Research Article

Erythrocyte Fraction in Thrombi Is Increased with Serum Iron by Influencing Fibrin Networks via Oxidative Stress

Mingli Liu, Minghui Chen, Zhongfei Hao, Qingbin Li, Yan Feng, Yongli Li, and Ruiyan Li

The Department of Neurosurgery, Second Affiliated Hospital of Harbin Medical University, Harbin Medical University, Harbin, Heilongjiang, China

Correspondence should be addressed to Yan Feng; 13936349639@163.com, Yongli Li; liyongli9999@aliyun.com, and Ruiyan Li; ruiyanli@yeah.net

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Objective. This study investigated whether the erythrocyte fraction in thrombi would be increased with serum iron via oxidative stress.

Methods. This study retrospectively enrolled patients with acute ischemic stroke treated using endovascular treatment in a single stroke center from October to December 2019. We examined the relationship between serum iron and erythrocyte-rich thrombi and the correlation of serum iron and the erythrocyte fraction in thrombi using clinical samples. Experiments in vivo and in vitro were performed to investigate the influence of oxidative stress on the correlation between serum iron concentration and erythrocyte fraction in thrombi.

Results. We found from the clinical samples that serum iron concentration was related to erythrocyte-rich thrombi and positively associated with the erythrocyte fraction in thrombi in vivo. Further, the tightness of the fibrin networks regulating the erythrocyte fraction in thrombi was increased with serum iron concentration in vivo. Additionally, the oxidative stress level was increased with serum iron concentration in vivo. Moreover, we found that the tightness of the fibrin networks increased with higher oxidative stress levels in vitro. Lastly, experiments in vivo with inhibiting oxidative stress showed that the erythrocyte fraction in thrombi and the tightness of fibrin networks significantly increased in the iron group than those in the iron with oxidative stress inhibitor group and control group. Conclusions. Oxidative stress played a role in the process that the erythrocyte fraction in thrombi was increased with serum iron by influencing fibrin networks.

1. Introduction

Serum iron concentration reflects the overall iron status in the body and is associated with thrombosis [1, 2]. Iron-induced vascular dysfunction is known to contribute to the incidence of ischemic cardiovascular events by increasing vascular oxidative stress [3]. However, these studies principally focused on the relationship between serum iron concentration and thrombosis caused by oxidative stress, rarely mentioning whether a correlation existed between serum iron concentration and the components of thrombi due to oxidative stress. Additionally, we previously found that serum iron levels might be positively associated with the erythrocyte fraction in thrombi [4]. Thus, the present study, based on our previous study, is aimed at investigating whether the erythrocyte fraction in thrombi was increased with increasing serum iron concentration due to oxidative stress.

2. Materials and Methods

2.1. Patient Enrollment. Consecutive patients with acute ischemic stroke (AIS) who received endovascular treatment (EVT) in the Second Affiliated Hospital of Harbin Medical University from October to December 2019 were enrolled for this retrospective study. This study was approved by
the ethics committee of the Second Affiliated Hospital of Harbin Medical University (KY2019-168) and conducted in accordance with the Declaration of Helsinki (1964). Each patient provided written consent. The authors had access to the information that could identify individual participants.

Patients in this study conformed to inclusion and exclusion criteria based on the latest guidelines on EVT for AIS [5]. Inclusion criteria are as follows: (1) AIS diagnosis, (2) large intracranial artery occlusions, (3) age ≥ 18 years, (4) premorbid modified Rankin scale (mRS) scores < 2, (5) National Institute of Health Stroke Scale (NIHSS) scores ≥ 6 and Alberta Stroke Program Early Computed Tomography Score (ASPECTS) ≥ 6, (6) MT within 6 h of stroke onset or treated 6–16 h based on DAWN or DEFUSE 3 eligibility criteria [6, 7], and (7) no findings on concomitant intracranial hemorrhagic disease, including aneurysm or arteriovenous malformations. The exclusion criteria are patients without retrieving thrombi. All patients were divided into two groups, namely, the erythrocyte-rich thrombi group and the fibrin-rich thrombi group, which was based on the erythrocyte fraction or fibrin fraction in thrombi that was more than 50%.

2.2. Endovascular Treatment Procedure. Before EVT, patients with AIS were diagnosed by neurologists based on their symptoms and by using medical imaging tools including computed tomography (CT) and magnetic resonance imaging (MRI). Moreover, physical examination and some other examinations including electrocardiography and blood tests were conducted. Based on the results, the neurorinterventionists decided whether patients with AIS should receive EVT.

In accordance with the latest guidelines for AIS, patients received intravenous treatment (IVT) with recombinant tissue-type plasminogen activator (rt-PA) prior to EVT within 4.5 h of the presentation of symptoms. Otherwise, they received EVT directly, without any IVT, beyond 4.5 h. Thrombectomy was performed using the Solumbra technique [8], wherein a stent retriever was combined with a noncontact aspiration technique using intracranial catheter support. Lastly, modified thrombolysis in cerebral infarction (mTICI) 2b or 3 was deemed as successful recanalization. All thrombi were fixed in 10% neutralized buffered formalin immediately after retrieval.

2.3. Clinical Data Collection. Clinical data including baseline characteristics and blood tests were collected in this study. The baseline characteristics included age, sex (male), hypertension, diabetes mellitus (DM), atrial fibrillation (AF), history of ischemic stroke, smoking (defined as a patient who smoked ≥ 1 cigarette daily and continuously for six months), NIHSS by physical examination, and ASPECTS by CT or MRI. Further, the number of patients who received rt-PA prior to EVT, the number of patients treated by EVT beyond the time window, symptom onset to groin puncture time (OTP), sites of occlusion judged using CT angiography (CTA), or magnetic resonance angiography (MRA) and confirmed using digital subtraction angiography (DSA) including internal carotid artery (ICA), middle cerebral artery (MCA), and basilar artery (BA) and stroke causes judged by TOSAT classification were also included. Blood tests consisted of some possible items associated with thrombi, including prothrombin time (PT), prothrombin activity (PTA), activated partial thromboplastin time (APTT), fibrinogen (FIB), and thrombin time (TT).

2.4. Animals. Ten-week-old male Sprague-Dawley (SD) rats were purchased from the Animal Center of the Second Affiliated Hospital of Harbin Medical University. The animal study was approved by the animal ethical committee of the Second Affiliated Hospital of Harbin Medical University (SYDW2021-017) and conducted in accordance with the international guidelines regarding animal experimentation. All rats were kept in the animal laboratory of the Second Affiliated Hospital of Harbin Medical University, and all rats were fed with a specific pathogen-free class of rat diet and free access to water.

SD rats (n = 90) were randomly divided into the high-iron (HI) group (n = 12), medium-iron (MI) group (n = 12), low-iron (LI) group (n = 12), normal-saline (NS) group (n = 12), negative control (NC) group (n = 12), sham-operated (Sham) group (n = 6), iron group (n = 6), iron with DL-cysteine group (n = 6), control group (n = 6), and control with DL-cysteine group (n = 6). Iron-dextrin (Sigma-Aldrich, St. Louis, MO) was administered in vivo by intraperitoneal injection every other day for 5 weeks, for a total of 18 times. The body weights of rats was measured before the injection. Iron-dextrin was administered at a dose of 150 mg/kg in the HI group, 100 mg/kg in the MI, iron, and iron with DL-cysteine groups, or 50 mg/kg in the LI group based on body weight. Rats in the NS and sham groups received the same dosage of NS solution as that of iron-dextrin in the HI group, and the rats in the control and control with DL-cysteine groups received the same dosage of normal saline solution as that of iron-dextrin in the iron and iron with DL-cysteine groups. Rats in the NC group did not receive any injections. Next, 6 rats were randomly selected each from the HI, MI, LI, NS, and NC groups and the 6 rats in the sham group and 24 rats in the iron group, iron with DL-cysteine group, control group, and control with DL-cysteine group were used to establish the carotid artery thrombus model. Blood samples were collected after 5 weeks from the remaining rats. No significant differences in behavioral phenotypes were recorded between each group of rats during the treatment phases.

2.5. FeCl3-Induced Carotid Artery Thrombus Model. An FeCl3-induced carotid artery thrombus model was used to create thrombi in the carotid artery in vivo and examine their components, based on a previously published protocol with slight modifications [9]. Briefly, rats were anesthetized and the left carotid artery was isolated. A 5 × 5 mm piece of filter paper presoaked in 10% FeCl3 solution was wrapped around the adventitia of the left carotid artery for 30 min. In the sham group, the left carotid artery was isolated for 30 min, but without the insertion of the filter paper. Some rats in the MI and NS groups received an injection of DL-cysteine hydrochloride (Yuanye Biotechnology, Shanghai,
Fisher Scientiﬁc, Waltham, USA) was dissolved in the diluting buffer. The concentration of H2O2 was added before fibrin clot formation, which resulted in the final concentrations of 0.9, 0.6, 0.3, and 0 mM H2O2 (for 0 mM, H2O2 was replaced with the same volume of deionized water).

2.10. Preparation of Buffers and Fibrin Clot Formation In Vitro. Both the diluting and percolating buffers were 0.05 M Tris-HCl and 0.15 M NaCl (pH 7.5; adjusted with HCl). Human fibrinogen lyophilized powder (Sigma-Aldrich, St. Louis, MO) was dissolved in the diluting buffer. The fibrin clot was mixed at room temperature for 1 h such that the final concentration of the solution was 2 mg/L human ﬁbrinogen, 20 mM CaCl2, and 1 U/mL human thrombin (Sigma-Aldrich, St. Louis, MO). Different concentrations of H2O2 were added before fibrin clot formation, which resulted in the final concentrations of 0.9, 0.6, 0.3, and 0 mM H2O2 (for 0 mM, H2O2 was replaced with the same volume of deionized water).

2.11. Fibrin Clot Permeability. Fibrin clot permeability was measured to assess the tightness of fibrin networks. Permeability was evaluated using a modiﬁed pressure-driven system in accordance with a previously described method [13]. Brieﬂy, a modiﬁed ﬂat-bottomed centrifuge tube containing the fibrin clot was connected to the outer cup of a syringe containing percolation buffer. The percolation buffer (10 mL) was used to wash away any soluble liquid in the ﬂat-bottomed tube after removing air from the system, and the time required for 50 mL of the percolation buffer to ﬂow through the fibrin clot was recorded. The permeation coefﬁcient (Ks) was calculated as follows: Ks = Q × L/η × A × Δp, where Q (mL) represents the ﬂow rate in time t (sec), L is the length of the fibrin clot (cm), η is the viscosity of the liquid (g/cm·s), A is the cross-sectional area (cm2), and Δp is the differential pressure (dyne/cm2). The experiment was performed three times for each group.

2.6. Blood Sample Collection. Clinical blood samples were collected from all enrolled patients within 2 h of EVT. Blood samples in vivo were collected from rats from the abdominal aorta following general anesthesia. All blood samples were collected in EDTA-containing anticoagulant tubes and immediately centrifuged, aliquoted, and stored at −80°C until analysis. Clinical blood samples were stored within 6 months, as well as blood samples in vivo within 1 week.

2.7. Hematoxylin and Eosin (HE) Staining. HE staining (Solarbio, Beijing, China) was performed following previously described standard procedures [10]. Brieﬂy, sections containing the retrieved thrombi and left carotid arteries with the thrombi were stained with HE at room temperature for 1 min. Hematoxylin stains the basophilic components while eosin stains acidophilic components. Images of HE sections were acquired at 100x or 200x magniﬁcation (sham group only at 100x magniﬁcation), captured using an Eclipse TI microscope (Nikon, Tokyo, Japan), and stored in a digital format. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to measure the area proportion consisting of red blood cells or ﬁbrin (100x magniﬁcation), which represented the erythrocyte or ﬁbrin fractions in thrombi. Histopathological analysis was conducted by investigators blinded to clinical and animal data.

2.8. Immunofluorescence Staining. Immunofluorescence staining was performed to examine the red blood cells and ﬁbrin (OGEN) in thrombi following a previously reported procedure [11]. Brieﬂy, antigen retrieval was performed on sections containing the left carotid artery and thrombi by heating to 95°C for 20 min in sodium citrate buffer (pH 6). The sections were then washed three times for 5 min with PBS, blocked in 10% normal sheep serum for 30 min, and incubated overnight at 4°C with ﬁbrinogen polyclonal antibody (1:300; Thermo Fisher Scientiﬁc, Waltham, USA) in PBS. The sections were washed in PBS three times for 5 min after warming to room temperature for 30 min. Lastly, the sections were incubated with Alexa Fluor 488 donkey anti-sheep IgG (1:500; Abcam, Cambridge, UK) in PBS at room temperature for 60 min and washed three times for 5 min. Negative controls of immunofluorescence staining were achieved by omission of the primary antibody and by using the isotype primary antibody (Supplemental Figure (available here)). Omission controls and isotype controls were performed by incubating a section with the diluent of the primary antibody (PBS) and the isotype primary antibody in PBS (sheep IgG isotype control antibody (Thermo Fisher Scientiﬁc, Waltham, USA)). The red blood cells were visualized at a wavelength of 555 nm based on their inherent ﬂuorescence. Images were acquired at 400x magniﬁcation using an Eclipse TI inverted ﬂuorescence microscope and processed using NIS-Elements F software (Nikon, Tokyo, Japan). ImageJ was used to analyze the relative tightness of ﬁbrin networks at 400x magniﬁcation represented by the average of ﬁbrin ﬁbers per ﬁeld (1 μm2) in the ﬁbrin networks. The average was calculated from the number of ﬁbrin ﬁbers from three different ﬁelds in the ﬁbrin networks. Histopathological analysis was conducted by investigators blinded to the animal data.

2.9. Analysis of Blood Samples. Serum iron levels in the enrolled patients were determined using colorimetry with 2,2-dipyrididine-bipyridine (Solarbio, Beijing, China) with a previously described method [12]. Serum iron levels in rats were measured colorimetrically using an assay kit (Jiancheng Bioengineering Institute, Nanjing, China). Plasma levels of lipid oxidation products, including malondialdehyde (MDA), and antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) were measured using the corresponding enzyme-linked immunosorbent assay (ELISA) kits (Mei-mian, Jiangsu, China). The increase in lipid oxidation products and decrease in antioxidative enzyme activity indicated increasing oxidative stress levels.
2.12. Statistical Analysis. Data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Unpaired Student’s t-test or Mann-Whitney U test was used to examine the differences between two groups after checking the normality of distribution using the Kolmogorov-Smirnov test or a one-way ANOVA for multiple groups with post hoc contrasts by the Student-Newman-Keuls test, as appropriate. Linear regression was used to establish the relationship between two groups of continuous variables. GraphPad Prism (version 8.0) and SPSS (version 22.0) were used for statistical analysis. At least three independent experiments in vitro were performed in each group, for which n represents the number of rats in each group or the number of experiment repeats. p < 0.05 was considered statistically significant.

3. Results

3.1. Correlation of Serum Iron in the Erythrocyte Fraction in the Thrombi from Clinical Samples. A total of 30 patients with AIS including 17 males and 13 females were enrolled from 37 patients with AIS who underwent EVT between October and December 2019. None of the patients had a history of coagulation abnormalities or iron imbalance-related disease. For EVT, a Solitaire FR (Medtronic, USA) was used for thrombectomy.

To determine the relationship between erythrocyte fraction in thrombi and serum iron, based on our previous study [4], HE images of the retrieved thrombi were used to measure and analyze the erythrocyte fractions (Figures 1(a) and 1(b)). The basic characteristics of all patients are listed
in Table 1, and no significant differences were found between patients in the erythrocyte-rich thrombi group and fibrin-rich thrombi group. Only the serum iron levels of patients in the erythrocyte-rich thrombi group were significantly higher than those in the fibrin-rich thrombi group instead of PT, PTA, APTT, FIB, and TT, indicating that serum iron might be associated with erythrocyte-rich thrombi (Table 2). Because serum iron concentration might be influenced by differences in gender and time of blood collection as reported in previous studies [14, 15], we categorized the enrolled patients into subgroups based on gender (male or female) and time of blood collection (daytime: 8:00 to 15:00 or night: 15:00 to 8:00) to further analyze the possible interference factors. No significant differences were apparent between the gender subgroups (Figure 1(c)) and blood collection time subgroups (Figure 1(d)), suggesting that serum iron concentration was not influenced by gender or the time of blood collection in this study. We further explored the relationship between serum iron and erythrocyte fraction in thrombi, and the results from the linear regression analysis indicated a positive association (Figure 1(e)). However, it remained unclear about the causal relationship between serum iron concentration and erythrocyte fraction in thrombi.

### Table 1: Analysis of baseline characteristic in all enrolled patients.

|                          | All patients (n = 30) | Erythrocyte-rich thrombi (n = 5) | Fibrin-rich thrombi (n = 25) | p value |
|--------------------------|-----------------------|----------------------------------|-----------------------------|---------|
| Age, mean ± SD           | 63.2 ± 1.8            | 59.6 ± 1.7                       | 63.9 ± 2.2                  | 0.391   |
| Male sex, n (%)          | 17 (56.7%)            | 3 (60.0%)                        | 14 (56.0%)                  | >0.999  |
| Smoking, n (%)           | 15 (50.0%)            | 3 (60.0%)                        | 12 (48.0%)                  | >0.999  |
| Hypertension, n (%)      | 13 (43.3%)            | 1 (20.0%)                        | 12 (48.0%)                  | 0.510   |
| DM, n (%)                | 8 (26.6%)             | 2 (40.0%)                        | 6 (24.0%)                   | 0.854   |
| AF, n (%)                | 10 (33.3%)            | 1 (20.0%)                        | 9 (36.0%)                   | 0.862   |
| History of ischemic stroke, n (%) | 6 (20.0%)       | 1 (20.0%)                        | 5 (20.0%)                   | >0.999  |
| NIHSS, mean ± SD         | 14.6 ± 0.5            | 15.8 ± 0.9                       | 14.4 ± 0.6                  | 0.238   |
| ASPECTS, mean ± SD       | 8.7 ± 0.2             | 9.2 ± 0.4                        | 8.6 ± 0.3                   | 0.373   |
| IVT, n (%)               | 3 (10.0%)             | 1 (20.0%)                        | 2 (8.0%)                    | 0.453   |
| EVT beyond time window, n (%) | 6 (20.0%)       | 1 (20.0%)                        | 5 (20.0%)                   | >0.999  |
| OTP (min)                | 294.8 ± 27.7          | 244.4 ± 44.5                     | 304.9 ± 32.0                | 0.344   |
| Oclusion sites, n (%)    |                       |                                  |                             | 0.711   |
| ICA                      | 9 (30.0%)             | 1 (20.0%)                        | 8 (32.0%)                   |         |
| MCA                      | 13 (43.3%)            | 3 (60.0%)                        | 10 (40.0%)                  |         |
| BA                       | 8 (26.7%)             | 1 (20.0%)                        | 7 (28.0%)                   |         |
| Stroke cause, n (%)      |                       |                                  |                             | 0.494   |
| LAA                      | 14 (46.7%)            | 2 (40.0%)                        | 12 (48.0%)                  |         |
| CE                       | 10 (33.3%)            | 1 (20.0%)                        | 9 (36.0%)                   |         |
| Undetermined and others  | 6 (20.0%)             | 2 (40.0%)                        | 4 (16.0%)                   |         |

DM: diabetes mellitus; AF: atrial fibrillation; NIHSS: National Institutes of Health Stroke Scale; ASPECTS: Alberta Stroke Program Early Computed Tomography Score; IVT: intravenous treatment; EVT: endovascular treatment; OTP: symptom onset to groin puncture time; ICA: internal carotid artery; MCA: middle cerebral artery; BA: basilar artery; LAA: large artery arteriosclerosis; CE: cardiogenic embolism.

### Table 2: Analysis of the factors in blood test on erythrocyte-rich thrombi.

|                          | Erythrocyte-rich thrombi (n = 5) | Fibrin-rich thrombi (n = 25) | p value |
|--------------------------|----------------------------------|-----------------------------|---------|
| Serum iron (μmol/L), mean ± SD | 16.2 ± 0.8                      | 6.4 ± 0.8                   | 0.001   |
| PT (sec), mean ± SD      | 11.2 ± 0.4                       | 11.2 ± 0.2                  | 0.982   |
| PTA, mean ± SD           | 95.0 ± 6.7                       | 96.8 ± 3.4                  | 0.826   |
| APTT (sec), mean ± SD    | 35.1 ± 2.8                       | 33.0 ± 1.1                  | 0.446   |
| FIB (g/L), mean ± SD     | 2.9 ± 0.2                        | 2.9 ± 0.1                   | 0.813   |
| TT (sec), mean ± SD      | 13.9 ± 0.8                       | 15.4 ± 0.9                  | 0.290   |

PT: prothrombin time; PTA: prothrombin activity; APTT: activated partial thromboplastin time; FIB: fibrinogen; TT: thrombin time.

3.2. Correlation between Serum Iron Levels and Erythrocyte Fraction in Thrombi In Vivo. Before further investigating the correlation between serum iron levels and erythrocyte
Fraction in thrombi, serum iron concentrations should be determined after receiving the injection in vivo [16]. The serum iron levels in the HI, MI, and LI groups were significantly different than those in the NC group and increased with an increase in iron-dextrin, but there were no significant differences between the NS and NC groups (Figure 2(a)). These results suggested that serum iron levels increased significantly with an increase in the dosage of iron-dextrin.

The thrombi produced in vivo in the carotid arteries in all groups except the sham group were stained using HE.
which demonstrated that surgery to isolate the left carotid artery did not result in thrombosis [17]. Significant differences in the erythrocyte fraction were found in all groups except for the NS group, in comparison with the NC group. The highest erythrocyte fraction in the thrombi was found in the HI group, and a significant decrease was found in the MI and LI groups (Figure 2(c)). These results indicated that the erythrocyte fraction in thrombi increased with serum iron, thereby establishing the primary and secondary relationships between serum iron and erythrocyte fraction in thrombi.

Previous studies have reported that red blood cells are captured by the fibrin network during thrombus formation [18, 19]. Thus, it could be deduced that the erythrocyte fraction in thrombi was regulated by the tightness of the fibrin networks. Immunofluorescence studies showed that red blood cells in the thrombi were scattered and embedded within the fibrin network (Figure 3(a)). Based on the immunofluorescence images, we also confirmed that the erythrocyte fraction in thrombi was positively associated with the average number of fibrin fibers per field (Figure 3(b)). Furthermore, there were significant differences in the average number of fibrin fibers per field in the HI, MI, and LI groups compared with those in the NC group. No significant differences were found between the NS and NC groups with respect to fibrin fibers. In addition, the average number of
Figure 4: Correlation of serum iron and oxidative stress level in vivo. (a) Significant differences in MDA concentration between the HI (5.99 ± 0.06 nmol/L), MI (4.97 ± 0.05 nmol/L), LI (3.63 ± 0.09 nmol/L), and NC groups (2.25 ± 0.10 nmol/L) and no significant differences between the NS (2.27 ± 0.09 nmol/L) and NC groups (p = 0.779). MDA concentration was increased in the presence of serum iron. (b) Significant differences in SOD concentration between the HI (0.95 ± 0.02 ng/mL), MI, (1.16 ± 0.01 ng/mL), LI (1.29 ± 0.01 ng/mL), and NC groups (1.38 ± 0.05 ng/mL) and no significant differences between the NS (1.37 ± 0.01 ng/mL) and NC groups (p = 0.346). SOD concentration decreased in the presence of serum iron. (c) Significant differences in CAT concentration between the HI (5.30 ± 0.21 ng/mL), MI (6.73 ± 0.14 ng/mL), LI (8.64 ± 0.09 ng/mL), and NC groups (11.63 ± 0.33 ng/mL) and no significant differences between the NS (11.23 ± 0.22 ng/mL) and NC groups (p = 0.206). CAT concentration decreased in the presence of serum iron. (d) Significant differences in GSH-PX concentration between the HI (0.80 ± 0.04 ng/mL), MI (1.21 ± 0.06 ng/mL), LI (1.78 ± 0.06 ng/mL), and NC groups (2.33 ± 0.08 ng/mL) and no significant differences between the NS (2.29 ± 0.02 ng/mL) and NC groups (p = 0.639). GSH-PX concentration was decreased in the presence of serum iron. Results are expressed as mean ± SEM, n = 6; ns: p > 0.05; **p < 0.001.

3.3. Role of Oxidative Stress on the Correlation between Serum Iron and Erythrocyte Fraction in Thrombi In Vivo and In Vitro. It was certain that investigating the influence of oxidative stress on the correlation of serum iron and erythrocyte fraction was to investigate whether oxidative stress could influence the relationship between serum iron and the tightness of fibrin networks. To examine the relationship between oxidative stress and serum iron levels, MDA, SOD, CAT, and GSH-PX levels in all groups were analyzed [20, 21]. MDA levels in the HI, MI, and LI groups were significantly different from those in the NC group, whereas there were no significant differences in MDA levels between the NS and NC groups. Additionally, the increase in MDA levels was significantly associated with increased serum iron levels (Figure 4(a)). Meanwhile, SOD, CAT, and GSH-PX levels in all groups, except the NS group, were significantly different compared with those in the NC group and revealed a significantly negative association between serum iron levels and SOD levels (Figures 4(b)–4(d)). The results suggested that oxidative stress was enhanced with an increase in serum iron levels.

The Ks value was calculated to determine the pore size within the fibrin networks, wherein a smaller pore size represented a tighter fibrin network [22]. Furthermore, H₂O₂ was added to the fibrin clots to simulate the influence of oxidative stress on the fibrin network formation. The higher the H₂O₂ concentration, the higher was the oxidative stress [23]. A modified pressure-driven system to evaluate the permeability of fibrin clots is shown in Figure 5(a). We found a significant difference in Ks values of the fibrin clots with fibrin fibers per field increased with an increase in serum iron levels (Figure 3(c)). These results suggested that the erythrocyte fraction in thrombi was increased with an increase in serum iron and was attributed to the regulation of the tightness of the fibrin network.
different H₂O₂ concentrations. Fibrin clots treated with higher H₂O₂ concentrations were determined to have lower Ks values (Figure 5(b)). The smaller pore size in the fibrin networks in the fibrin clots resulting from higher H₂O₂ concentrations revealed that a tighter fibrin network was formed as the H₂O₂ concentration was increased. These results indicated that an increase in oxidative stress, as found with higher levels of serum iron, resulted in the formation of tighter fibrin networks.

To further determine the role of oxidative stress, experiments in vivo were performed wherein oxidative stress was inhibited using DL-cysteine hydrochloride [24]. HE images showed no obvious thrombi were found in the severed carotid arteries in control with DL-cysteine group, and it might be attributed to DL-cysteine which influenced FeCl₃-induced carotid artery thrombus model by inhibiting oxidative stress (Figure 6(a)). Findings from HE staining revealed that the erythrocyte fraction in thrombi in the iron group was significantly higher than that in the iron with DL-cysteine group and the control group, and that there were no significant differences between the iron with DL-cysteine group and the control group (Figure 6(b)). Immunofluorescence images showed fibrin networks in the iron group, iron with DL-cysteine group, and the control group (Figure 6(c)). The fibrin networks in the iron group were significantly tighter than those in the iron with DL-cysteine group and the control group, whereas the tightness of fibrin networks in the iron with DL-cysteine group and the control group was not significantly different (Figure 6(d)). These results suggested that inhibiting oxidative stress during iron overload may reduce the erythrocyte fraction and the tightness of fibrin networks in thrombi, which further indicated that the involvement of oxidative stress led to an increase in the erythrocyte fraction in thrombi in the presence of serum iron and could influence the fibrin networks.

4. Discussion

In this study, we investigated the relationship between the erythrocyte fraction in thrombi and serum iron from patients and also studied this aspect using experiments in vivo and in vitro. As red blood cells in thrombi were captured by the fibrin networks, we further explored the relationship between fibrin networks and serum iron. Although previous studies have reported that iron can lead to the formation of denser fibrin networks in vitro [25, 26], these studies utilized inorganic iron ions to influence the fibrin networks, which are likely in a different form than those in the body. Additionally, the published studies have not investigated whether oxidative stress is involved in the influence of iron on fibrin networks. Therefore, in this study, we used serum iron, which is similar to iron in the body, as a marker to investigate our hypothesis.

Under physiological conditions, various proteins react with iron to generate significant quantities of ROS through biological processes [27]. A previous study has established that iron overload increases local ROS generation in vessel walls via the production of both OH⁻ and H₂O₂ at the site of thrombi [3]. It was affirmed that iron played a role leading to oxidative stress in the body. Nevertheless, it was speculated in the study that ROS might be generated in blood and could directly influence fibrin networks as they were
being formed. This speculation was explained by previous findings that iron induces neutrophils in the blood to release ROS or that iron-mediated Fenton reactions could increase ROS production [28, 29]. It should be also noted that thrombi were not found in the control with DL-cysteine group. This phenomenon could be explained based on the previous study that oxidative stress leads to thrombosis in a model of FeCl₃-induced carotid artery thrombus [30] but DL-cysteine inhibits oxidative stress and prevents thrombus formation in the control with DL-cysteine group. However, the involvement of oxidative stress could be still proven by results from the other groups based on experiments in vivo with the inhibition of oxidative stress, because a similar thrombus model was used in our study.

Previous studies have reported that the production of two-stranded protofibrils and the formation of fibrin networks are involved in the fibrin polymerization process [31, 32]. Removal of the central region in fibrinopeptides and the intrinsic flexible region of the αC connector facilitates the transition from intramolecular to intermolecular interactions [33]. The crystal packing of fragment D and its covalently bound dimer might be promoted through
end-to-end associations [34]. Monomeric molecules can be connected through D-E-D interactions to form two-stranded protofibrils and the extended γ-chain in the cross-links are present externally after forming the two-stranded protofibrils [35]. First, the αC-αC domains are un tethered in the lateral connections of the protofibrils. Subsequently, the untethered αC domains from one protofibril interact with the another αC domains [36]. As the two-stranded protofibrils propagate longitudinally and reach a certain length, they aggregate laterally to form thicker fibers or multiple strands of fibers that combine to form twisted fibrin fibers and bundles and then branch off at several points to form interwoven fibrin networks [37, 38]. However, a lack of interaction sites in the αC domain of fibrinogen due to oxidative stress is considered the principal cause for impaired lateral association [39]. Dissociation of the αC domain increases intermolecular binding and enhances the interaction between the mutually aligned γ-chains [40]. Additionally, the end-linked dimers might be able to alter the conformations of the D and DD regions during fibrin assembly and lead to the formation of highly branched thinner fibers as opposed to the normal fibrin architecture that is observed due to the two-dimensional stacking behavior influenced by the primary orientation of fibrinogen [41]. Findings from atomic force microscopy from a previous study suggest that oxidative stress influences the connections of protofibrils and the crosslinking patterns between the α- and γ-chains, resulting in more branching and denser aggregation [42].

5. Limitations

There were some limitations in this study. First, statistical bias may have been present owing to the availability of limited samples from a single stroke center. However, our previous study that had enrolled more patients revealed similar findings and the current study further strengthens our previous findings. Although Ks values were used in this study to estimate the tightness of fibrin networks after H2O2 modifications in experiments in vitro, we did not obtain direct images using scanning electron microscopy. This would be needed in our further study. Lastly, we were only able to investigate the oxidative stress involved in the biological process and did not examine other factors such as ROS generation in blood or the possible signaling pathways. These mechanisms will be studied in our future studies.

6. Conclusions

Our findings demonstrated that oxidative stress was involved in the biological process that erythrocyte fraction in thrombi was increased with serum iron by influencing fibrin network formation.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

The study design was done by Yan Feng, Yongli Li, and Ru-yan Li. Data collection was done by Mingli Liu, Minghui Chen, and Zhongfei Hao. The operators of endovascular treatment were Ruiyan Li, Yan Feng, and Qingbin Li. Experiments in vivo were done by Mingli Liu. Experiments in vitro were done by Mingli Liu. Data analysis was done by Mingli Liu, Minghui Chen, and Zhongfei Hao. Writing was done by Mingli Liu. Revisions were done by Qingbin Li and Yongli Li. All authors read and approved the final manuscript. Mingli Liu, Minghui Chen, and Zhongfei Hao contributed equally.

Supplementary Materials

Supplemental Figure: omission of the primary antibody and the isotype primary antibody for negative controls of immunofluorescence staining. (Supplementary Materials)

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