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Original Article

Analyses of hemorrhagic diathesis in high-iron diet-fed rats

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Abstract: Iron overload has been well recognized to cause oxidant-mediated cellular/tissue injury; however, little is known about the effects of iron overload on the blood coagulation system. We encountered an unexpected bleeding tendency in rats fed a high-iron diet in a set of studies using iron-modified diets. In this study, we investigated the mechanism of hemorrhagic diathesis induced by dietary iron overload in rats. Six-week-old F344/DuCrlCrlj male rats were fed a standard (containing 0.02% iron) or a high-iron diet (containing 1% iron) for 6 weeks and were then sampled for hematological, blood biochemical, coagulation, and pathological examinations. Serum and liver iron levels increased in rats fed the high-iron diet (Fe group) and serum transferrin was almost saturated with iron. However, serum transaminase levels did not increase. Moreover, plasma prothrombin time and activated partial thromboplastin time were significantly prolonged, regardless of the presence of hemorrhage. The activity of clotting factors II and VII (vitamin K-dependent coagulation factors) decreased significantly, whereas that of factor VIII was unaltered. Blood platelet levels were not influenced by dietary iron overload, suggesting that the bleeding tendency in iron-overloaded rats is caused by secondary hemostasis impairment. In addition, hemorrhage was observed in multiple organs in rats fed diets containing more than 0.8% iron. Our results suggest that iron overload can increase the susceptibility of coagulation abnormalities caused by latent vitamin K insufficiency. (DOI: 10.1293/tox.2020-0004; J Toxicol Pathol 2021; 34: 33–41)

Key words: iron overload, hemorrhage, partial thromboplastin time, prothrombin time

Introduction

Iron is an essential micronutrient necessary for hemoglobin synthesis, redox reactions, and other fundamental metabolic processes[1–4]. Since mammals do not have major physiological pathways for iron excretion, iron metabolism needs to be tightly regulated in the body to prevent iron overload or deficiency[1–3, 5]. Impairment of iron metabolism, caused primarily or secondarily by certain disorders (e.g., hereditary hemochromatosis and chronic liver diseases), results in excess iron accumulation in the body[4, 6]. Under physiological conditions, circulating iron binds to the iron transporter transferrin with approximately 30% saturation in humans[1, 3]. Once the body iron level exceeds transferrin capacity, the toxic non-transferrin bound iron (NTBI) level increases. NTBI is readily taken up by certain cells (e.g., hepatocytes, cardiomyocytes, and neurons), inducing oxidant-mediated cellular/tissue injury[4, 7–9].

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Acute iron poisoning is highly fatal in children and results in acute gastroenteritis, hepatic necrosis, shock syndrome, and plasmatic coagulation defect[10]. Coagulopathy in acute iron poisoning in humans occurs within the first 24 hours after iron ingestion without severe hepatic dysfunction[11]. On the contrary, studies on the influence of chronic iron overload (e.g., hemochromatosis or dietary iron overload) on blood coagulation are limited. Day *et al*. have shown that chronic iron loading accelerates thrombotic response after arterial injury through a pro-oxidant mechanism[12]. However, no studies have focused on the bleeding tendency under chronic iron overload conditions in humans and animals till date.

While studying the effects of iron-modified diets on rats, we encountered an unexpected bleeding tendency in some rats fed a high-iron diet. In order to elucidate the mechanism of hemorrhagic diathesis, we investigated the hematological, biochemical, and coagulation parameters in rats with dietary iron overload. In addition, bleeding frequency was evaluated in a series of studies using iron-modified diets.

Materials and Methods

Animals

The following three experiments were performed in this study: 1) main experiment for hematological, biochemical,

and coagulation analyses, 2) coagulation activity analysis, and 3) RNA sequencing. In the main experiment, 6-week-old male F344/DuCrlCrlj rats (Charles River Laboratories Japan, Yokohama, Japan) were divided into control (Cont; n=6) and high-iron (Fe; n=6) groups. Rats in the Cont group were fed a standard diet (DC-8, containing 0.02% iron; CLEA Japan, Tokyo, Japan), while rats in the Fe group were fed a high-iron diet containing 1.0% iron (Oriental Yeast Co. Ltd., Tokyo, Japan) for 6 weeks. The gross appearance of visceral organ/tissues was examined at necropsy. Nutritional information of the diet fed is shown in Table 1. Two additional experiments for coagulation analysis (Cont; n=4, Fe; n=6) and RNA sequencing (Cont; n=3, Fe; n=3) were conducted in the same manner as the main experiment. In all the experiments, rats were maintained in specific pathogen-free conditions with controlled temperature (21 ± 3°C) and 12-hour light-dark cycle. Food and water were provided *ad libitum*. Rats were euthanized under deep isoflurane anesthesia, and the blood and liver were collected. All animal procedures were approved by the Institutional Animal Care and Use Committee (code nos. 27-18, 28-20) and were performed according to the institutional Guidelines for Animal Experimentation.

Hematological, blood biochemical, and coagulation analyses

In the main experiment, the iron-overloaded rats with hemorrhagic evidence (n=3; Fe-hemo group, the details of hemorrhagic evidence are described in results) were analyzed separately from the iron-overloaded rats without hemorrhagic evidence (n=3; Fe group) for blood biochemical, hematological, and coagulation analyses. Serum was separated from the blood collected from the abdominal aorta by centrifugation (1,500× *g*, 10 min, 4°C). Moreover, the plasma was separated from 3 mL blood collected in a tube with sodium citrate (Terumo, Tokyo, Japan) by centrifugation (1,500× *g*, 10 min, 4°C). The serum and plasma samples (Cont, n=6; Fe, n=3; Fe-hemo, n=3) were subjected to biochemical (serum iron, total iron binding capacity [TIBC, to calculate transferrin saturation], aspartate aminotransferase [AST], alanine aminotransferase [ALT], and albumin) and coagulation analyses (activated partial thromboplastin time [APTT], prothrombin time [PT], fibrinogen, and antithrombin III [AT-III]), respectively. Also, 500 µL blood was collected in a tube with EDTA (Terumo) for hematological analyses (red blood cell count [RBC], hemoglobin concentration [HGB], hematocrit value [HCT], reticulocytes [RET, absolute number and ratio], mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], and platelet [PLT]). The activities of coagulation factors II, VII, and VIII (Cont n=4; Fe n=6) were also analyzed similarly in an additional experiment. The biochemical parameters and activity of coagulation factors were analyzed by SRL Inc. (Tokyo, Japan). Hematological and coagulation tests were performed at Sysmex Corp. (Hyogo, Japan).

Table 1. Nutritional Information of Control and High-iron Diets

Diet	Control	High-iron
protein (g%)	25.20	22.00
fat (g%)	4.30	5.00
carbohydrate (g%)	52.30	57.00
protein (kcal%)	28.00	24.40
fat (kcal%)	11.30	12.50
carbohydrate (kcal%)	60.70	63.20
total calories (kcal/100g diet)	344.50	361.00
iron (%)	0.02	1.00
vitamin K (µg/100 g diet, estimated value)	500.00[a]	18.20[a, b]

[a]calculated by supplier, possibly reduced during solidification and drying processes. [b]comparable to AIN-76 (17.00 µg/100g diet), calculated by supplier.

Liver iron content

Samples from the right lateral liver lobe of randomly selected rats (Cont, n=3; Fe, n=2; Fe-hemo, n=1) were subjected to liver iron analysis using atomic absorption spectrophotometry (SRL Inc.) in the main experiment.

Histopathology

Samples from the left lateral liver lobe of randomly selected rats (Cont, n=3; Fe, n=2; Fe-hemo, n=1) were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, cut at 5 µm, and stained with hematoxylin and eosin (HE) for histopathological examination and Prussian blue for Fe^{3+} detection.

RNA sequencing

Samples from the right medial liver lobe of rats (Cont; n=3, Fe; n=3) were immersed in RNAlater reagent (Qiagen; Hilden, Germany) and stored at −80 °C before use. Total RNA was extracted using SV Total RNA Isolation System (Promega; Madison, WI, USA). The extracted samples for each group were then pooled into a single tube. RNA sequencing and data analysis were performed using the Illumina HiSeq platform (Illumina, Inc.; San Diego, CA, USA) at Hokkaido System Science Co., Ltd. (Sapporo, Japan). Genes related to blood coagulation were selected; the fold changes in the Fe group compared to that in the Cont group are shown in Table 2.

Evaluation for bleeding frequency in rats fed iron-modified diet

Bleeding frequency was evaluated in a series of studies using iron-modified diet to investigate the relationship between bleeding frequency and iron content in the diet. The data of the rats fed iron-modified diets from 2012 to 2018 were collected, including the data described in this study (6 week-dosing group in Exp. 2). In the experiments except for Exp. 2, detailed gross examination was performed only on the liver. In addition, if gross findings were observed in organs/tissues other than the liver at necropsy, the findings were recorded. The hemorrhage frequency in F344 rats fed diets containing 0.0%, 0.5%, 0.8%, and 1.0% iron and the

composition of these diets are shown in Supplementary Table 1. Details of the experiments are shown in Supplementary Table 2. The experiments listed in Supplementary Table 2 were conducted in the same manner as described above, according to the institutional Guidelines for Animal Experimentation.

Statistical analysis

Data are presented as means with individual values. Statistical analyses were performed using the Prism ver. 7.0e (GraphPad; San Diego, CA, USA) with Tukey's multiple comparison or Student's *t*-test. $P<0.05$ was considered statistically significant.

Table 2. mRNA Expression Profiles in Liver of Iron Overload Rat

Gene symbols	Gene names	Relative value*
Coagluation factors		
FGA	fibrinogen alpha chain	1.87
Fgb	fibrinogen beta chain	2.14
Fgg	fibrinogen gamma chain	1.35
F2	coagulation factor II (prothrombin)	1.15
F3	coagulation factor III (tissue factor)	0.98
F5	coagulation factor V	1.10
F7	coagulation factor VII	0.94
F8	coagulation factor VIII	1.22
F9	coagulation factor IX	1.26
F10	coagulation factor X	1.34
F11	coagulation factor XI	0.94
F12	coagulation factor XII	0.96
F13b	coagulation factor XIII β subunit	0.97
Vwf	von Willebrand factor	0.80
Reguratory factors		
Serpinc1	serpin family C member 1	1.01
Proc	protein C	0.94
Pros1	protein S	1.04
Plg	plasminogen	1.06
Vitamin K related factors		
Gccx	gamma-glutamyl carboxylase	1.15
Vkorc1	vitamin K epoxide reductase complex, subunit 1	0.76

*Relative value compared with that of control rats.

Results

Focal hemorrhagic patches were observed in the lungs of a Fe rat in the main experiment (Fig. 1A). Histologically, the lung lesion consisted of diffuse and extensive hemorrhage filling the bronchioles and alveoli without the infiltration of inflammatory cells or hemosiderin-laden macrophages, suggesting a fresh hemorrhagic lesion (Fig. 1B). The RBC count and serum albumin and iron levels were decreased in this rat compared to those in rats with hyperferremia, probably resulting from blood loss due to hemorrhage. Two other Fe rats had the same clinicopathologic findings, although no gross bleeding was noted. These animals were considered to have normocytic normochromic anemia due to bleeding. Therefore, they (n=3; Fe-hemo rats) were analyzed separately from other Fe rats for blood biochemical, hematological, and coagulation (APTT, PT, fibrinogen, and AT-III) analyses.

The serum iron level in the Fe rats increased by 3.4 times compared with that in the Cont rats (Fig. 2A), and transferrin saturation increased to nearly 100% (Fig. 2B). These parameters decreased to control levels in the Fe-hemo rats (Fig. 2A and B). In Fe + Fe-hemo rats, the liver iron level increased by 4.7 times compared with that in the Cont rats (Fig. 2C). Serum AST levels decreased in the Fe + Fe-hemo rats, while ALT levels remained unaltered (Fig. 2D and E). Serum albumin levels increased in the Fe rats, which decreased to control levels in the presence of hemorrhage (Fig. 2F). Histologically, in HE-stained sections, no abnormality was observed in the liver of all rats (Fig. 3A, B, Supplementary Fig. 1A and B), consistent with the absence of serum transaminase elevation. Iron deposition was greater in the liver of the Fe rats than that in the liver of the control rats (Fig. 3C and D), with more intense staining in the periportal area than in the centrilobular area. Hepatocellular iron staining was less intense in the liver of the Fe-hemo rats than that in the liver of the Fe rats, while iron staining in the sinusoidal cells was similar between the two groups (Supplementary Fig. 1C and D).

Hematologic tests revealed increases in MCV and MCH, and a slight increase in RET (absolute number and

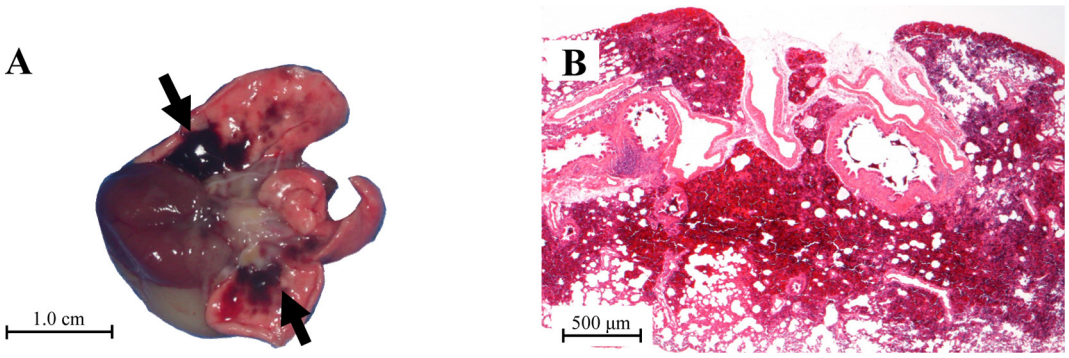

Fig. 1. Hemorrhagic lesions in rats fed the high-iron (1.0%) diet in the main experiment. Hemorrhage is seen in the lung (A, macroscopy; B, microscopy). Hemorrhagic sites are indicated by arrows.

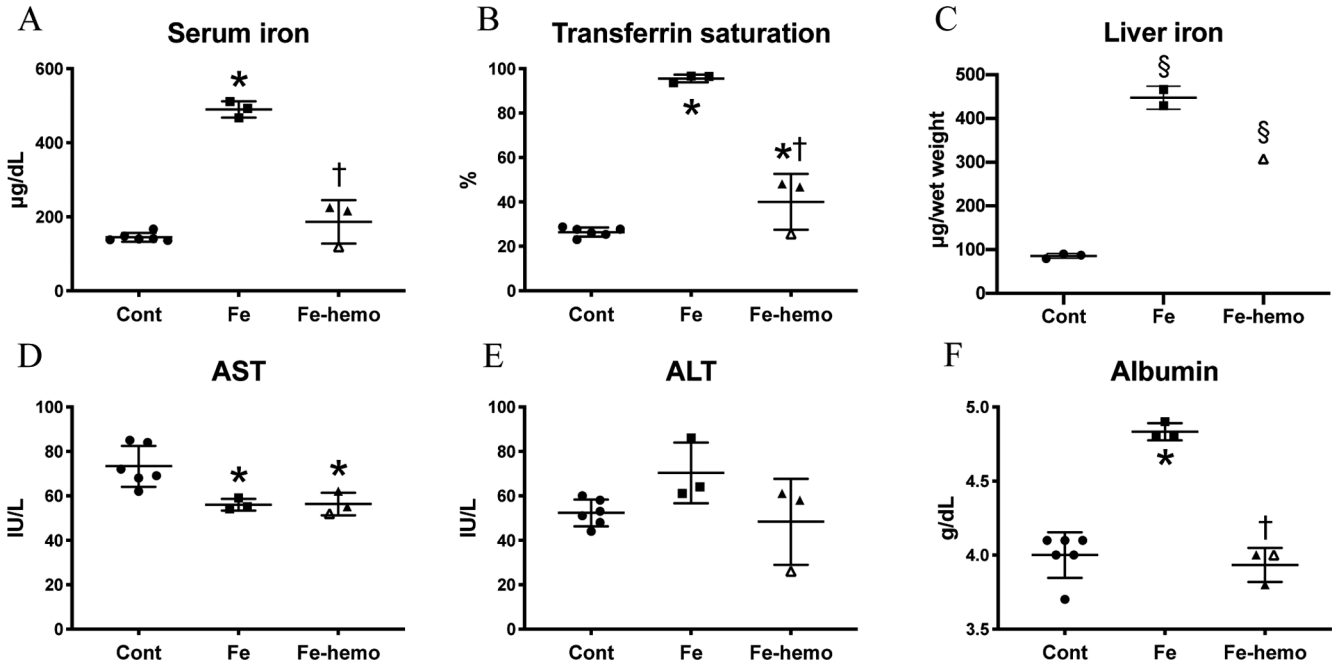

Fig. 2. Blood biochemical parameters of rats fed the high-iron diet. In Fe group, rats with or without hemorrhage were separately analyzed. Fe-hemo: iron-overloaded rats with hemorrhagic evidence. Data are shown as mean with individual data. For liver iron analysis, three animals each were selected from control group (Cont) and Fe + Fe-hemo groups (Fe, n=2; Fe-hemo, n=1). White triangle indicates data from the animal with lung hemorrhage. In the figure (C), Fe and Fe-hemo are shown separately, but the statistical analysis was performed together as the Fe + Fe-hemo group (n=3 in total). *P<0.05 vs. Cont, †P<0.05 vs. Fe, by Tukey's multiple comparison (Cont, n=6; Fe, n=3; Fe-hemo, n=3); §P<0.05 vs. Cont, by Student's *t*-test (Cont, n=3; Fe + Fe-hemo, n=3).

ratio) in the Fe and Fe-hemo rats compared with those in the Cont rats (Fig. 4D–G). In the Fe rats, the presence of hemorrhage led to decreases in RBC, HGB, HCT, and MCHC, and an increase in MCV (Fig. 4A–C, F and H). However, PLT did not change (Fig. 4I).

Coagulation tests revealed prolonged APTT and PT and decreased activity of coagulation factors II and VII in the Fe rats compared with those in the Cont rats (Fig. 5-1A, B, 5-2A and B). APTT and PT were also prolonged in the Fe-hemo rats. APTT and PT could not be measured in one rat with severe decrease in RBC and HGB because of no coagulation during analysis, suggesting coagulation factor depletion due to hemorrhage. There were no differences in fibrinogen, AT-III, and coagulation factor VIII activity between the rats of Cont and Fe groups (Fig. 5-1C, D, and 5-2C). The fibrinogen and AT-III were increased in the Fe-hemo rats (Fig. 5-1C and D), suggesting hemorrhage-related tissue damage or inflammation in iron-overloaded rats with hemorrhage.

To identify the molecular changes related to the coagulative abnormalities, RNA-sequencing was conducted using liver samples of the Cont and Fe rats. Among the 20 genes involved in the coagulation system, only the fibrinogen beta chain gene increased by more than 2-fold in the iron-overloaded rat liver compared to the control rat liver (Table 2).

To investigate the association between bleeding frequency and iron content in the diet, bleeding frequency was evaluated in a series of studies using iron-modified diets. Bleeding tendency was observed in rats fed diets containing more than 0.8% iron (Supplementary Table 1 and 2). Bleeding frequency was higher in the 1.0% iron diet-fed rats (17.5%, n=63) than in the 0.8% iron diet-fed rats (9.1%, n=55), when data from all experiments were included. When limited to 4-week feeding experiments, the difference became insignificant (11.1% in the 1.0% iron diet-fed rats [n=27] vs. 9.1% in the 0.8% iron diet-fed rats [n=55], Supplementary Table 1). Hemorrhage was observed in the thymus, lungs, nasal cavity, periocular region, abdominal cavity, peri-epididymal tissue, urinary bladder, skeletal muscles (latissimus dorsi and tibialis anterior), tail, penis, and prostate (Supplementary Fig. 2A–D and Supplementary Table 2). The thymus (25.0%) and lungs (18.8%) were frequently affected (Supplementary Table 3). Hemorrhage was observed between days 22 and 90 (Supplementary Table 2). A series of experimental data revealed that high iron content may be related to bleeding tendency.

Discussion

The most important finding to understand the mechanism of the bleeding tendency in the iron-overloaded rats is the prolongation of APTT and PT. In contrast, the number of blood platelets was not influenced by iron overload, suggesting that the bleeding tendency in the iron-overloaded rats can be caused by an impairment of secondary hemostasis.

Clinically, prolonged APTT and PT indicate abnormal-

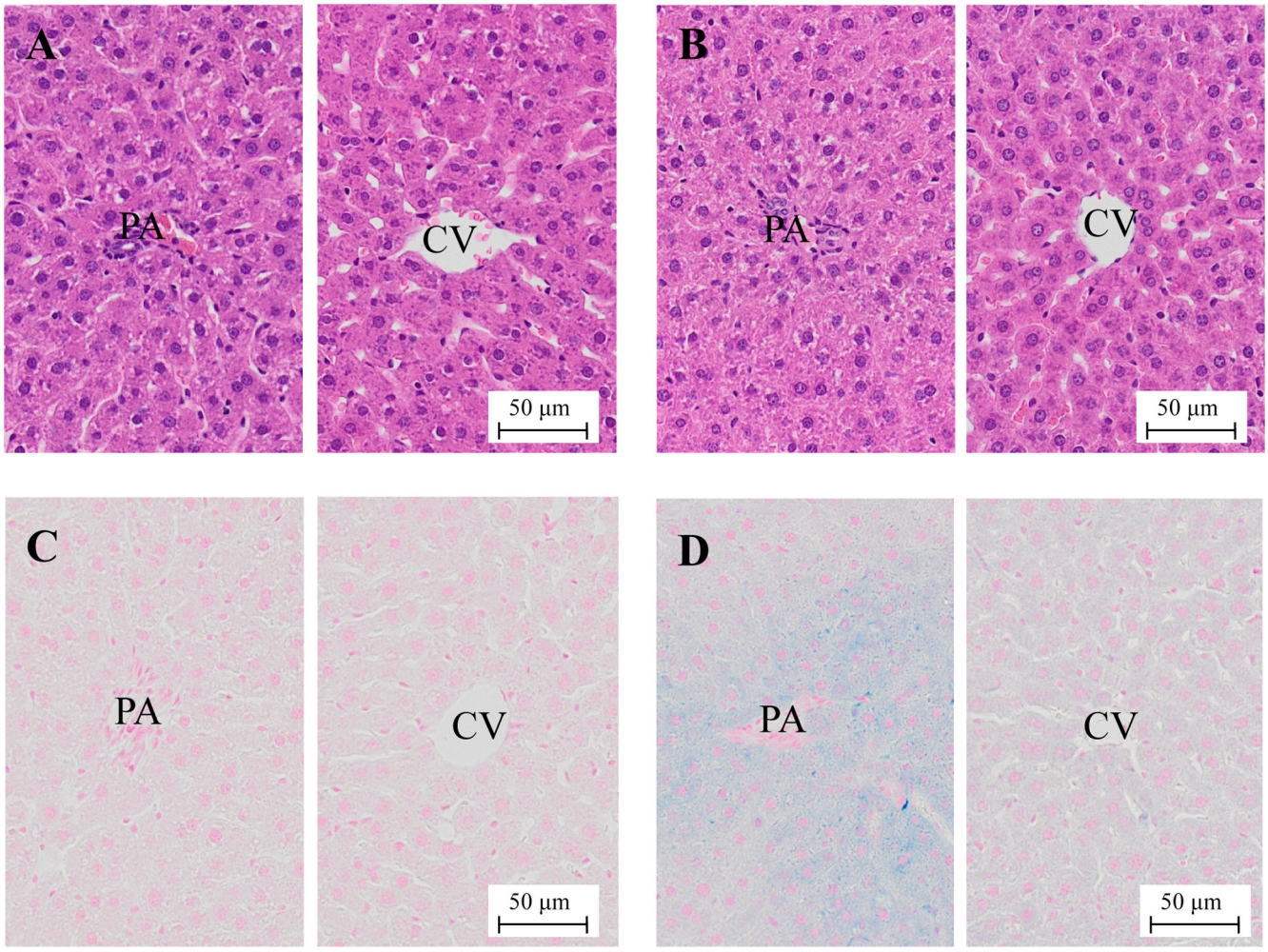

Fig. 3. Liver histopathology of rats fed the high-iron diet. Hematoxylin and eosin (HE; A, B) and Prussian blue (C, D) images of control (Cont; A, C) and Fe (B, D) groups. No histopathological abnormality is observed in the liver of Fe rats. Iron staining is more intense in Fe rat liver than that in Cont rat liver, with the peri-portal region being more intensely stained than the centrilobular region in Fe rat liver. PA, portal area; CV, central vein.

ities in several coagulation factors II, V, X, and fibrinogen (the common pathway), vitamin K deficiency, and liver diseases[13, 14]. In this study, the iron-overloaded rats had iron deposits, but no abnormality in liver histology or serum transaminase levels, suggesting that the high-iron diets were non-hepatotoxic. RNA sequencing of the liver revealed that iron overload does not affect the expression of most coagulation factors at the transcription level. It is considered that the coagulopathy in our iron-overloaded rats is not caused by liver disorder or underproduction of coagulation factors.

On the contrary, decreased activity of coagulation factors II and VII, but not of factor VIII, was observed in the iron-overloaded rats. Factors II, VII, XI, and X are vitamin K-dependent molecules and need vitamin K for their biological activities[15, 16]. Thus, it is possible that vitamin K deficiency can be involved in the bleeding in the iron-overloaded rats of this study. The amount of vitamin K in the high-iron diet (18.2 µg/100 g) is lower than that in the control diet in this study. However, it is comparable to that in AIN-76 (17.0 µg/100 g), a standard diet published by American Institute of Nutrition (AIN), calculated on the basis of the nutritional requirements of rats announced by the National Academy of Sciences-National Research Council (NAS-NRC)[17]. Although the vitamin K content of AIN-76 is lower than that of reformulated AIN-93M (83.4 µg/100 g)[18, 19], AIN-76-based diets are still widely used for rodent studies. Additionally, the vitamin K content in the 0.8% and 1% high-iron diet was the same as that in the iron-deficient and 0.5% high-iron diet; however, the rats fed the iron-deficient or 0.5% iron diet did not develop hemorrhage. Therefore, it is considered that the vitamin K content is not a single causative factor for bleeding, and that iron overload in combination with latent vitamin K insufficiency may lead to coagulation abnormality (bleeding tendency). Another possible explanation of the bleeding mechanism might be that the dietary citrate iron interferes with vitamin K absorption, or that iron overload itself interferes with the vitamin K cycle, but this study could not find evidence to support these mechanisms.

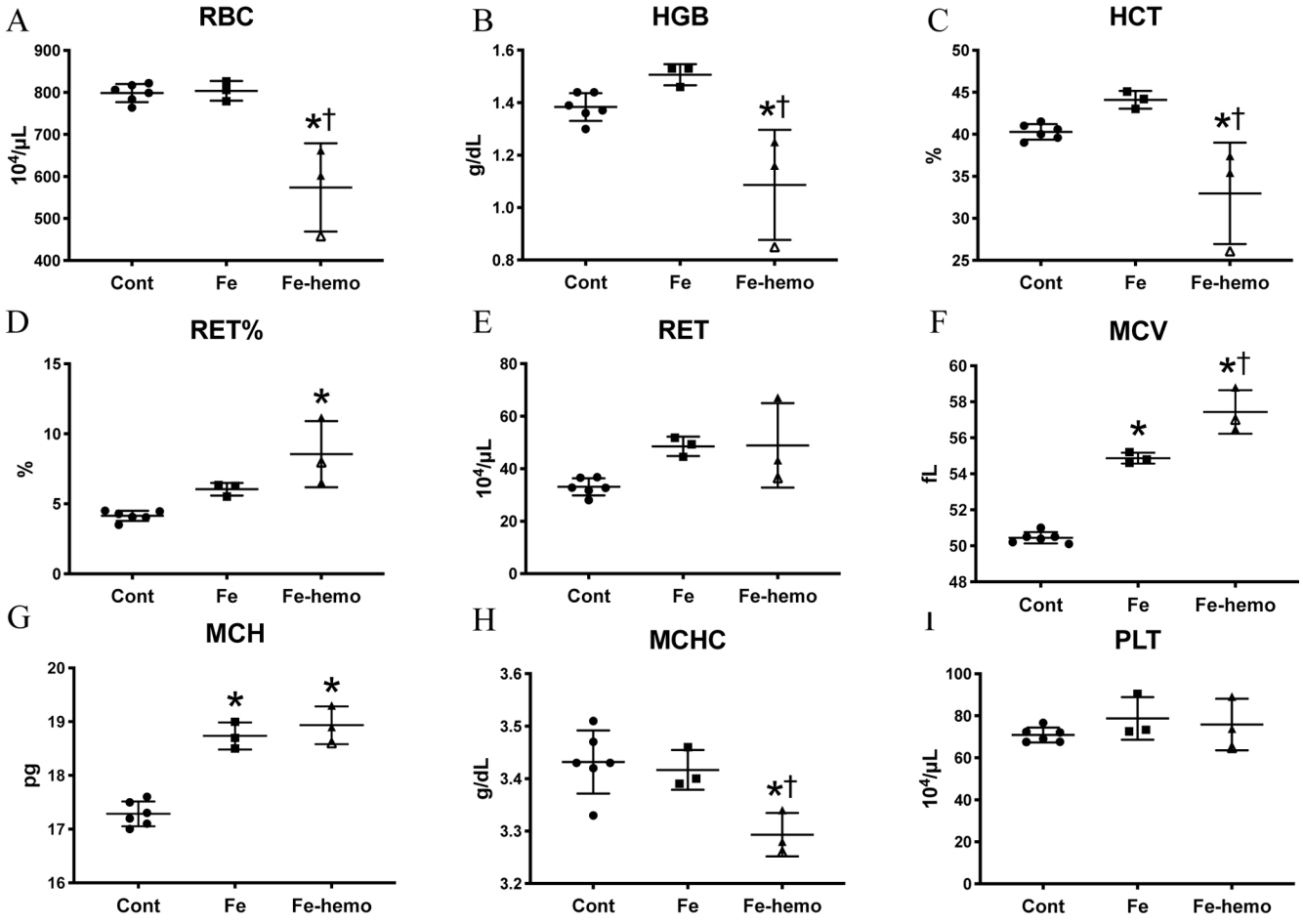

Fig. 4. Hematologic parameters of rats fed the high-iron diet. In Fe group, rats with or without hemorrhage were separately analyzed. Fe-hemo: iron-overloaded rats with hemorrhagic evidence. Data are shown as mean with individual data. White triangle indicates data from the animal with lung hemorrhage. *P<0.05 vs. control (Cont), †P<0.05 vs. Fe, by Tukey's multiple comparison.

On the contrary, previous studies on rabbits[20] and humans[11] have demonstrated early coagulopathy without severe hepatic dysfunction in acute iron poisoning. An *in vitro* study using human plasma demonstrated that NTBI functionally inhibits human thrombin and factor Xa[21]. This coagulopathy is considered to occur due to the susceptibility of serine proteases to NTBI[10, 21]. Despite the difference between acute iron poisoning and chronic iron overload, these data may support the results of this study and raise the hypothesis that hemorrhagic diathesis in rats fed a high-iron diet is caused by the combination of NTBI overproduction due to iron overload and latent vitamin K insufficiency.

Experiments using other diets (e.g., high-fat and high-cholesterol diets) having the same vitamin K content were also conducted; however, no rats had clinicopathological findings suggestive of bleeding tendency. It is necessary to consider whether vitamin K requirement was substantially satisfied in our iron-overloaded rats. To the best of our knowledge, no studies have reported bleeding tendency under conditions of chronic iron overload in humans. Several studies used genetic or induced iron-overloaded animal models[12, 22–26], but none showed bleeding tendency or coagulation abnormality, raising the question of what is different between the dietary model of this study and other iron-overloaded models. Of these studies, some studies using rat models have reported elevated transferrin saturation compared to that in each control; about 76% in TfR2 mutant iron-overloaded Sprague-Dawley rat[23], 92.5% in intraperitoneally administered dextran iron-induced iron-overloaded albino rats[24], and 95.9% in orally administered citrate iron-induced iron-overloaded Wistar rats[25]. This study showed that dietary citrate iron-induced iron-overloaded F344 rats had the same degree of transferrin saturation (95.6%). There are no studies reporting coagulation parameters in iron-overloaded rat models and only one study reporting coagulation parameters in iron-overloaded mice, which shows that APTT and PT do not change in the non-dietary dextran iron-induced iron-overloaded mice[12]. If coagulopathy is not accompanied by iron overload in other rat models, the hemorrhagic phenotype difference might be due to the difference in strain background (F344 vs. other strains). In addition, iron administration routes and iron sources are responsible for iron distribution in the liver; subcutaneous iron dextran injection induces iron deposition in both hepatocytes and

Fig. 5-1

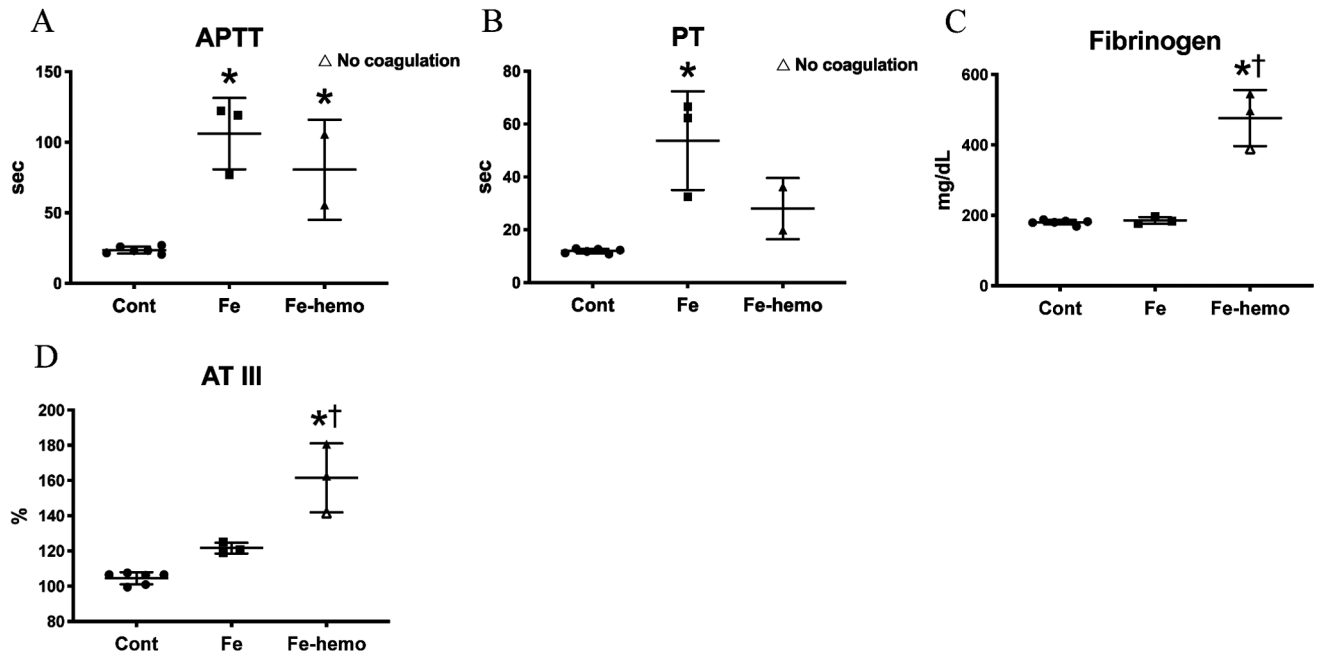

Fig. 5-2

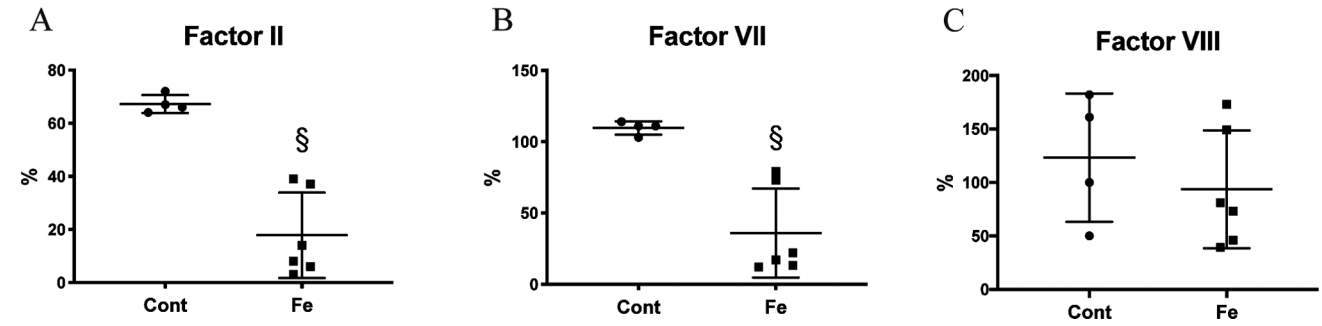

Fig. 5. Blood coagulation parameters of rats fed the high-iron diet. Activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen, and AT-III were examined (Fig. 5-1: Cont, n=6; Fe, n=3, Fe-hemo, n=3). Moreover, the activity of coagulation factors was also examined (Fig. 5-2: Cont, n=4; Fe, n=6). Data are shown as mean with individual data. White triangle indicates data from the animal with lung hemorrhage. APTT and PT were unmeasurable in this rat because of the absence of coagulation during the analysis. *P<0.05 vs. Cont, †P<0.05 vs. Fe, by Tukey's multiple comparison. §P<0.05 vs. Cont, by Student's *t*-test.

sinusoidal cells, while oral carbonyl iron administration induces only hepatocellular iron deposition[26], suggesting that characteristic iron kinetics due to differences in administration routes (dietary vs. non-dietary) and iron sources (citrate iron, dextran iron, carbonyl iron) could also be a candidate factor. Taken together, if vitamin K requirement was satisfied in iron-overloaded rats, it is possible that a high degree of hyperferremia with saturated transferrin-iron binding (indicating an increase in NTBI), genetic background, and/or characteristic iron kinetics of chronic oral administration can be related to the unique coagulation abnormality.

This study could not explain the development of hemorrhage only in some rats (<20%). Shortened lifespan and lethal hemorrhage have been reported in a hemophilia A mouse model; however, actual mortality due to hemorrhage is constant after birth, and the survival period reaches 2 years at longest[27]. It is considered that the occurrence of severe bleeding in animals with potential coagulation abnormalities can depend on both endogenous pathophysiological background and exogenous factors (e.g., activity of individual animals). Further comparative studies between iron-overloaded rats with and without hemorrhage are needed to elucidate the factors triggering hemorrhage.

This study showed increased MCV and MCH, and slightly increased numbers and ratio of reticulocytes in iron-overloaded rats, which have also been reported in the pa-

tients with hemochromatosis[28–31]. MCV and MCH elevation may reflect increased iron uptake and hemoglobin synthesis by immature erythroid cells, as indicated by the reticulocyte increase. These findings suggest that the rat model used in this study has phenotypes of erythrocyte indices relevant to iron overload in humans.

In conclusion, hemorrhage was sporadically observed in F344 rats fed a 1.0% iron-containing diet for 6 weeks, which is characterized by hepatic iron overload without liver injury, saturated iron-transferrin binding, prolonged PT and APTT, and decreased activities of vitamin K-dependent coagulation factors (II, VII). Bleeding tendency in rats was also observed in a series of studies using more than 0.8% iron-containing diets. This is the first report of hemorrhagic diathesis in rats with dietary iron overload and suggests that iron overload can increase the susceptibility to coagulation abnormality caused by latent vitamin K insufficiency.

Disclosure of Potential Conflicts of Interest: The authors indicate that they have no potential conflicts of interest.

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