INTRODUCTION

Among the in vivo thrombosis models, the ferric chloride (FeCl₃) model has been widely used for its simplicity, effectiveness, and reliability. This model can be easily applied to a variety of vessels of different diameters, and it also displays a high degree of reproducibility and sensitivity to both anticoagulants and antiplatelet. In this model, the application of FeCl₃ to the surface...
of a vessel induces thrombosis. Despite previous efforts to characterize the model, it still lacks standardization.1,3 A wide range of FeCl₃ concentrations (2.5%–80%) have been reported for use in various strains of mice or rats.1,4 However, limited information is available on the concentration-dependent effects on and sex-based differences in thrombus formation and stability.2 Methodological differences in the FeCl₃-induced thrombosis model may significantly affect experimental results.16 Moreover, they complicate inter-study comparisons and challenge researchers in determining the optimal conditions for their research.

Several mechanisms of FeCl₃-induced thrombosis have been suggested, including oxidative stress–induced vascular injury and red blood cell (RBC)-mediated platelet recruitment,11-14 However, the exact mechanisms of FeCl₃-induced thrombosis remain uncertain.15 Knowledge of the thrombus composition is important as it represents the primary thrombus characteristics and may reflect some aspects of the thrombosis mechanism. Although several studies have reported the presence of platelets, RBCs, and fibrin in FeCl₃-induced thrombi, limited information is available about the composition of each component.16 Moreover, whether the composition differs according to FeCl₃ concentration remains unknown. Therefore, despite the wide use of the FeCl₃-induced thrombosis model, several issues are still uncertain. In this study, using the carotid artery thrombosis model, we examined the effects of various FeCl₃ concentrations on the formation and stability of thrombi in two different strains of mice. We also showed the ultrastructural features of thrombi with some speculation on the mechanism of FeCl₃-induced thrombosis.

MATERIALS AND METHODS

Animals
All animal procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine and performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (2018-0331). Seven- to 9-week-old mice from the Institute of Cancer Research (ICR) (male 32–34 g, female 27–29 g) and C57BL/6N mice (male 20–22 g, female 18–20 g) were purchased from Orient Bio Inc. (Seongnam, South Korea) to be used in the study.

Study design
First, to compare the effects of different concentrations of FeCl₃ on thrombus formation in the two different mouse strains, the time to occlusion and thrombus area were determined, and the thrombus composition was evaluated. Then, the effects of different concentrations of FeCl₃ on thrombus stability were compared. The ultrastructural morphology of the thrombi was examined by transmission electron microscopy (TEM) in ICR mice treated with 10% and 50% FeCl₃.

FeCl₃-induced arterial thrombosis model
The animals were anesthetized with 5% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen; anesthesia was maintained with 2% isoflurane during the operative procedure. Body temperature was monitored and maintained at 37.0°C±0.2°C using a homeothermic blanket control unit and a heating pad (Harvard Apparatus, Holliston, MA, USA). A midline cervical incision was made, and the left common carotid artery (CCA) was isolated under a surgical microscope. Carotid blood flow was monitored using an ultrasonic Doppler flow probe (MA0.7PSB; Transonic Instruments, Ithaca, NY, USA) connected to a Transonic TS420 blood flow meter (Transonic Instruments) and an iWorx IX-304T data acquisition system (iWorx Systems, Inc., Dover, NH, USA). Baseline flow was recorded for 5 min before the FeCl₃ treatment. Vascular injury was induced on the CCA by placing a filter paper (1×0.5 mm) saturated with different concentrations [10%, 20%, 30%, 40%, and 50% (w/v)] of FeCl₃ (F2877; Sigma-Aldrich Inc., St. Louis, MO, USA) for 5 min. The CCA was washed and excised at the end of the flow recording for 25 min after the FeCl₃ treatment.

Determination of time to and duration of occlusion
Time to occlusion was defined as the time from FeCl₃ application to the ceased blood flow (0 mL/min). The duration of occlusion was defined as the time from the initial occlusion to flow increase to 10% of the baseline flow. Recanalization was defined as the restoration of blood flow back to at least 50% of the baseline level.

Measurement of thrombus size
The excised CCA was fixed in 4% paraformaldehyde and embedded in paraffin. CCA paraffin blocks were sectioned longitudinally into 4-μm slices and stained with hematoxylin and eosin. The thrombus area was measured in the slide representing the largest part of the thrombus under a light microscope (Zeiss Imager D2, Carl Zeiss Imaging Solution, Oberkochen, Germany) and Zeiss AxioVision software (AxioVs40 V 4.8.1.0; Carl Zeiss Imaging Solution) using Image J software.

Immunohistochemistry of thrombus
Sectioned slices were deparaffinized and subjected to heat-induced epitope retrieval with retrieval solution (IHHC World, Inc., MD, USA). After blocking with 5% horse serum, the sections were incubated with primary antibodies against fibrinogen/fibrin (ab834269; Abcam, Cambridge, UK), CD41 (ab63983; Abcam) for platelets, TER119 (BL116202; Biologend, San Diego, CA, USA) for RBCs, Ly6G (BL127602; Biologend) for neutrophils, and coagulation factor XIIIa (FXIIIa, PA5-22110; Invitrogen, Carlsbad, CA, USA). Endogenous peroxidase activity was blocked using 0.03% hydrogen peroxide. The sections were incubated for 30 min with 1:200-diluted biotin–conjugated secondary antibodies [goat anti-rabbit immunoglobulin G (IgG) antibody, BA-1000, and goat anti-rabbit IgG antibody, BA-9401; Vector Laboratories, CA, USA].
Burlingame, CA, USA) and then with horseradish peroxidase-conjugated streptavidin-biotin complex (ABC Elite Kit; Vector Laboratories). After counterstaining with hematoxylin, the colors were developed using NovaRed (NovaRed Kit; Vector Laboratories).

**Measurement of thrombus composition**

Thrombus composition was measured as previously described. Briefly, images of immunostained thrombi were captured using a Zeiss light microscope and AxioVision software and processed using Image J software. After manual drawing of the thrombus contour, imaging analysis was performed semi-automatically using the color devolution module for Nova Red in Image J. Pixel density was determined using the auto threshold. The fraction (%) of each component (platelet, RBC, and fibrin) was calculated as the pixel density percentage for the entire thrombus.

**Assessment of thrombus stability**

Blood flow was measured for 2 h. All measurements were standardized by subtracting the minimum blood flow. Blood flow restoration for 2 h was calculated by dividing the average blood flow by baseline flow and represented as a percentage.

**Transmission electron microscopy**

The excised arteries containing thrombi of ICR mice were immediately fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. The thrombi were post-fixed with 1% OsO4 for 2 h. The thrombi were dehydrated, treated with propylene oxide for 10 min, and incubated overnight to allow penetration. The thrombi were then embedded using a Poly/Bed 812 Kit (Polysciences, Bergstrasse, Germany) and subjected to thermal polymerization in an electric oven (TD-70; DOSAKA, Kyoto, Japan) for 12 h. The excised arteries containing thrombi of ICR mice were immediately fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 200 mM NaCl, and 0.02% NaN3) for 24 h at 37°C. The gels were stained with 0.1% Amido black for 30 min and de-stained with methanol and acetic acid. The gels were scanned using a flatbed scanner (Epson Perfection V800 Photo; Seiko Epson Co., Nagano, Japan) and analyzed by ImageJ software.

**Statistical analyses**

Statistical analyses were performed using IBM SPSS statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). Three-or two-way analysis of variance (ANOVA) was performed for multiple analyses involving concentration, sex, and strain, followed by the post-hoc Bonferroni method. For trend analyses, ANOVA $p$ values for trend tests were calculated using the general linear mode. Correlation analysis was performed using Pearson’s correlation coefficient. Values are presented as mean± standard deviation. $p$ values <0.05 were considered significant.

**RESULTS**

**Thrombus formation by FeCl3 concentration and mouse strain**

A total of 50 ICR mice and 50 C57BL/6N mice were used to determine the time to occlusion according to the various concentrations of FeCl3 (10%, 20%, 30%, 40%, and 50%, w/v) (five male and five female mice at each FeCl3 concentration). In both ICR and C57BL/6N mice, higher concentrations of FeCl3 induced a faster thrombotic occlusion. Although the time to occlusion was shortened in a dose-dependent manner in both ICR and C57BL/6N mice ($p$ for trend <0.001 for both strains), the dose-dependency tended to be dampened from 30% to 50% in C57BL/6N mice (Fig. 1A). A significant interaction was observed between concentration and strain (F=2.784, $p=0.032$). No difference in time to occlusion was found between male and female mice ($p=0.975$) (Supplementary Tables 1 and 2, only online).

Higher concentrations of FeCl3 produced larger thrombi in both ICR and C57BL/6N mice (Fig. 1B and Supplementary Table 3, only online). Thrombus size (area) increased in a dose-dependent manner in ICR mice ($p$ for trend <0.020), but not in C57BL/6N mice ($p$ for trend=0.111).

**Changes of thrombus composition by FeCl3 concentration**

In the present study, topical application of FeCl3-soaked filter paper on mouse carotid artery induced thrombus formation via the diffusion of FeCl3 through the vessel wall (Fig. 2A). We evaluated the distribution of each thrombus component in the
resulting thrombi (Fig. 2B). Fibrin and platelet counts were evenly distributed throughout the thrombus. RBCs were seen as small clusters across the thrombus and predominantly accumulated near the regions of vascular injury induced by the FeCl₃ treatment. Neutrophils were mostly located in the periphery of the thrombus.

![Image](https://example.com/image1)

**Fig. 1.** Time to occlusion and thrombus area by different FeCl₃ concentrations. (A) Overall time to occlusion was shortened in a dose-dependent manner in ICR mice and C57BL/6N mice. However, it tended to be dampened from 30% to 50% in C57BL/6N mice. (B) Thrombus size (area) increased in a dose-dependent manner in ICR mice but not in C57BL/6N mice (p for trend=0.020 for ICR, p for trend=0.111 for C57BL/6N). Bars represent the mean ± standard deviation. Ten mice (five male, five female) of each strain were used at each concentration. ICR, Institute of Cancer Research.

![Image](https://example.com/image2)

**Fig. 2.** Composition of FeCl₃-induced mouse carotid artery thrombi at various FeCl₃ treatment concentrations. (A) Tracings of the representative thrombus in the cross-section of mouse carotid artery (left) and schematic of thrombus formation by FeCl₃ (right). (B) Representative immunohistochemistry of fibrin, platelet, RBCs, and neutrophils in mouse thrombus. Fibrin and platelets are evenly distributed. However, many RBCs are seen as clusters or near the FeCl₃-treated area. Neutrophils are located at the periphery of the thrombus. Immunoreactivity is shown as red (NovaRed stain). Scale bar=100 µm. (C) Comparison of thrombus composition at various FeCl₃ concentrations for the two mouse strains. %Areas for fibrin, platelets, and RBCs are shown, while cells/mm² are shown for neutrophils. RBC content increased with increasing FeCl₃ concentrations (p for trend <0.001 for ICR mice and C57BL/6N mice). Ten mice (five male, five female) were used of each strain at each concentration. Bars represent means ± standard deviation. ICR, Institute of Cancer Research; RBCs, red blood cells.
We investigated whether the concentration of FeCl₃ affected the histological composition of the thrombus. As the concentration of FeCl₃ increased, RBC content increased in both mouse strains ($p$ for trend <0.001 for both strains) (Figs. 2C and 3). Other components (fibrin, platelets, and neutrophils) did not change with different FeCl₃ concentrations (Fig. 2C). There were no sex-based differences in the thrombus composition (Supplementary Tables 4 and 5, only online).

**Association between RBC content and time to occlusion and thrombus size**

Since the FeCl₃ concentration was associated with time to occlusion and RBC content, we investigated whether the RBC content in the thrombus correlates with time to occlusion. There was an inverse correlation between time to occlusion and RBC content in the entire mouse population ($r=0.65$, $p<0.001$) (Fig. 4A). An inverse correlation was observed in ICR mice (male, $r=0.75$, $p<0.001$; female, $r=0.83$, $p<0.001$) and C57BL/6N mice (male, $r=0.63$, $p=0.001$; female, $r=0.62$, $p<0.001$) (Supplementary Fig. 1, only online). There was no significant difference between the two strains ($p=0.131$). No significant association was observed between RBC content and thrombus size ($r=0.19$, $p<0.059$) (Fig. 4B).

**Ultrastructural morphology of thrombus**

The ultrastructure of the thrombus was examined using TEM. Mouse thrombi induced by 50% FeCl₃ showed increased RBC accumulation and larger RBC aggregates compared to those induced by 10% FeCl₃ (Fig. 5A and B). Polyhedral RBC and intermediate forms of RBCs were observed after 10% and 50% FeCl₃ treatment. RBCs were surrounded by closely attached platelets. RBCs were attached to and surrounded by aggregates of degranulated platelets. These features were observed in RBCs adjacent to the injured vessels as well as those in the middle of the thrombus (Fig. 5A-C). Platelets were aggregated and packed throughout the thrombus, and some were attached to the endothelium. Platelets adjacent to the FeCl₃-treated endothelium also showed degranulation, although to a lesser degree than those attached to RBCs (Fig. 5D). Fibrin fibers were interposed between RBCs and platelets. Degranulated platelets were more frequently noted in thrombi induced by 50% FeCl₃ than in those treated with 10% FeCl₃ (Fig. 5).

**Thrombus stability according to FeCl₃ concentration and mouse strain**

For this experiment, blood flow was monitored for 2 h after the FeCl₃-induced thrombosis. Only male mice were used, as there...
were no sex-based differences in the time to occlusion results. For both mouse strains, the mean blood flow for 2 h of recording decreased with higher FeCl₃ concentrations \((p_{\text{for trend}}=0.005 \text{ for ICR, } p_{\text{for trend}}=0.001 \text{ for C57BL/6N})\) (Fig. 6A), suggesting that the thrombus was more frequently resolved at lower concentrations. However, the duration of occlusion was prolonged at higher concentrations \((p_{\text{for trend}}<0.001 \text{ for ICR, } p_{\text{for trend}}=0.002 \text{ for C57BL/6N})\) (Fig. 6B). At every FeCl₃ concentration, at least one of five C57BL/6N mice had recanalization during 2 h of monitoring, and all C57BL6 mice treated with 10% FeCl₃ showed recanalization. However, among ICR mice, none showed recanalization after 40% or 50% FeCl₃ treatment (Table 1).

We further examined whether spontaneous recanalization at lower FeCl₃ concentrations and the difference between mouse strains were associated with endogenous fibrinolysis or fibrin retraction. In fibrin zymography, there were no differences in the fibrinolytic activities of tissue-type plasminogen activator and urokinase-type plasminogen activator between ICR and C57BL/6N mice \((p=0.321)\) or between 10% and 50% FeCl₃ concentrations \((p=0.988)\) (Fig. 6C and D). However, the optical density of FXIIIα in thrombus, which plays a role in thrombus (fibrin) stability, was significantly lower in C57BL/6N mice than in ICR mice \((p<0.001)\) (Fig. 6E and F).
DISCUSSION

This study demonstrated that FeCl₃ has dose-dependent effects on arterial thrombosis. The time to occlusion was shortened with increasing FeCl₃ concentration from 10% to 50%. Moreover, thrombus size increased with increasing FeCl₃ concentration. Previous studies using a fluorescent video microscope showed that higher FeCl₃ concentrations induced faster thrombus formation in the range of 2.5% to 20% of FeCl₃.²⁴ Findings in this study are consistent with those of previous studies and further demonstrated the dose-dependency at much higher FeCl₃ concentrations.

However, the reason why higher FeCl₃ concentrations accelerate thrombosis remains unknown. The thrombus composition may reflect some aspects of the thrombosis mechanism. This study showed that FeCl₃-induced thrombus is primarily fibrin- and platelet-rich, which suggests that platelets are engaged in FeCl₃-induced thrombosis. However, the compositions...
of the platelets and fibrin were independent of FeCl₃ concentration. They were not localized to the area of FeCl₃ treatment but were more evenly distributed throughout the entire thrombus. In contrast, the proportion of RBCs increased with increasing FeCl₃ concentration, the amount of RBC was inversely correlated with time to occlusion, and RBCs were predominantly localized near FeCl₃ treatment area. These findings suggest that, while platelets are involved in FeCl₃-induced thrombosis, RBCs play an important role in the FeCl₃ concentration-dependent acceleration of thrombosis.

The exact mechanisms of thrombosis in the FeCl₃-induced model remain uncertain. FeCl₃-induced oxidative stress was suggested to result in vascular injury and lead to platelet adhesion to the injury site and subsequent aggregation of platelets with thrombus formation. Recent studies suggested that RBCs may be the initial cells to participate in thrombosis by binding to the FeCl₃-treated endothelial surfaces. The studies showed that, upon FeCl₃ treatment, RBCs adhered to Fe³⁺ ions via their physiochemical properties and formed initial aggregates by recruiting platelets. Mice with a high hematocrit exhibited a faster time to artery occlusion in the FeCl₃ model. In a previous study using a microfluidic device designed to replicate the endothelium-blood environment, the flocculation activity of FeCl₃ increased with increasing FeCl₃ concentrations, aggregating RBCs and other blood components. Those findings, along with ours, suggest that RBCs and platelets play an important role in FeCl₃-induced thrombosis.

Furthermore, we found that platelets attached or adjacent to RBCs were extensively degranulated. Platelets have dense granules containing countless small molecules. These granules are released upon platelet activation and recruit more platelets to the vessel injury. Previous studies demonstrated that RBCs activate adjacent platelets by releasing substances such as ADP and thromboxane A₂. Our findings indicate that platelets adjacent to RBCs extensively release granules immediately after FeCl₃ treatment. The released platelet granules may contribute to the activation, aggregation, and further recruitment of platelets.

However, it is unknown how FeCl₃ affects RBCs and how RBCs interact with platelets. FeCl₃ may generate free radicals, and exposure of RBCs to FeCl₃ is known to result in lipid peroxidation. In this study, FeCl₃ rendered RBC morphological changes in polyhedral or intermediate forms. RBC fragmentation was seen in the FeCl₃ model. Oxidative stress induced by FeCl₃ treatment might be associated with the changes in RBC morphology. Phosphatidylserine is exposed when cells are damaged by oxidative stress or shear stress. Externalization of phosphatidylserine on RBC membranes generates thrombin burst. Thrombin produces fibrin and further activates platelets. The presence of fibrin fibers between RBC aggregates on TEM in this study suggests that thrombin might play a role in fibrin production. These findings suggest that the damage to RBCs induced by FeCl₃ might contribute to thrombosis by platelet activation and thrombin generation.

However, in this study, RBCs comprised only about 10% of the thrombi. Additionally, degranulation of some platelets in the absence of RBCs was also observed near the FeCl₃-treated endothelium, implying that FeCl₃ might also affect platelets. The externalization of phosphatidylserine on activated platelets is also known to possess hypercoagulability. These findings suggest that FeCl₃ may affect various cells in the blood and mediate thrombosis via more diverse and complex mechanisms.

Most previous studies on FeCl₃-induced thrombosis focused on the initial stage of thrombosis and measured the time to occlusion to determine effective thrombosis. The stability of a thrombus induced by FeCl₃ is less well known. In this study, the stability of the thrombus differed by FeCl₃ concentration and mouse strain. In both ICR and C57BL/6N mice, higher FeCl₃ induced the formation of a more stable thrombus in a dose-dependent manner. However, we found that ICR and C57BL/6N mice showed different thrombus stabilities, especially at lower concentrations of FeCl₃. ICR mice produced more stable thrombi with less variation than C57BL/6N mice. This information on thrombus stability may be helpful for designing mechanistic studies that require stable clots and evaluating the efficacy of thrombolytic drugs.

Resolution of the thrombus that formed after FeCl₃ treatment might be related to spontaneous fibrinolysis or insufficient clot contraction. We evaluated the key mediators of these processes, which include fibrinolytic activity by plasminogen activators and fibrin stability by FXIIIa. In this study, fibrinolytic activities did not differ by FeCl₃ concentration or mouse strain. However, the immunoreactivity of FXIIIa was higher in ICR mouse thrombus than in C57BL/6N mouse thrombi. FXIIIa catalyzes the cross-linking of fibrin fibers, contributing to enhanced thrombus stability and resistance to thrombolysis. The less stable thrombus in C57BL/6N mice may be partly explained by the lower levels of FXIIIa in their thrombi.

This study had some limitations. Although we visualized direct evidence of platelet activation in relation to RBCs by showing the degranulation of platelets adjacent to them, the mechanism of RBC–platelet interaction by FeCl₃ remains unknown. Also, the response to FeCl₃ may differ according to filter paper size and exposure duration. Furthermore, although we investigated the role of RBC in the FeCl₃ model, the contribution of neutrophils, which have an emerging role in arterial thrombosis, was not evaluated in this study. Neutrophils, which are known to accumulate with thrombus age, may be better studied in future studies.

### Table 1. Recanalization Rate During 2 h of Monitoring after Occlusion

| Mouse strain | Ferric chloride concentration | 10% (n=5) | 20% (n=5) | 30% (n=5) | 40% (n=5) | 50% (n=5) |
|--------------|--------------------------------|----------|----------|----------|----------|----------|
| Institute of Cancer Research | 3 (60) | 2 (40) | 2 (40) | 0 (0) | 0 (0) |
| C57BL/6N | 5 (100) | 4 (80) | 1 (20) | 1 (20) | 1 (20) |

Values in parentheses are percentages. Recanalization was defined as blood flow restored to at least 50% of the baseline level after occlusion.
thrombi that are more aged than the very fresh thrombi analyzed in this study. Lastly, as we tested two mouse strains, the responses to FeCl₃ in other mouse strains and other species are unknown.

Nonetheless, our findings provide evidence of RBC-associated thrombus acceleration and platelet activation to the recent notion that emphasizes the active role of RBCs in thrombosis.²³ Our results provide some insight into optimizing experiments using FeCl₃. FeCl₃ did not significantly alter thrombus composition across all concentrations (10%–50%), except for RBCs. Furthermore, all animals had occlusions within these concentration ranges. Thus, any FeCl₃ concentration tested in our study may be used to evaluate thrombus formation. However, in cases requiring stable thrombus, including mechanistic studies using aged thrombi and evaluating thrombolytic agents, higher concentrations are needed. Of the two mouse strains tested in this study, ICR mice were more reliable than C57BL/6J mice in terms of better dose-dependency and thrombus stability.

In conclusion, we showed that the FeCl₃ model produces thrombi rich in fibrin and platelets. The visualization of possible RBC–platelet interactions in mouse thrombi may help expand our understanding on the mechanisms of FeCl₃-induced thrombosis. The FeCl₃ model showed different responses in thrombus formation and stability according to FeCl₃ concentration and mouse strain. These findings may help researchers plan future experiments using the FeCl₃ model.

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