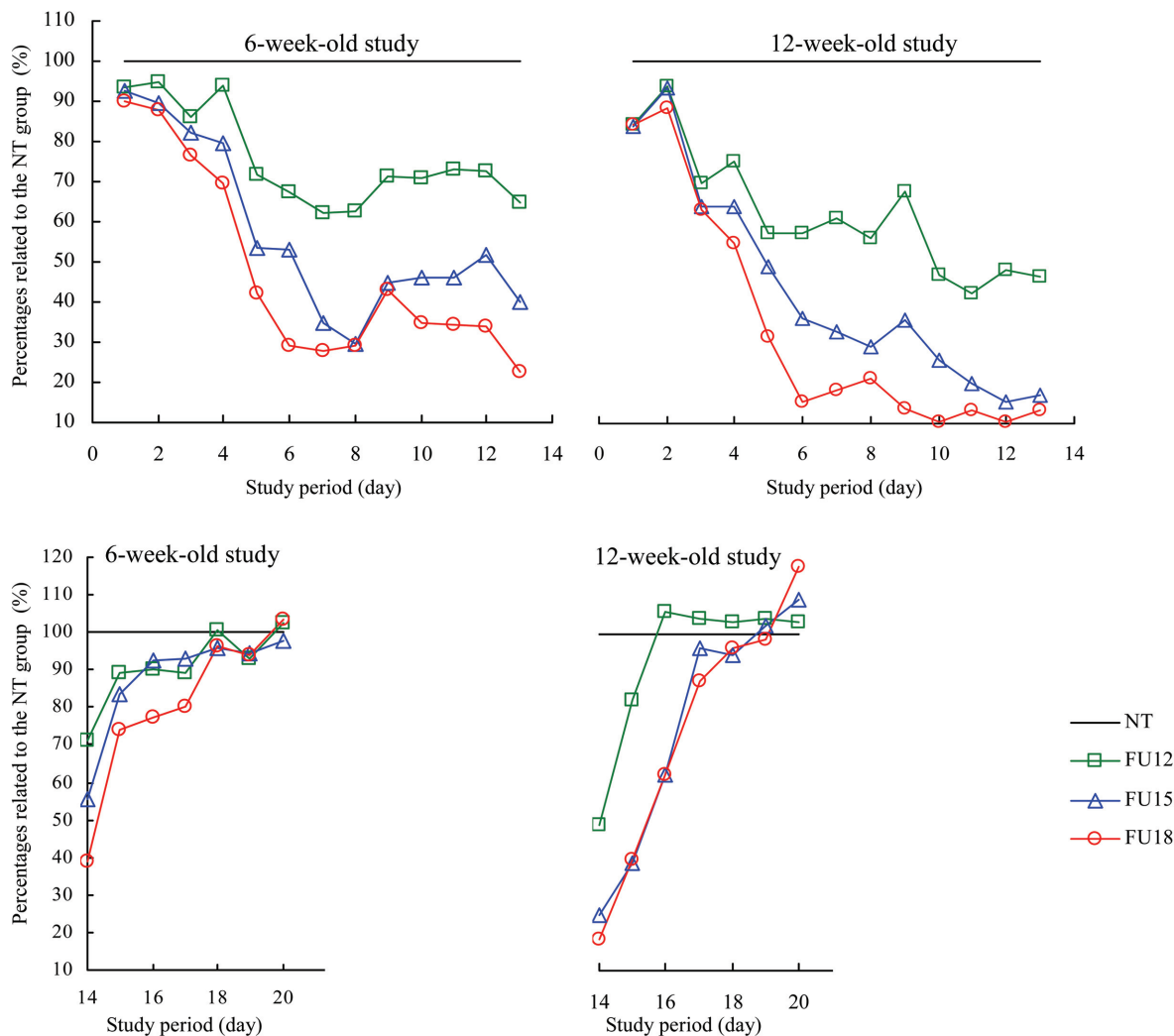


Fig. 2. Food consumption changes in the 5-FU treated groups during the administration and recovery periods. Data are expressed as percentages relative to the NT group and were calculated using the mean values for each group. The data of the 6-week-old study were reprinted from our previous report³. In the 5-FU treated groups, a decrease in food consumption was seen during the administration period in both the 6- and 12-week-old studies. The decrease in food consumption recovered during the recovery period and was immediately comparable to that in the NT group.

Food consumption (Fig. 2)

In the 5-FU treated groups, a decrease in food consumption was seen during the administration period. The decrease in food consumption recovered during the recovery period and was immediately comparable to that in the NT group. At the end of the administration period, the ratios of the total amount of food consumption in each 5-FU treated group (FU12, FU15 and FU18), compared with the NT group, were 38%, 56% and 65%, respectively. At the end of the recovery period, the ratios of the total amount of food consumption (during the administration and recovery periods) in each 5-FU treated group (FU12, FU15 and FU18), compared with the NT group, were 27%, 45% and 52%, respectively.

In the pair-feeding groups, a few rats left some food behind during the early stage of the administration period but

subsequently consumed all the available food thereafter. The food was consumed within about one hour in the pair-feeding groups.

Body weight (Fig. 3)

In the 5-FU treated and pair-feeding groups, a decrease in body weight was seen during the administration period. Thereafter, the body weight gain recovered during the recovery period. The body weights in the pair-feeding groups showed similar patterns to those in the 5-FU treated groups.

Hematology (Figs. 4 and 5)

At the end of the administration period, decreased white blood cell count was not observed in this 12-week-old study, though a decrease in the white blood cell count (with an

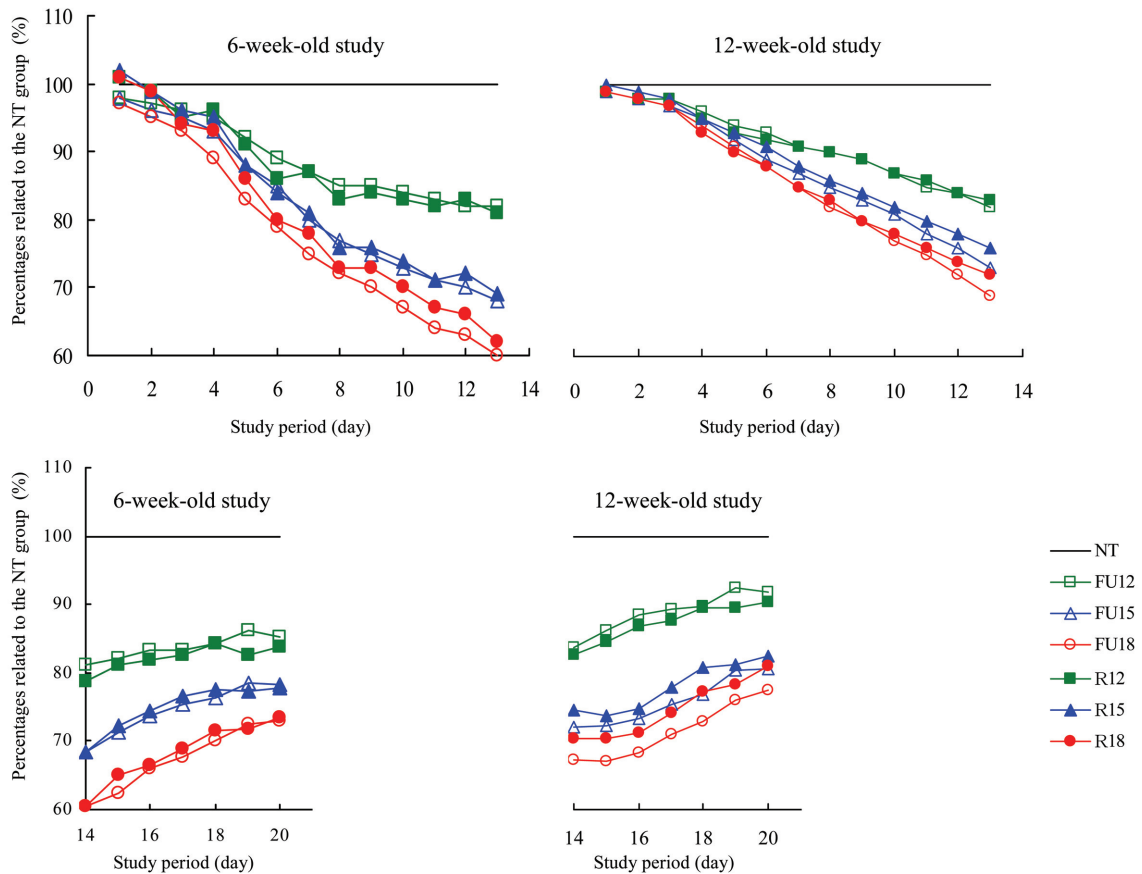


Fig. 3. Body weight changes in the 5-FU treated and pair-feeding groups during the administration and recovery periods. Data are expressed as percentages relative to the NT group and were calculated using the mean values for each group. The data of the 6-week-old study were reprinted from our previous report³. In the 5-FU treated and pair-feeding groups, a decrease in body weight was seen during the administration period in both the 6- and 12-week-old studies; thereafter, the body weight gain recovered during the recovery period. The body weights from baseline to the day before necropsy was performed at the end of the administration period in the NT group increased from 230 g to 330 g in the 6-week-old study and from 459 g to 485 g in the 12-week-old study, respectively.

unchanged differential count) was observed in the FU15, FU18 and R18 groups in the 6-week-old study³. A decrease in reticulocytes was observed in the FU12, FU15, FU18, R12, R15 and R18 groups. At the end of the recovery period, a change in reticulocytes that exceeded the normal level was observed in the FU15 and FU18 groups, though a decrease or increase in reticulocytes was not seen in the pair-feeding groups. In addition, an increase in platelets was observed in the FU12, FU15 and FU18 groups.

At the end of the administration period, an increase in hemoglobin was observed in the FU15, FU18, R12, R15 and R18 groups, but no apparent difference was observed between the 5-FU treated and pair-feeding groups. Increases in the red blood cell and hematocrit levels related to an increase in hemoglobin were seen in the pair-feeding groups. The elevated values recovered at the end of the recovery period; however, decreased hemoglobin, red blood cell and hematocrit levels were observed in the FU15 and/or FU18 groups.

Bone marrow cytology (Fig. 6)

At the end of the administration period, a decrease in myeloid cells was not observed in this 12-week-old study, though a decrease in myeloid cells was observed in the FU15, FU18 and R18 groups in the 6-week-old study³. A decrease in erythroid cells was noted in the FU18, R12, R15 and R18 groups, while a decrease in lymphoid cells was noted in the FU18 and R18 groups; no apparent differences between the 5-FU treated and pair-feeding groups were observed. A high M/E ratio was observed in the FU18, R15 and R18 groups. Abnormal granulocytes with hypersegmented nuclei and/or polyploidy nuclei (with a frequency of 1% or less) were only observed in the 5-FU treated groups (Fig. 7). At the end of the recovery period, the changes observed at the end of the administration period were reduced or had disappeared.

Organ weight (Fig. 8)

At the end of the administration period, reduction in the

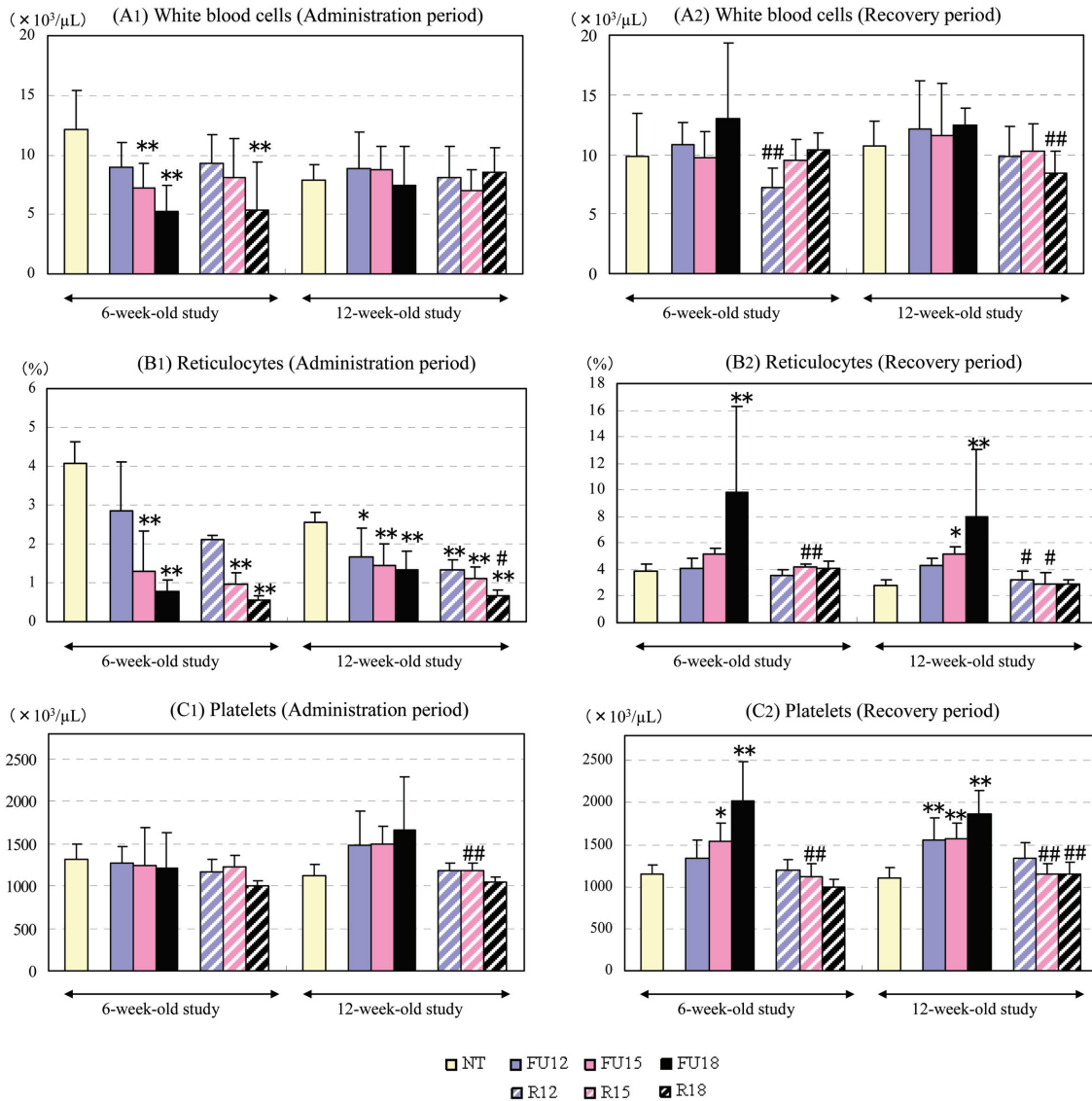


Fig. 4. Hematological changes in white blood cells (A), reticulocytes (B) and platelets (C) at the end of the administration (A1, B1 and C1) and recovery (A2, B2 and C2) periods. Data are expressed as means \pm S.D. (n=5 or 6). The data of the 6-week-old study were reprinted from our previous report³. Statistical significance was analyzed using the Dunnett’s test or Dunnett’s-type test (* p<0.05, ** p<0.01), compared with the NT group. Moreover, the differences in values between the 5-FU treated and pair-feeding groups were analyzed using a Student’s *t*-test or Aspin-Welch’s *t*-test (# p<0.05, ## p<0.01).

thymus weight was observed in the FU18 group. The thymus weights did not decrease in the pair-feeding groups in this 12-week-old study, while the reduction in the thymus weight in the FU18 group suggests the influence of 5-FU dosing. At the end of the recovery period, the changes observed at the end of the administration period were reduced or had disappeared. In addition, an increase in the spleen weight was observed in the FU18 group.

Necropsy

No abnormalities were seen in any group at the end of the administration period.

Histopathology (Figs. 9 and 10, Tables 1 and 2)

Slightly decreased hematopoiesis in the bone marrow was observed at the end of both the administration and recovery periods in the NT group.

At the end of the administration period, decreased hematopoiesis in the bone marrow that was indicative of an effect of 5-FU administration or dietary restriction was observed in the FU15, FU18 and R18 groups. This finding was persisted at the end of the recovery period. A difference between the 5-FU treated and pair-feeding groups was noted since decreased hematopoiesis was more frequently noted in the 5-FU treated groups. In addition, though slight

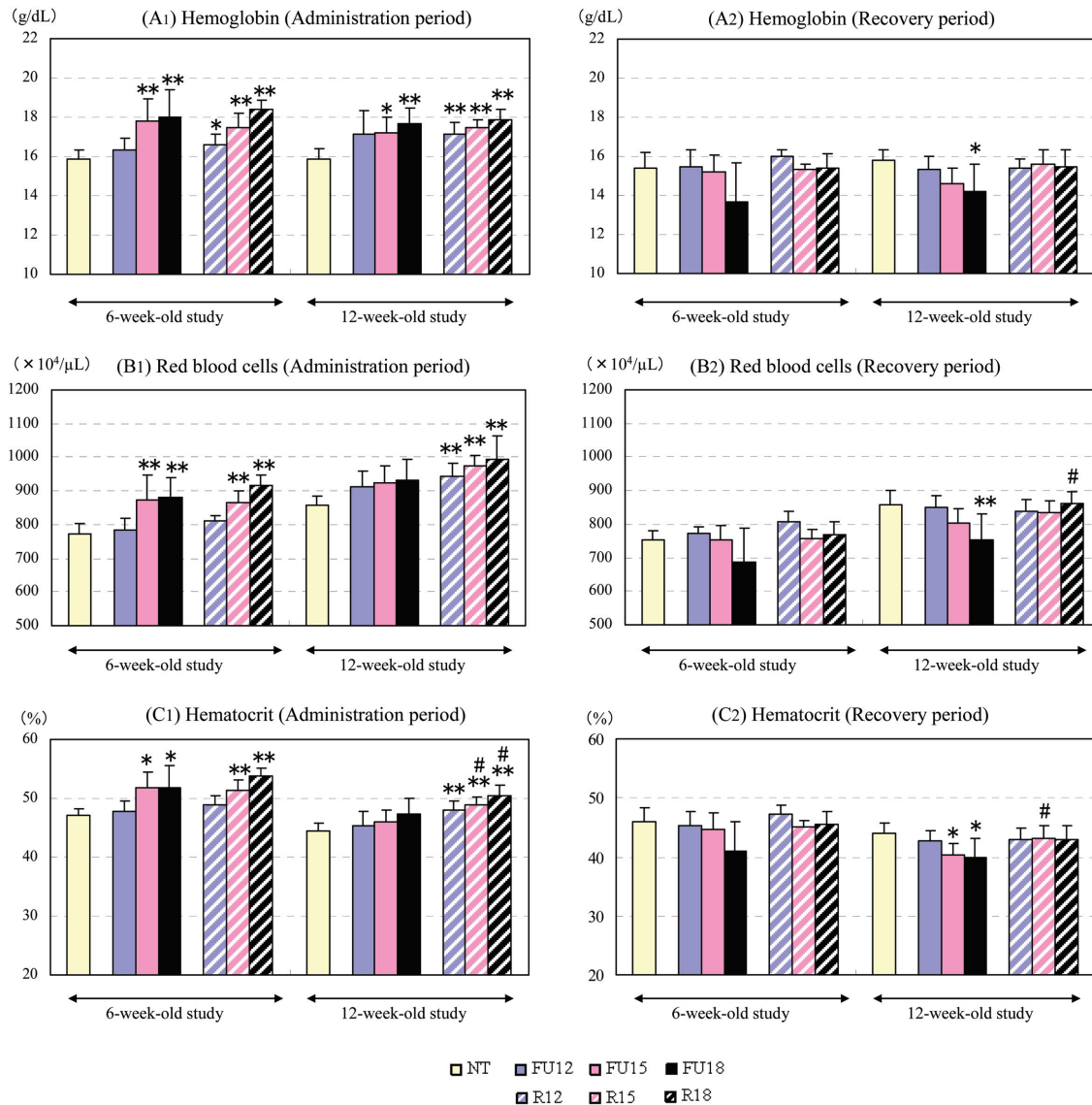


Fig. 5. Hematological changes in hemoglobin (A), red blood cells (B) and hematocrit (C) at the end of the administration (A1, B1 and C1) and recovery (A2, B2 and C2) periods. Data are expressed as means \pm S.D. ($n=5$ or 6). The data of the 6-week-old study were reprinted from our previous report³. Statistical significance was analyzed using the Dunnett's test or Dunnett's-type test (* $p<0.05$, ** $p<0.01$), compared with the NT group. Moreover, the differences in values between the 5-FU treated and pair-feeding groups were analyzed using a Student's *t*-test or Aspin-Welch's *t*-test (# $p<0.05$, ## $p<0.01$).

telangiectasis in the bone marrow was observed in the FU12, FU18 and R18 groups. The telangiectasis disappeared at the end of the recovery period. No apparent differences in telangiectasis were observed between the 5-FU treated and pair-feeding groups.

At the end of the administration period, atrophy of the thymus was observed in the 5-FU treated and pair-feeding groups (data not shown). Moreover, atrophy of the white pulp of the spleen was observed mainly in the dead animals. At the end of the recovery period, increased extramedullary hematopoiesis in the spleen was found only in the 5-FU treated groups. Nothing of note was found in other organs.

Discussion

Age-related differences between the young rats (6-week-old study) and adult rats (12-week-old study) were analyzed in the hematological examination values. The data of the young rats were reprinted from our previous report³ since our hypothesis was verified by comparison with that previous report. In the 5-FU treated groups, a decrease in food consumption was seen during the administration period in both the 6- and 12-week-old studies. The body weights in the pair-feeding groups were comparable to those in the 5-FU treated groups in both studies. The degree of decreased

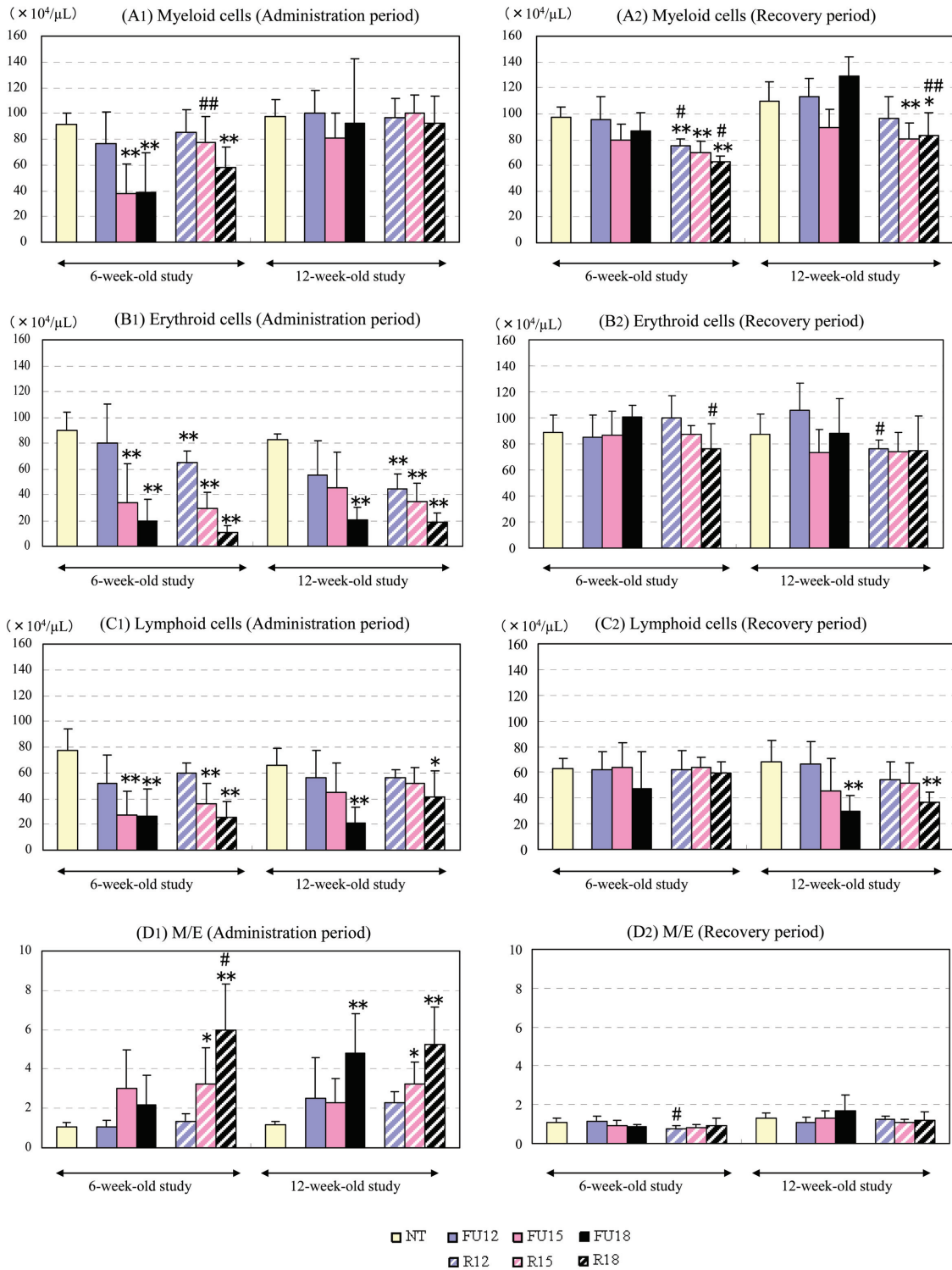


Fig. 6. Bone marrow cytological changes in myeloid cells (A), erythroid cells (B), lymphoid cells (C) and M/E ratio (D) at the end of the administration (A1, B1, C1 and D1) and recovery (A2, B2, C2 and D2) periods. Data are expressed as means \pm S.D. (n=5 or 6). The data of the 6-week-old study were reprinted from our previous report³. Statistical significance was analyzed using the Dunnett's test or Dunnett's-type test (* p<0.05, ** p<0.01), compared with the NT group. Moreover, the differences in values between the 5-FU treated and pair-feeding groups were analyzed using a Student's *t*-test or Aspin-Welch's *t*-test (# p<0.05, ## p<0.01).

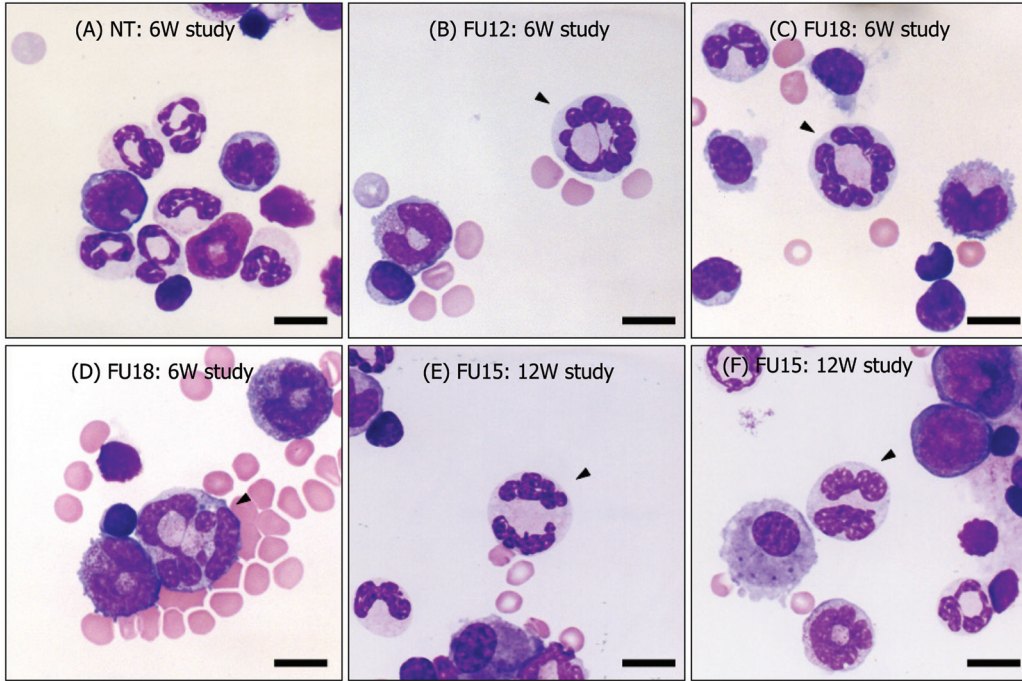


Fig. 7. Bone marrow cytology. Representative images of the NT group in the 6-week-study (A) and the 5-FU treated groups in the 6-week-old (B, C and D) and 12-week-old (E and F) studies are shown. The images of the 6-week-old study were reprinted or reshot from the slides in our previous report³. In the 5-FU treated groups at the end of the administration period, granulocytes with hypersegmented nuclei (B) or polyploidy nuclei (C, D, E and F) were observed, and the frequency in the marrow nucleated cells was 1% or less. May-Grünwald and Giemsa stain. Original magnification: $\times 1,000$. Bar = 10 μm .

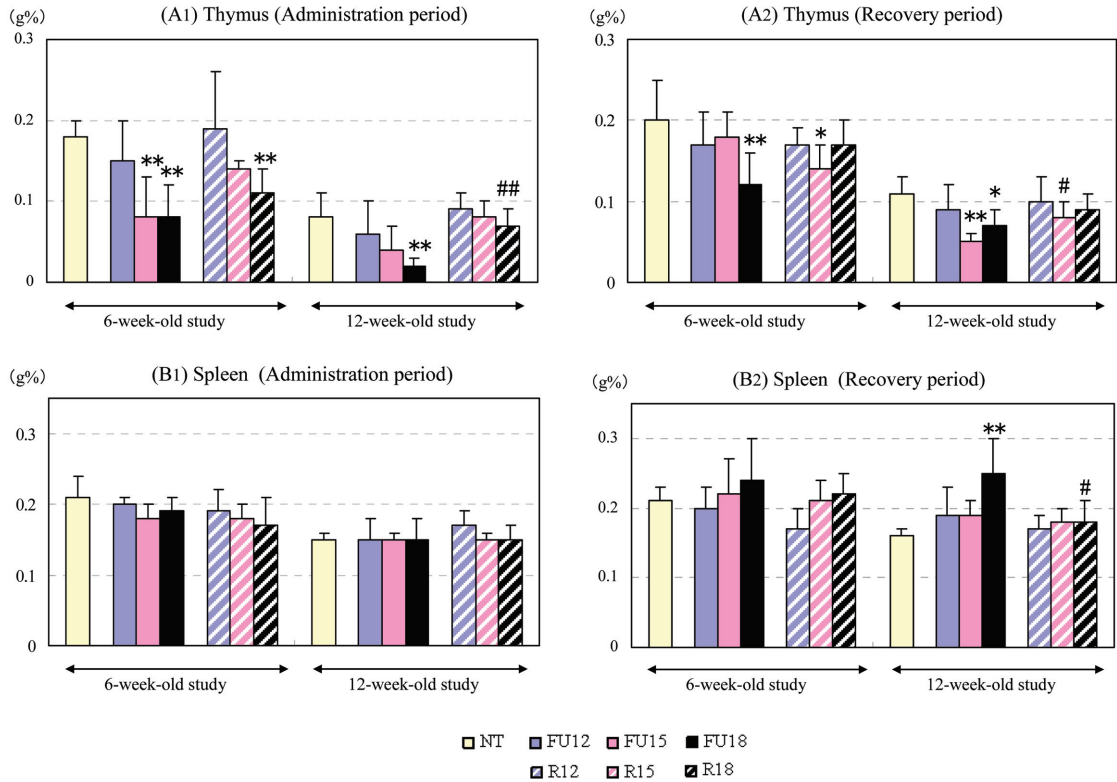


Fig. 8. Relative organ weight changes in the thymus (A) and spleen (B) at the end of the administration (A1 and B1) and recovery (A2 and B2) periods. Data are expressed as means \pm S.D. ($n=5$ or 6). The data of the 6-week-old study were reprinted from our previous report³. Statistical significance was analyzed using the Dunnett's test or Dunnett's-type test (** $p<0.01$), compared with the NT group. Moreover, the differences in values between the 5-FU treated and pair-feeding groups were analyzed using a Student's *t*-test or Aspin-Welch's *t*-test (## $p<0.01$).

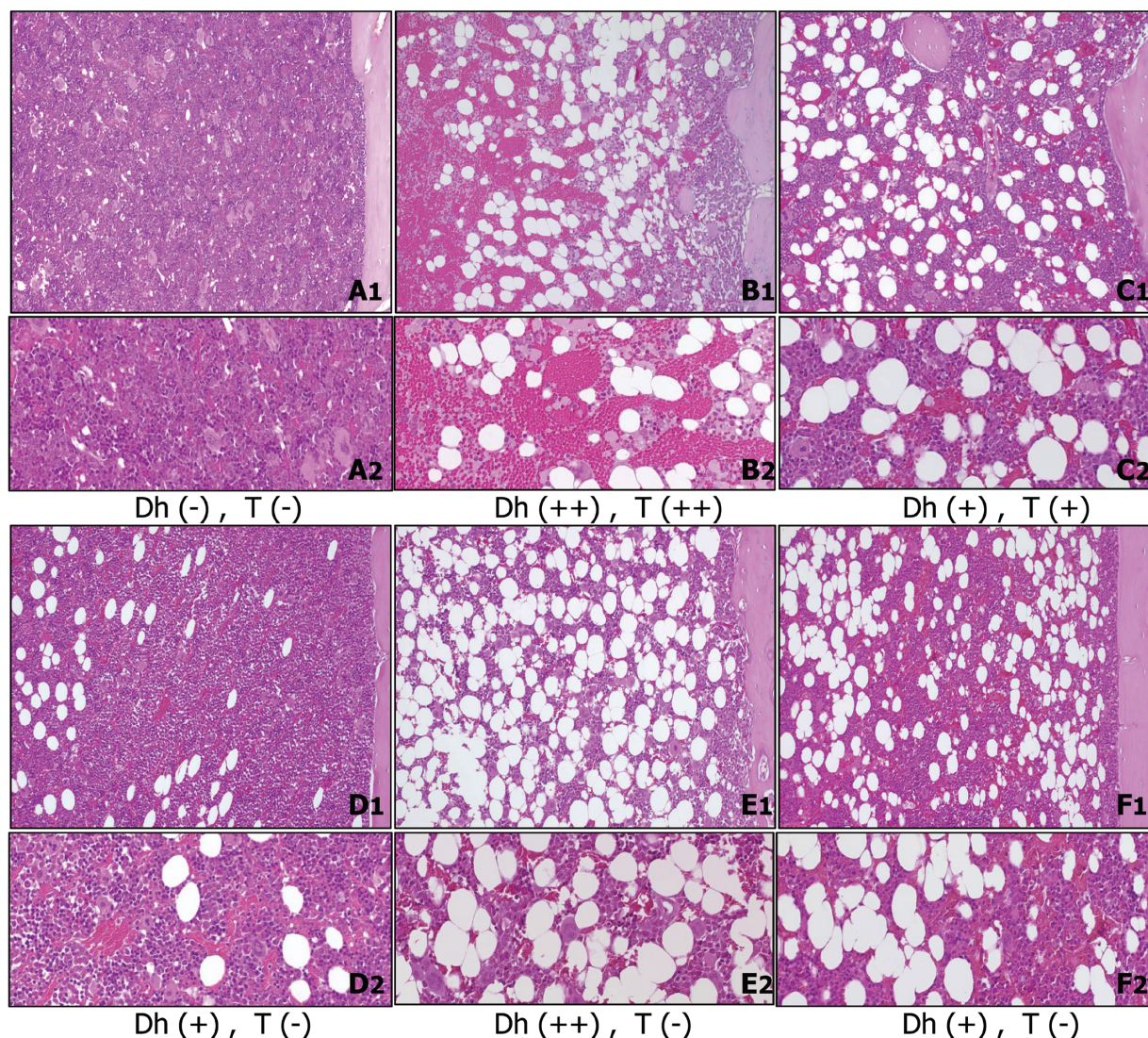


Fig. 9. Histopathology of the bone marrow at the end of the administration period. Representative images of the NT group in the 6-week-old (A) and 12-week-old (D) studies, the 5-FU treated groups in the 6-week-old (B) and 12-week-old (E) studies and the pair-feeding groups in the 6-week-old (C) and 12-week-old (F) studies are shown. The images of the 6-week-old study were reprinted or reshot from the slides in our previous report³. The images marked with a letter followed by a 1 indicate the low magnification: $\times 70$. The images marked with a letter followed by a 2 indicate the high magnification: $\times 140$. H&E stain. Dh: decreased hematopoiesis; not remarkable (-), slight (+) and moderate (++) images are shown. T: telangiectasis; not remarkable (-), slight (+) and moderate (++) images are shown. Telangiectasis with marked hemorrhage was categorized as severe (+++; image not shown).

food consumption was remarkable in the 12-week-old study. On the other hand, the degree of decreased body weight was remarkable in the 6-week-old study. In young animals that are still growing, energy is necessary not only for maintaining their body, but also for growth. Therefore, the young rats were perhaps more susceptible to body weight loss in response to a reduction in food consumption.

In this study, the following granulocytic or lymphocytic changes were considered to be characteristic of age-related differences. Decreases in peripheral white blood cells and marrow myeloid cells were observed in the 5-FU treated and pair-feeding groups at the end of the administration period only in the 6-week-old study. In addition, reductions in the

thymus weight in the pair-feeding groups were only observed in the 6-week-old study. Decreased peripheral white blood cells and reductions in thymus weight showed a strong effect of undernutrition, since no apparent differences were observed between the 5-FU treated and pair-feeding groups or the changes were only observed in the pair-feeding groups. No decrease in the white blood cell count was observed in the 12-week-old study. However, the influence of 5-FU was significant in the 12-week-old study. The degree of decreased food consumption and the number of deaths were remarkable or comparable in the 12-week-old study. The immune system, with its ongoing cellular proliferation and differentiation, lymphocyte trafficking and

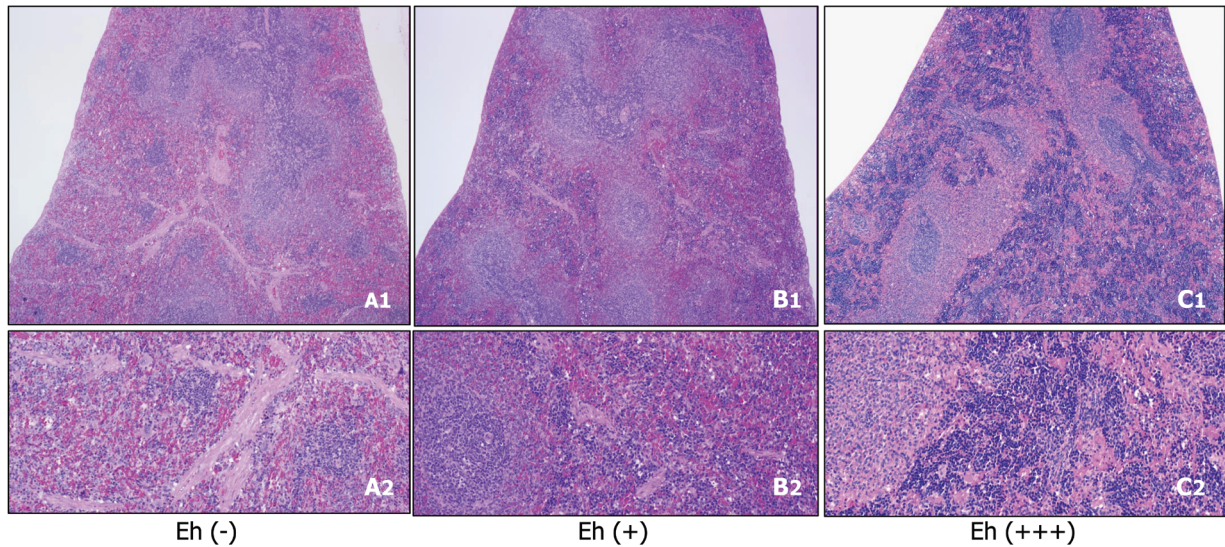


Fig. 10. Histopathology of the spleen at the end of the recovery period. Representative images of the NT group in the 6-week-old study (A) and the 5-FU treated groups in the 6-week-old (B) and 12-week-old (C) studies are shown. The images of the 6-week-old study were reshoot from the slides in our previous report³. The images marked with a letter followed by a 1 indicate the low magnification: $\times 28$. The images marked with a letter followed by a 2 indicate the high magnification: $\times 70$. H&E stain. Eh: increased extramedullary hematopoiesis; not remarkable (-), slight (+) and severe (+++) images are shown.

Table 1. Histopathological Findings of the Bone Marrow at the End of the Administration and Recovery Periods

Groups	NT			FU12			FU15			FU18				R12			R15			R18						
	Grade	-	+	++	-	+	++	-	+	++	-	+	++	+++	-	+	++	-	+	++	-	+	++			
6-week-old study																										
Administration period																										
Number of animals		6			6			6			6 ^{a, b)}				6			6						6		
Decreased hematopoiesis	6	0	0	5	0	1	1	4	1	0	3	3		2	4	0	3	2	1	3	2	1	3	2	1	
Telangiectasis	6	0	0	6	0	0	2	3	1	1	2	2	1	6	0	0	4	2	0	2	4	0	2	4	0	
Recovery period																										
Number of animals		6			6			6			5 ^{a)}				6			5 ^{c)}						5 ^{c)}		
Decreased hematopoiesis	6	0	0	5	0	1	3	2	1	3	1	1		5	1	0	1	4	0	0	1	4	0	0	1	4
Telangiectasis	6	0	0	6	0	0	6	0	0	5	0	0	0	6	0	0	5	0	0	5	0	0	5	0	0	
12-week-old study																										
Administration period																										
Number of animals		6			7 ^{a)}			7 ^{a)}			6				6			6						6		
Decreased hematopoiesis	1	5	0	2	5	0	1	3	3	0	3	3		0	6	0	0	6	0	0	0	6	0	0	5	1
Telangiectasis	6	0	0	5	2	0	7	0	0	5	1	0	0	6	0	0	6	0	0	5	1	0	5	1	0	
Recovery period																										
Number of animals		6			5 ^{a)}			5 ^{a)}			6				6			6						6		
Decreased hematopoiesis	0	6	0	1	4	0	0	2	3	0	4	2		0	6	0	0	4	2	0	4	2	0	3	3	
Telangiectasis	6	0	0	5	0	0	5	0	0	6	0	0	0	6	0	0	6	0	0	6	0	0	6	0	0	

The histopathological changes in the femoral bone marrow were graded into 4 categories: not remarkable (-), slight (+), moderate (++) and severe (+++). The number of animals affected at each grade is shown. The data of the 6-week-old study were reprinted from our previous report³. A moderate or greater reduction in hematopoiesis in the 12-week-old study and a slight or greater reduction in hematopoiesis in the 6-week-old study were regarded as suggesting the influence of 5-FU administration or dietary restriction.

^{a)} Animals that died were included in the administration period (therefore, the number of animals reported in the recovery period is lower). ^{b)} One animal that died in the FU18 group in the 6-week-old study was not examined histopathologically because of advanced autolysis. ^{c)} One animal in each of the R15 and R18 groups in the 6-week-old study was not evaluated after the recovery period because of sampling failure (missing).

gene amplification, is highly susceptible to toxic insults, particularly in the thymus and bone marrow where rapid cell turnover occurs²¹. For the individual dose-response relationship, thresholds for most toxic effects certainly exist, although interindividual variability in response and qualitative changes in response pattern with dose make it difficult to establish a true no-effects threshold for any chemical²². Since some reports on the thresholds for

changes in dietary restricted rats have been published^{9,23}, the changes in this study, such as the differences in the reductions of white blood cells, suggest that the thresholds differed according to age.

In addition, the following erythroblastic changes were considered to be characteristic of age-related differences. A decrease in reticulocytes was observed at the end of the administration period in the 5-FU treated and pair-feeding

Table 2. Histopathological Findings of the Spleen at the End of the Administration and Recovery Periods

Groups	Grade	NT			FU12			FU15			FU18				R12			R15			R18		
		-	+	++	-	+	++	-	+	++	-	+	++	+++	-	+	++	-	+	++	-	+	++
6-week-old study																							
Administration period																							
Number of animals		6			6			6			6 ^{a, b)}				6			6			6		
Increased extramedullary hematopoiesis		6	0	0	6	0	0	6	0	0	6	0	0	0	6	0	0	6	0	0	6	0	0
Atrophy of the white pulp		6	0	0	6	0	0	6	0	0	4	1	1	0	6	0	0	6	0	0	6	0	0
Recovery period																							
Number of animals		6			6			6			5 ^{a)}				6			6			6		
Increased extramedullary hematopoiesis		6	0	0	5	1	0	3	3	0	0	5	0	0	6	0	0	6	0	0	6	0	0
Atrophy of the white pulp		6	0	0	6	0	0	6	0	0	5	0	0	0	6	0	0	6	0	0	6	0	0
12-week-old study																							
Administration period																							
Number of animals		6			7 ^{a)}			7 ^{a)}			6				6			6			6		
Increased extramedullary hematopoiesis		6	0	0	7	0	0	7	0	0	6	0	0	0	6	0	0	6	0	0	6	0	0
Atrophy of the white pulp		6	0	0	7	0	0	6	1	0	6	0	0	0	6	0	0	6	0	0	6	0	0
Recovery period																							
Number of animals		6			5 ^{a)}			5 ^{a)}			6				6			6			6		
Increased extramedullary hematopoiesis		6	0	0	2	3	0	0	4	1	0	3	2	1	6	0	0	6	0	0	6	0	0
Atrophy of the white pulp		6	0	0	5	0	0	5	0	0	6	0	0	0	6	0	0	6	0	0	6	0	0

The histopathological changes in the spleen were graded into 4 categories: not remarkable (-), slight (+), moderate (++) and severe (+++). The number of animals affected at each grade is shown. The data of the 6-week-old study were reprinted from our previous report³.

^{a)} Animals that died were included in the administration period (therefore, the number of animals reported in the recovery period is lower). ^{b)} One animal that died in the FU18 group in the 6-week-old study was not examined histopathologically because of advanced autolysis.

groups in both the 6- and 12-week-old studies, and the degree of this change was remarkable in the 6-week-old study. Since a decrease in marrow erythroid cells was also observed, no age-related differences were observed in this study. As for the reductions in reticulocytes and marrow erythroid cells observed at the end of the administration period, no apparent differences were observed between the 5-FU treated and pair-feeding groups in either the 6- or 12-week-old studies. The influence of undernutrition on erythroblastic changes cannot be disregarded. The plasma erythropoietin level is known to be high in infant rats^{24,25}. Naeshiro et al. reported that overnight fasting influenced the erythropoietin level more severely in male F344 rats aged 6 weeks than those aged 10 weeks²⁴. The large number of erythroblastic cells in infant rats gradually decreases^{26,27}. Moreover, the granulocyte/erythroid ratio (G/E ratio) reportedly increases at 9 weeks of age and becomes a constant value thereafter, suggesting that 9 weeks of age is the maturational age in rats using marrow cells as an indicator, and this transition is comparable to the sexual maturation time of male rats²⁶. The influences of undernutrition were strongly apparent on erythroblastic changes in both the 6- and 12-week-old studies but were more severe in the young rats (6-week-old study) in which hematopoiesis maturation had not yet been completed. The histopathological examinations showed that decreased hematopoiesis in the bone marrow attributable to administration of 5-FU or dietary restriction was remarkable in the 6-week-old study, but reductions in hematopoiesis were rare in the pair-feeding groups in the 12-week-old study. This fact suggests that, similar to the effect on the hematological changes, the effect of undernutrition was small in the 12-week-old study.

In this study, the following changes were considered

characteristic of the effects of 5-FU. Abnormal granulocytes with hypersegmented nuclei and/or polyploidy nuclei in morphologic observations of the bone marrow were only noted in the 5-FU treated groups in both the 6- and 12-week-old studies. This change originates from the inhibition of DNA synthesis by 5-FU. Morphologic observations are thought to be useful for identifying marrow toxicity since they can reveal the direct effects of the drug. No age-related differences in abnormal morphologies were noted in this study. A decrease in marrow myeloid cells was observed only in the 5-FU treated groups in the 6-week-old study. Decreased hematopoiesis in the bone marrow was observed remarkably in the 5-FU treated groups in the 12-week-old study. The cause of these age-related differences is uncertain. Histopathological examinations revealed the presence of telangiectasis in the bone marrow in the 5-FU treated and pair-feeding groups in both the 6- and 12-week-old studies. In particular, the influence of 5-FU treatment on telangiectasis was apparent in the 6-week-old study, since moderate or severe changes were only observed in the 5-FU treated groups. It has been reported that telangiectasis in the bone marrow is caused by a blood vessel disorder²⁸ or a decrease in the blood cell count²⁹. Myeloid hypoplasia induced by a prazosin analogue in rats has previously been reported to result in a normal proportion of fat cells with a reduced density of hematopoietic cells and a greatly expanded sinusoidal volume filled with red blood cells³⁰. In this study, we speculated that the telangiectasis represented the filling of a deficit with red blood cells and that age-related differences in its appearance might have been related to a reduction in marrow myeloid cells. As mentioned above, the influence of undernutrition on erythroblastic changes at the end of the administration period cannot be disregarded; however, the effect of 5-FU became obvious

after its withdrawal, and no age-related differences in the detection of erythroblastic changes were observed. At the end of the recovery period, increased reticulocyte and/or platelet counts with large individual variations were observed in the FU15 group in the 12-week-old study and in the FU18 group in both the 6- and 12-week-old studies, and this finding was more noticeable in the 12-week-old study than in the 6-week-old study. In addition, increased extramedullary hematopoiesis in the spleen with an increase in the organ weight was found only in the 5-FU treated groups at the end of the recovery period and was more frequent and severe in the 12-week-old study than in the 6-week-old study. Extramedullary hematopoiesis tends to be decreased in adult animals, but can increase in any animal when there is increased demand for these cells as in the case of anemia, inflammation, decreased production by the bone marrow or neoplasia³¹. The age-related difference of the rebound reactions found in this study might relate to the maturity level of the spleen and/or the degree of decreased bone marrow hematopoiesis following 5-FU administration.

In conclusion, this study showed several characteristic age-related differences between rats treated with 5-FU dosing or dietary restriction. Many of the changes observed in the 5-FU treated rats in the 12-week-old study, as well as those in the 6-week-old study, were thought to have been caused by the reduction in food consumption associated with the 5-FU treatment. Generally, the influences of undernutrition were more apparent in young rats (6-week-old study) that were in a stage of active growth (increasing body weight) and in which hematopoiesis maturation had not yet been completed. These facts suggest that adult rats with lowering of body weight gain, rats at 12 weeks of age as an example, are more suitable than young rats for evaluation of 5-FU-induced myelotoxicity.

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