

Original

## Evaluation of Short-term Myelotoxicity Study in Dietary Reduced Rats

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**Abstract:** This study attempted to prove our hypothesis that a short-term toxicity study, using a 4-day dosing regimen as an example, is suitable for evaluating myelotoxicity in rats. We compared the hematological, bone marrow cytological and histopathological results of 5-fluorouracil (5-FU) treated and pair-feeding groups after a 4-day administration period. Several experimental groups were defined for this 4-day study as well as for our previously reported 14-day study (Miyata *et al.*, 2009); these included 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 the FU12, FU15 and FU18 groups, respectively) and a nontreated control group. Although severe reductions in body weight gain and food consumption were reported in the 14-day study, only slight reductions were observed in the 4-day study. In the 4-day study, a decrease in blood reticulocytes and a decreasing trend of marrow erythroid cells were only observed in the FU18 group, and no effects were observed in the pair-feeding groups. The erythroblastic changes observed in this 4-day study were thought to reflect the direct influence of 5-FU administration. Since concerns regarding the influence of secondary changes related to undernutrition were minimized in the 4-day study, it was thought to clarify the direct influence of 5-FU administration on erythroblastic cells. Thus, a 4-day study protocol might be helpful for distinguishing secondary changes related to undernutrition. (*J Toxicol Pathol* 2010; 23: 31–37)

**Key words:** myelotoxicity, short-term toxicity study, dietary restriction, 5-fluorouracil, pair-feeding, rat

### Introduction

The rat is a widely used animal model for safety assessments of such things as pharmaceuticals. Dietary restriction is currently one of the most effective methods for extending the lifespan of rodents and delaying the onset of age-related diseases<sup>1–3</sup>. Meanwhile, many reports concerning hematological changes in dietary restricted rats have been published<sup>4–9</sup>. Young rats exhibiting remarkable growth are generally used in short-term repeated-dose toxicity studies, and suppression of body weight gain and decreased food consumption are often recognized in the drug administration groups in such toxicity studies. Consequently, whether hematological, bone marrow cytological or histopathological changes are caused directly by the drug being tested 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 for 14 days in young rats (6 weeks old at the start of experimentation)<sup>10</sup>. Furthermore, we also reported that adult rats (12 weeks old at the start of experimentation) with a minimal body weight gain were more suitable than young rats for hematological evaluations in a 14-day study period<sup>11</sup>. However, the age-related differences between the young and adult studies were not very large, possibly because these age groups were rather close. Thus, we suspected that further rat studies were needed to evaluate myelotoxicity. Though a 14-day period was selected in our previous studies, we hypothesized that a shorter period, such as 4 days, might be useful for evaluating myelotoxicity, since the influence of undernutrition would be reduced.

The anti-cancer drug 5-FU belongs to a category of chemotherapy agents called antimetabolites and functions as a pyrimidine analog<sup>12, 13</sup>; the myelotoxic effect of 5-FU on the bone marrow is a serious adverse effect associated with

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its clinical use<sup>14</sup>. DNA and RNA synthesis is inhibited by 5-FU not only after repeated dosing, but also after single dosing<sup>15</sup>. In this study, we selected 5-FU as a positive control for myelotoxicity.

In the present study, 5-FU treated and pair-feeding groups (animals that were given the same amount of food as the average amount of food consumed by the 5-FU treated animals) were used. We compared the hematological, bone marrow cytological and histopathological results of the 5-FU treated and pair-feeding groups after a 4-day administration period. Here, we discuss the differences in the hematological and/or myelotoxic effects under decreased food consumption based on the common general toxic parameters. Our hypothesis that a 4-day period may be suitable for evaluating myelotoxicity was verified by referring to our previous 14-day study results.

## 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. Since this 4-day study was a comparative experiment, the same dosages as used in our previous 14-day study<sup>10</sup> were chosen; therefore, dosages of 12, 15 and 18 mg/kg/day were selected.

### *Animals and housing conditions*

A total of 42 male CrI: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 and pair-feeding, the animals were 6 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 animals were divided into the following 7 groups: NT, FU12, FU15, FU18, R12, R15 and R18 (6 rats/group). 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 4 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 allotment of chow was supplied without removing the food that had not been consumed. The next day of initial administration of 5-FU was set as the start date of pair-feeding.

### *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 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. 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 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 coriphosphine-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 weight) 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 using 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), while the other tests were conducted using significance levels of 1% and 5% (two-tailed). Statistical analyses of the clinical observations and necropsy and histopathology results were not performed.

## Results

### *Mortality and clinical signs*

No deaths and no abnormalities were seen in the present 4-day study.

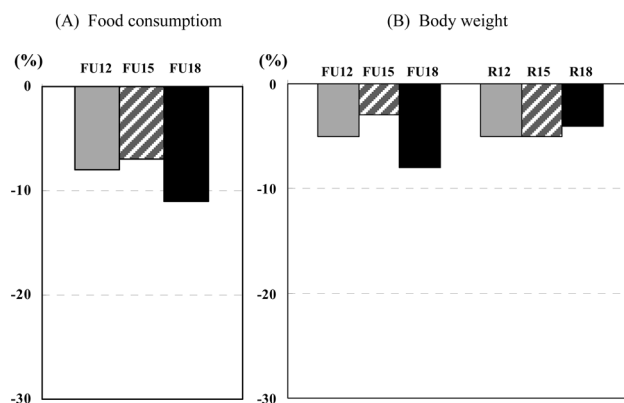
### *Food consumption and body weight*

In the 5-FU treated groups, a decrease in food consumption was seen 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 -8%, -7% and -11%, respectively (Fig. 1A). In the pair-feeding groups, one rat left some food on Day 2 but subsequently consumed all the available food thereafter.

Body weight in the FU18 group was lowest at the end of the administration period, but it was not a marked change since it was only less than 10% compared with the corresponding NT group. No statistically significant difference was observed between the 5-FU treated and pair-feeding groups (Fig. 1B).

### *Hematology and bone marrow cytology*

The principal results are shown in Figs. 2 and 3. In the hematological analysis of the peripheral blood samples, a statistically significant decrease in the number of reticulocytes was observed in the FU18 group (Fig. 2A). In the bone marrow analysis, a decreasing trend of erythroid cells was observed in the FU18 group, although this change was not statistically significant (Fig. 3A). Meanwhile, no effects were observed in the pair-feeding groups in the 4-day study. Individual data regarding the numbers of blood reticulocytes and marrow erythroid cells in the FU18 group are shown in Fig. 4. Decreases in the numbers of these cells were particularly remarkable in 2 of the 6 rats. In addition, abnormal granulocytes with hypersegmented nuclei and/or polyploidy nuclei (with a frequency of 1% or less) were observed in one rat in the FU18 group. This rat was one of the two rats that exhibited remarkable decreases in the numbers of reticulocytes and erythroid cells, as mentioned above. A statistically significant difference in the number of marrow lymphoid cells was observed between the 5-FU



**Fig. 1.** Food consumption and body weight changes during the administration period. Data are expressed as percentages relative to the NT group and were calculated using the mean values for each group.

treated and pair-feeding groups, but this change was not meaningful, as it was within the physiological range (Fig. 3C).

### *Organ weight*

A decrease in the thymus weight was observed in the FU18 group (Fig. 5A). No change was observed in the spleen weight (Fig. 5B).

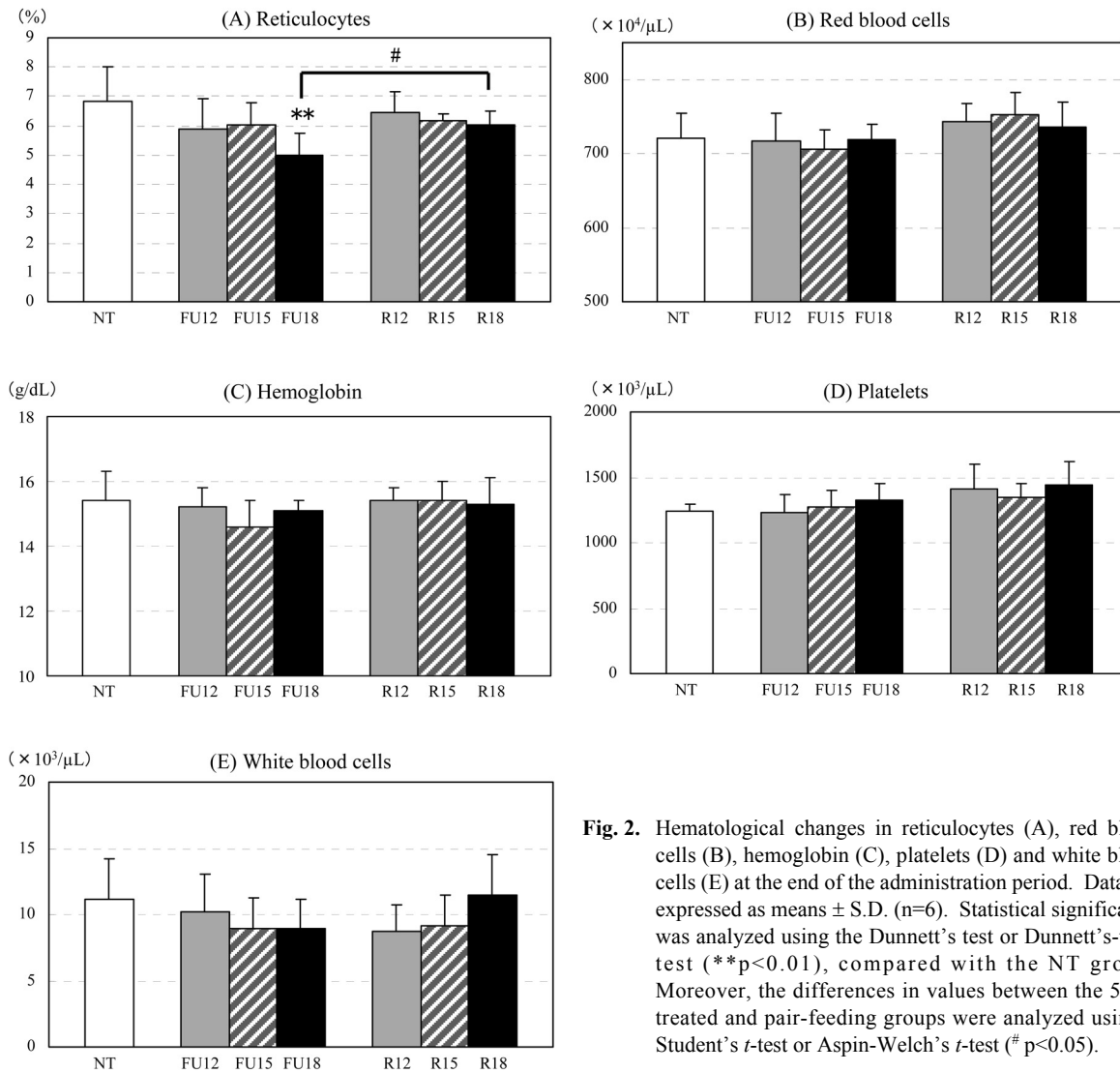
### *Necropsy and histopathology*

No abnormalities were seen in either the macroscopical or microscopical examinations performed in all the groups. Representative tissue images of the bone marrow are shown in Fig. 6.

## Discussion

We compared the hematological, bone marrow cytological and histopathological results of the 5-FU treated and pair-feeding groups after a 4-day administration period. Differences between the present study data (4-day period) and previously reported study data (14-day period)<sup>10</sup> were then compared.

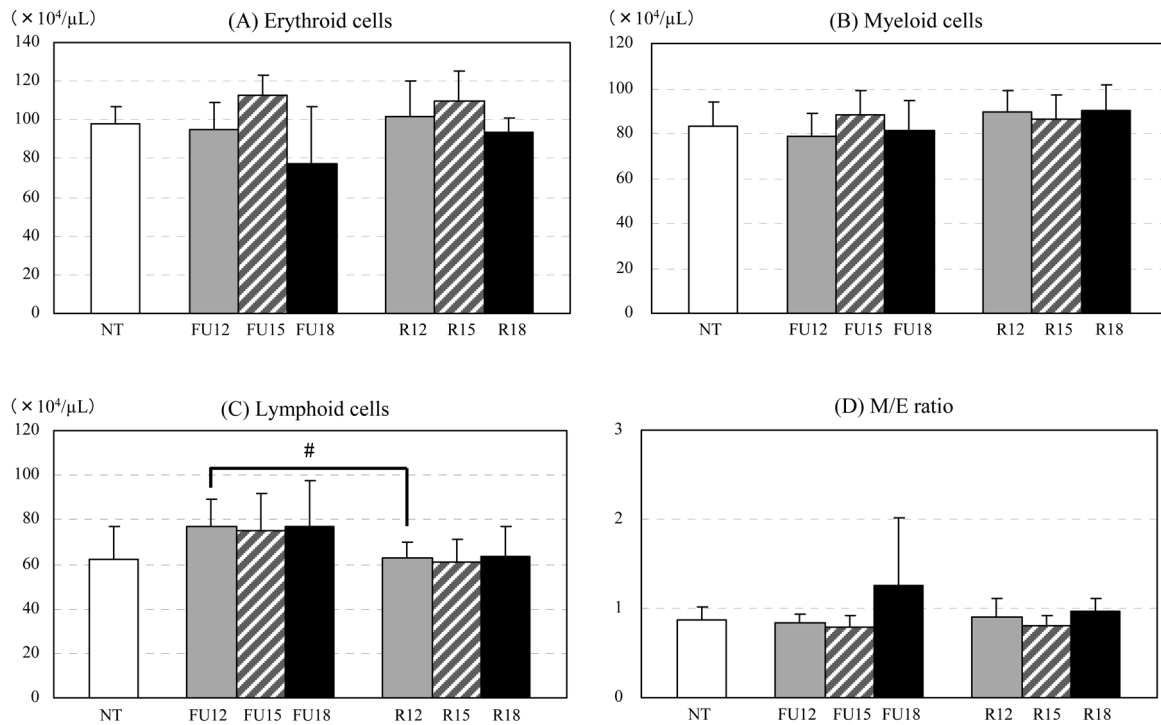
Although severe reductions in body weight and food consumption were reported in the 14-day study, only slight reductions were observed in the present 4-day study. A decrease in the numbers of blood reticulocytes and a decreasing trend of marrow erythroid cells were observed in the FU18 group in the 4-day study. Furthermore, the decreases in the numbers of these cells were particularly remarkable in 2 of the 6 rats. Meanwhile, no effects were observed in the pair-feeding groups in this 4-day study. On the other hand, no differences in these decreases of the erythroblastic cells were found in the 5-FU treated or pair-feeding groups in the 14-day study due to severe undernutrition<sup>10</sup>. The erythroblastic changes observed in



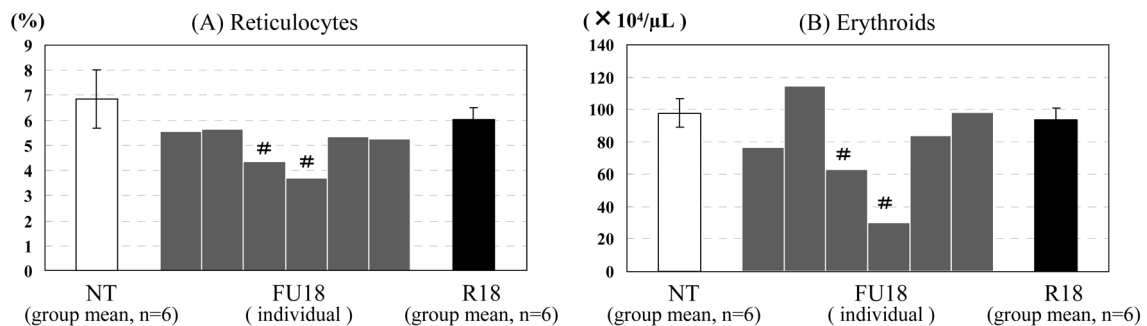
**Fig. 2.** Hematological changes in reticulocytes (A), red blood cells (B), hemoglobin (C), platelets (D) and white blood cells (E) at the end of the administration period. Data are expressed as means  $\pm$  S.D. (n=6). Statistical significance was analyzed using the Dunnett's test or Dunnett's-type 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<0.05).

this 4-day study were thought to reflect the direct influence of 5-FU administration. Ogawa *et al.* reported that dietary restriction strongly influences the reticulocytes in peripheral blood<sup>5</sup>. We previously reported that the influence of undernutrition on erythroblastic changes cannot be disregarded in 14-day toxicity studies<sup>10, 11</sup>. Moreover, the susceptibility of plasma erythropoietin levels to feeding conditions<sup>16</sup> originates from the decrease in the dietary protein content<sup>17-19</sup>. We considered that undernutrition, especially a reduction in dietary protein, might also result in a decrease in blood reticulocytes and marrow erythroid cells. Since the influence of secondary changes related to undernutrition was reduced in the 4-day study, it was thought to clarify the direct influence of 5-FU administration on erythroblastic cells. Thus, evaluations performed at a time when the influence of undernutrition had not yet appeared were useful for evaluating the erythroblastic cells. In addition, the direct influence of 5-FU administration was also detected in the marrow cytological examination performed in the 4-day study. Abnormal granulocytes with

hypersegmented nuclei and/or polyploidy nuclei (with a frequency of 1% or less) were observed in one rat in the FU18 group; however, a reduction in myelo-lymphoid cells was not observed. Though those changes were diminutive, but they were considered the effects of 5-FU based on our previous 14-day study results. These cytological anomalies were not observed in any of the nontreated and pair-feeding regimens in our previous studies<sup>10, 11</sup>. The administration period might be short in dosage used in this 4-day study so that marrow cytological change may change clearly. The number of reticulocytes is larger in rats than in dogs or monkeys<sup>20, 21</sup>; thus, rats appear to exhibit erythrokinetic activity. This fact might explain the difference in cytopenia receptivity. In this 4-day study, no histopathological changes were detected, though telangiectasis and decreased hematopoiesis in bone marrow were reported in the previous 14-day study<sup>10</sup>. The above pathological differences between the two studies were likely due to the short dosing period, low dosage of 5-FU and small influence of undernutrition in the 4-day study.



**Fig. 3.** Bone marrow cytological changes in erythroid cells (A), myeloid cells (B), lymphoid cells (C) and M/E ratio (D) at the end of the administration period. Data are expressed as means  $\pm$  S.D. ( $n=6$ ). Statistical significance was analyzed using the Dunnett's test or Dunnett's-type test (not significant:  $p \geq 0.05$ ), 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 ( $\# p < 0.05$ ).

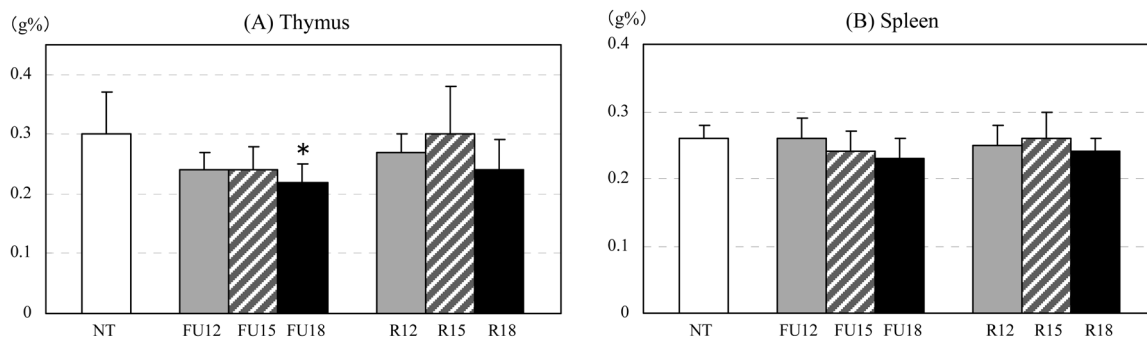


**Fig. 4.** Individual hematological and bone marrow cytological data in blood reticulocytes (A) and marrow erythroid cells (B) at the end of the administration period. Data from the FU18 group are expressed as individual values. The compared data from the NT and R18 groups are expressed as means  $\pm$  S.D. ( $n=6$ ). The decreases in the numbers of blood reticulocytes and marrow erythroid cells were particularly remarkable in 2 rats (indicated by '#').

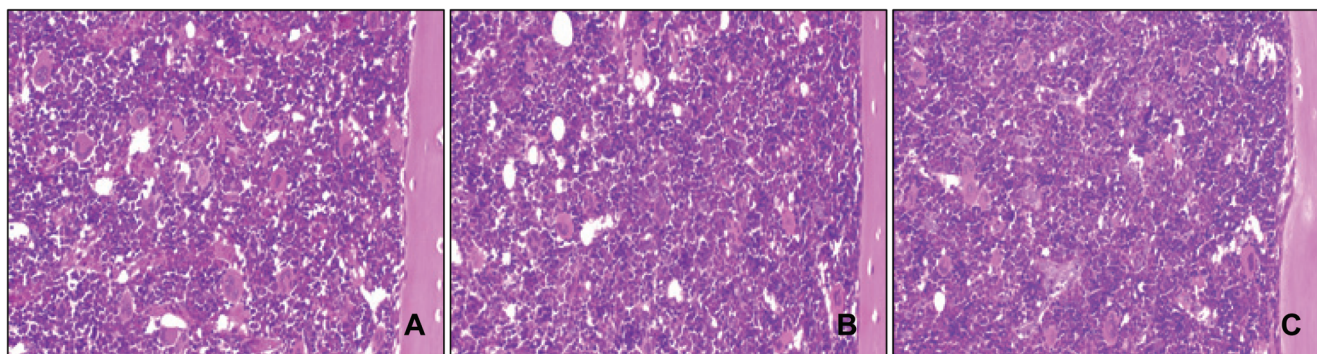
Matsumoto *et al.* reported that 4-day toxicity studies are effective based on the total generation time of marrow cells<sup>22</sup>, and the occurrence of hemocytopenia is seen within about 4 days in tests for several myelotoxic chemicals<sup>22-25</sup>. Since examinations performed within a 4-day period are not yet influenced by undernutrition, the influence of 5-FU on erythroblastic cells was successfully detected in our 4-day study. The pair-feeding examination performed in the present study also suggested that the 4-day toxicity study was useful for detecting 5-FU toxicity. Nonspecific stress

responses commonly lead to erroneous identification of test compounds as immuno-myelotoxic. In conclusion, in repeated-dose toxicity studies of drugs in rats, weight loss and decreased food consumption are often recognized in drug administration groups. Consequently, 4-day toxicity studies for hematological evaluations might be helpful for distinguishing secondary changes arising from undernutrition.

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**Fig. 5.** Relative organ weight changes in the thymus (A) and spleen (B) at the end of the administration period. Data are expressed as means  $\pm$  S.D. (n=6). Statistical significance was analyzed using the Dunnett's test or Dunnett's-type test (\*  $p < 0.05$ ), 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 (not significant:  $p \geq 0.05$ ).



**Fig. 6.** Histopathology of the bone marrow at the end of the administration period. Representative images of the NT group (A), FU18 group (B) and R18 group (C) are shown. No abnormalities were seen in the microscopical examinations performed in all the groups. Magnification:  $\times 140$ . H&E stain.

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