

Thrombopoietin accumulation in hepatocytes induces a decrease in its serum levels in a sinusoidal obstruction syndrome model

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Abstract. Sinusoidal obstruction syndrome (SOS) is a type of fatal hepatic injury, which predominantly occurs following exposure to drugs, such as oxaliplatin, or bone marrow transplantation. Extravasated platelet aggregation (EPA) plays an important role in the development of SOS in rat and mouse models. Furthermore, platelets invading the space of Disse adhere to hepatocytes and are phagocytized in patients with SOS. Aging platelets and platelets in patients with sepsis are phagocytized by hepatocytes through Ashwell-Morell receptors, and thrombopoietin (TPO) is produced by the JAK2-STAT3 signaling pathway. The purpose of the present study was to examine the significance of TPO as a biomarker of SOS. SOS was induced in Crl:CD1(ICR) female mice by intra-peritoneal administration of monocrotaline (MCT). TPO levels were measured in the serum and liver tissue. Pathological and immunohistochemical studies of the liver were performed to analyze the expression levels of TPO. TPO mRNA expression levels were measured using reverse transcription-quantitative PCR. In the SOS model, the platelet counts in peripheral blood samples were significantly decreased at 24 and 48 h after MCT treatment as compared with that at 0 h. In addition, a pathological change in hepatic zone 3 was observed in the SOS model group. Furthermore, the protein levels of TPO in liver tissue were significantly increased in the SOS model group compared with those in the control group, which was confirmed by immunohistochemistry. By contrast, serum TPO protein levels were significantly decreased in the SOS model group compared with those in the control group. These results indicated that EPA may induce sinusoidal endothelial

fenestration in a mouse model of SOS, preventing TPO from translocating into the blood. In conclusion, serum TPO levels may be reduced in a mouse model of SOS owing to the accumulation in hepatocytes, suggesting that TPO could be a useful biomarker of SOS.

Introduction

Sinusoidal obstruction syndrome (SOS), also called central venous occlusion, is caused by severe liver damage following liver transplantation, hematopoietic stem cell transplantation and some anticancer drug treatments (1-8). SOS develops mainly in zone 3 of the hepatic acinus and exhibits portal hypertension symptoms, such as splenomegaly and thrombocytopenia (9,10); notably, zone 3 is more susceptible to drugs due to the low amount of glutathione, which is responsible for drug detoxification (8,11).

The presence of platelet aggregation in the space of Disse around zone 3 has been reported in the livers of transplant recipients who developed SOS, and in patients with colorectal liver metastasis who received preoperative chemotherapy with oxaliplatin-based regimens (1-3). This phenomenon is known as extravasated platelet aggregation (EPA) (1-3).

In a monocrotaline (MCT)-induced SOS rat model, EPA has been detected in the space of Disse, and the effect of anti-platelet drugs (PDEIII inhibitors) on the prevention and reduction of SOS has been reported (12,13). In addition, the severity of SOS can be reduced by the protective effect of recombinant thrombomodulin on liver endothelial cells (LSECs) in a SOS mouse model (14,15). Based on these findings, LSEC detachment and EPA may serve important roles in the mechanism of SOS; however, it is difficult to predict the onset of SOS, determine its severity and assess therapeutic approaches. Some biomarkers of SOS have been reported, such as urine metabonomic profiles; however, none are specific (16). Therefore, it is necessary to identify biomarkers to determine the status of SOS.

We previously reported that platelets invading the space of Disse not only adhere to hepatocytes but are also taken up into the hepatocytes of patients who develop SOS after liver transplantation (2). Several reports on the clearance of platelets have described mechanisms involving Fc receptor-mediated

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splenic macrophages, integrins, apoptosis and Ashwell-Morell receptors (AMR) (17). Aging platelets and platelets in patients with sepsis are phagocytized by hepatocytes through AMR and thrombopoietin (TPO), which is produced by the JAK2-STAT3 signaling pathway (18).

TPO is a hematopoietic factor that promotes the proliferation and differentiation of megakaryocytes. TPO was successfully cloned by multiple groups in 1994 (19). TPO is produced mainly by hepatocytes, where the amino acid sequence in the N-terminal region binds to cell surface-expressed receptors (c-Mpl) on platelets and megakaryocytes (19). Upon binding to the megakaryocyte receptor, TPO induces activation of multiple signaling pathways, including JAK, STAT and MAP kinases, suppresses megakaryocyte apoptosis, and increases megakaryocyte number, size and nuclear ploidy; as a result, platelet production is enhanced (19). Two TPO receptor agonists, romiplostim and eltrombopag, are currently approved for the treatment of idiopathic thrombocytopenic purpura, and their use is increasing worldwide. In previous years, it has also been suggested that TPO receptors may be an effective treatment for aplastic anemia (19,20).

The aim of the present study was to examine the significance of TPO levels as a biomarker of SOS in a drug-induced SOS mouse model.

Materials and methods

Reagents. A 20 mg/ml solution of MCT (Wako Pure Chemical Industries, Ltd.) was prepared by dissolving 1,000 mg MCT in 1.0 N HCl, and the pH was adjusted to 7.4 with 0.5 N NaOH. The solution was diluted in phosphate-buffered saline (PBS; pH 7.4), to increase the total volume to 50 ml (21).

Animal model of SOS. A total of 54 female Crl:CD1 mice (Charles River Laboratories, Inc.) weighing 20–30 g at 6–8 weeks of age were used in this study. The Animal Research Committee of Kanazawa University (Kanazawa, Japan) approved all experiments (approval no. 183934). Mice were maintained under the following conditions: Temperature, 23±3°C; humidity, 55±10%; 12-h light/dark cycle, 8:45 lights on, 20:45 lights off; access to food and water, *ad libitum*. The mice were randomly divided into two groups: SOS model and control (n=27/group). After fasting for 12 h, MCT (270 mg/kg) was administered via intraperitoneal injection in the SOS model group, and the control group received the same volume of PBS (14,15,22). The mice were sacrificed 12, 24 and 48 h after MCT or PBS treatment (n=16 mice sacrificed at each time point, n=8/group), and blood samples (~1 ml) and liver tissues were collected. The blood samples were centrifuged at 1,500 × g for 15 min at 4°C to collect serum. Liver tissue lysate was collected as follows: Liver tissue was homogenized with RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM sodium chloride; 0.5 w/v% sodium deoxycholate; 0.1 w/v% sodium dodecyl sulfate; 1.0 w/v% NP-40 substitute) containing a protease inhibitor; while cooling on ice, sonication at 19.5–20.5 kHz was performed approximately three times (10–15 sec each). After storage on ice, the sample was centrifuged at 15,000 × g for 15 min at 4°C and the supernatant was collected. All animals were monitored for health and behavior once a day. Animals were euthanized by decapitation, and the

cause of death was respiratory and cardiac arrest. In addition, 15 mice were used for surgical stabilization and optimization of SOS modeling.

Biochemical analysis. Platelet counts were measured using an automated blood cell counter (Celltac α MEK-6458; Nihon Kohden). Serum and liver tissue concentrations of TPO were measured using ELISA (cat. no. MTP00; R&D Systems, Inc.). ELISA was performed according to the manufacturer's protocol.

Liver histology. The liver tissues were fixed in 10% neutral buffered formalin at room temperature overnight and embedded in paraffin. Slides were prepared (4 μm) and were stained with hematoxylin and eosin. Hematoxylin staining was performed for 4 min and eosin staining was performed for 2 min at room temperature. All sections were examined using a BX51 light microscope (Olympus Corporation).

Immunohistochemistry. The liver tissues were fixed in 4% paraformaldehyde in PBS for 3 days at room temperature and embedded in O.C.T. compound solution (Sakura Finetek Japan Co., Ltd.) and 30% sucrose in 0.1 M phosphate buffer (pH 7.4) containing 0.05% NaN₃. All tissue samples were sectioned at 6 μm using a cryostat (Thermo Fisher Scientific, Inc.). Slides were immunostained with a primary antibody against TPO (1:200; cat. no. ab216884; Abcam). Briefly, deparaffinized sections were autoclaved with 10% citric acid buffer (pH 8.0) at 120°C for 15 min. After treatment with Peroxidase Blocking Solution containing 10% Na₃PO₄, 5% H₂O₂, 3% NaH₂PO₄ and H₂O (Agilent Technologies, Inc.) for 5 min at room temperature, sections were incubated with a primary antibody at 4°C overnight. After the sections were washed in PBS, the sections were incubated with EnVision+ Single Reagent (HRP, Rabbit) (cat. no. K4003, not diluted; Agilent Technologies, Inc.) for 45 min at room temperature. Immunoreactivity was visualized using EnVision reagent (Agilent Technologies, Inc.), and the slides were developed with diaminobenzidine and counterstained with hematoxylin for 1 min at room temperature. All sections were examined using a BX51 light microscope (Olympus Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from liver tissue using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using the Affinity Script qPCR cDNA Synthesis Kit (Agilent Technologies, Inc.) according to the manufacturer's protocol. Amplification and real-time fluorescence detection were performed using the Mx3005P Real-Time QPCR system (Stratagene; Agilent Technologies, Inc.) with the following protocol: Activation step (95°C, 10 min), followed by 40 cycles of denaturation (95°C, 30 sec), annealing (55°C, 60 sec), and extension (72°C, 60 sec). The relative abundance of transcripts was normalized according to the expression of β-actin using the 2^{-ΔΔC_q} method (23). The primer sequences were as follows: TPO forward, 5'-CACAGCTGTCCCAAG CAGTA-3' and reverse, 5'-CATTCACAGGTCCGTGTGTC-3'; and β-actin forward, 5'-TCCATCGAAGTGTGACGT-3' and reverse, 5'-GAGCAATGATCTTGATCTTCAT-3'.

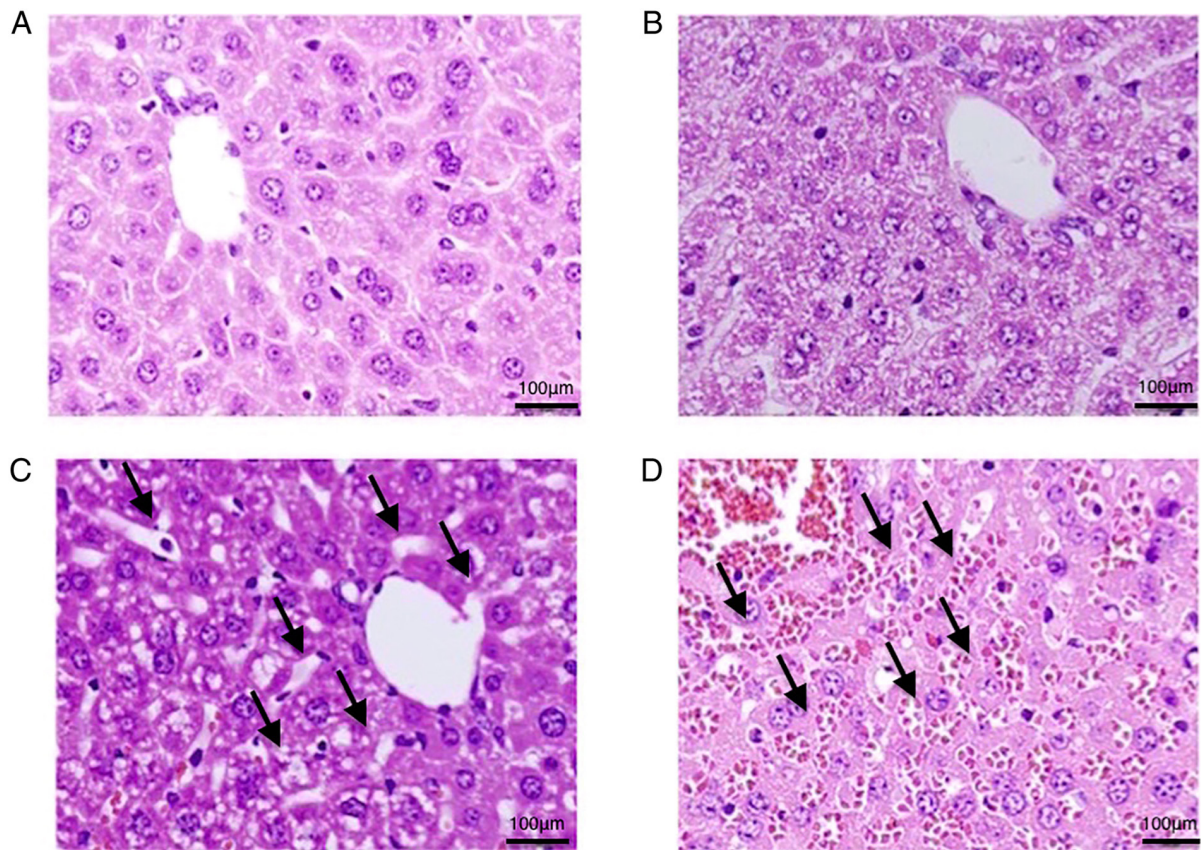


Figure 1. Pathological findings in the liver after induction of sinusoidal obstruction syndrome with MCT. The liver tissue samples were collected at (A) 0, (B) 12, (C) 24 and (D) 48 h after MCT administration. Liver tissue sections were stained with hematoxylin and eosin. Black arrows indicate sinusoid dilation in C and red blood cells in the dilated sinusoid in D. These changes were not observed 0 or 12 h after MCT treatment. In both groups, tissues from three animals were stained at each time point and representative images are shown. MCT, monocrotaline.

Statistical analysis. All results are expressed as the mean \pm standard deviation. The groups were compared using one-way ANOVA followed by Dunnett's test or two-way ANOVA followed by Tukey's test. All analyses were performed using SPSS II 23.0 software (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Pathological findings in the SOS model. Sinusoid dilation 24 h after MCT treatment was observed in zone 3. Red blood cells could be observed in the dilated sinusoid at 48 h, indicating congestion. These findings are pointed to by black arrows. These changes were not observed at 0 and 12 h (Fig. 1).

Platelet counts are decreased in the SOS model. The platelet counts in peripheral blood samples were significantly decreased after 24 h in the SOS model (0 h, $79.8 \pm 4.0 \times 10^4$; 12 h, $63.8 \pm 9.7 \times 10^4$; 24 h, $41.2 \pm 10.3 \times 10^4$ and 48 h, 21.6×10^4 ; $P = 0.337$, $P = 0.0231$ and $P < 0.001$, respectively) (Fig. 2). Pathological changes and thrombocytopenia was confirmed in the SOS model, which is consistent with the results of previous reports (14,15,22).

TPO mRNA expression are not affected in the SOS model. TPO mRNA expression levels in liver tissues were not

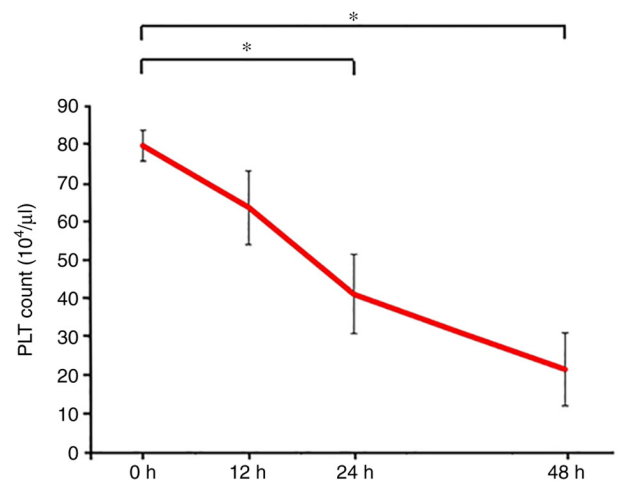


Figure 2. PLT counts in peripheral blood in the sinusoidal obstruction syndrome model. Blood samples were collected at 0, 12, 24 and 48 h after MCT treatment. PLT counts significantly decreased at 24 and 48 h after MCT administration as compared with that at 0 h. * $P < 0.05$. Data were analyzed by one-way ANOVA and Dunnett's test. Error bars indicate standard error. MCT, monocrotaline; PLT, platelet.

significantly different between the SOS model and control groups. Non-significant increases in TPO levels were detected in both groups at 48 h (vs. control group at 48 h, $P = 0.0731$; vs. 0h SOS model group, 0.521; Fig. 3).

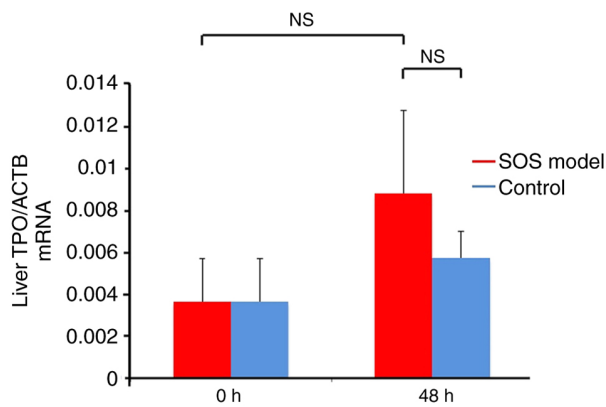


Figure 3. Liver TPO mRNA expression levels. Liver tissue samples were collected 0 and 48 h after MCT or PBS administration. TPO mRNA expression levels in the SOS model showed no significant difference as compared with those in the control group, but there was a tendency to increase over time. Data were analyzed by two-way ANOVA and Tukey's test. Error bars indicate standard error. NS, not statistically significant; SOS, sinusoidal obstruction syndrome; MCT, monocrotaline; ACTB, β -actin; TPO, thrombopoietin.

TPO protein levels are increased in the SOS model. The TPO protein levels in liver tissue increased over time, especially at 48 h, in the SOS model group. By contrast, TPO protein levels did not increase in the control group (12 h: 4.37 ± 2.09 pg/mg vs. 1.46 ± 0.52 pg/mg, $P=0.891$; 24 h: 6.18 ± 1.11 pg/mg vs. 1.15 ± 0.33 pg/mg, $P=0.480$; 48 h: 9.61 ± 2.71 pg/mg vs. 1.49 ± 0.34 pg/mg, $P=0.037$; Fig. 4).

To verify these findings, immunohistochemistry was also performed. The TPO protein expression levels were verified by immunohistochemistry in the SOS model group 48 h after treatment (Fig. 5). A representative image of a slide created from multiple mice is shown. Similar images were seen in all SOS model mice.

TPO serum levels are decreased in the SOS model. In contrast to TPO protein levels in liver tissues, the serum levels of TPO were significantly decreased in the SOS model group at 48 h compared with in the control group. By contrast, in the control group, TPO serum levels in peripheral blood showed no change over time. (12 h: $1,439.3 \pm 163.5$ pg/ml vs. $1,575.2 \pm 196.0$ pg/ml, $P=0.999$; 24 h: 987.4 ± 112.2 pg/ml vs. $1,909.7 \pm 369.3$ pg/ml, $P=0.091$; 48 h: 596.0 ± 133.8 pg/ml vs. $1,644 \pm 313.5$ pg/ml, $P=0.022$; Fig. 6).

Discussion

SOS is a fatal complication following liver transplantation, hematopoietic stem cell transplantation and treatment with anticancer drugs. Morbidity ranges from 5 to >50%, and the mortality rate of severe SOS is as high as 80% (24).

In the present study, dilation of the sinusoid was observed in the liver in a SOS mouse model after 24 h and congestion of the sinusoid was observed after 48 h. In our previous studies, destruction and fibrosis of LSECs were detected 48 h after SOS induction (12-15,22).

Decreased platelet counts in the peripheral blood are generally thought to result from hypersplenism due to portal hypertension in patients with SOS; however, thrombocytopenia has been reported to occur before splenomegaly (3). The cause of early thrombocytopenia in patients with SOS could

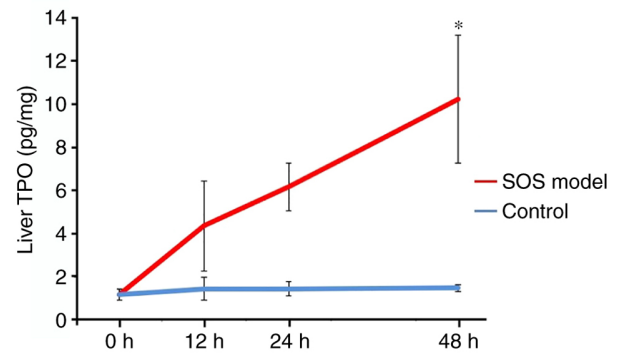


Figure 4. Liver TPO protein levels. Liver tissue samples were collected at 0, 12, 24 and 48 h after MCT or PBS administration. TPO levels increased significantly at 48 h in the MCT group as compared with those in the control group. * $P<0.05$ vs. control. Data were analyzed by two-way ANOVA and Tukey's test. Error bars indicate standard error. SOS, sinusoidal obstruction syndrome; MCT, monocrotaline; TPO, thrombopoietin.

be explained by the consumption of platelets due to EPA in the liver.

Biomarkers that predict the onset of SOS and the effectiveness of therapies following SOS onset have not yet been established. To the best of our knowledge, the present study was the first to focus on TPO, which is involved in platelet homeostasis. Aging platelets and senescent platelets in a septic environment are desialylated, internalized by hepatocyte AMR and induce TPO expression via the JAK2-STAT3 signaling pathway (18). Platelets that invade the space of Disse not only adhere to hepatocytes but are taken up by hepatocytes in the liver of patients with SOS following liver transplantation (2). The present study hypothesized that when EPA-induced platelets are taken up by hepatocytes, the serum levels of TPO may increase. In the present study, peripheral blood platelet counts were significantly decreased 24 h after induction of SOS in a mouse model, as previously reported (15). Unexpectedly, serum TPO levels were significantly reduced in the SOS mouse model, despite increased TPO levels in the liver. This discrepancy may be due to the prevention of TPO produced in the liver from being transferred to the blood under SOS conditions, resulting in TPO protein accumulation in liver tissue. Although slightly increased, the mRNA expression levels of TPO were not significantly altered in the SOS model. Thus, TPO changes in the liver may be largely due to the influence of physiological expression. That is, although the increase in TPO production by platelet uptake in the SOS model was not significant, the blood concentration decreased as a result of inhibition of the secretion of physiologically produced TPO.

Unlike general capillary endothelial cells, LSECs do not have a basement membrane. Instead, the space of Disse is present between sinusoidal endothelial cells and hepatocytes. In addition, the combined sinusoidal endothelial cells have a number of fenestrae with a diameter of ~100 nm in the cell gap, through which various substances are exchanged between blood flowing through the sinusoid and hepatocytes (25-27). Narita *et al* (21) reported that overexpression of CD34 within liver parenchyma was associated with an abnormal indocyanine green retention rate. This association was due to SOS-induced liver damage caused by chemotherapy for colorectal liver metastasis. The SOS was caused by impaired

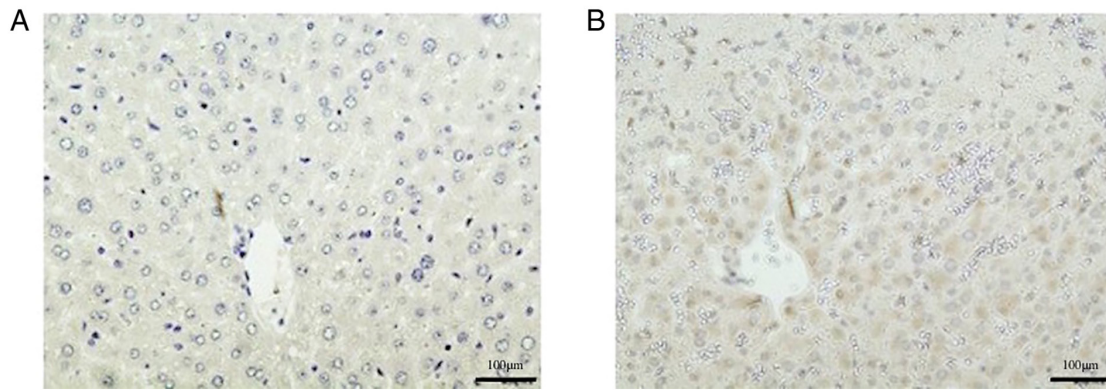


Figure 5. Immunohistochemistry of TPO. Liver tissue samples were collected at (A) 0 and (B) 48 h after monocrotaline administration. Liver sections were stained with an antibody against TPO. Staining was enhanced at 48 h as compared with that at 0 h. TPO is shown as brown staining. Representative images of three slides. TPO, thrombopoietin.

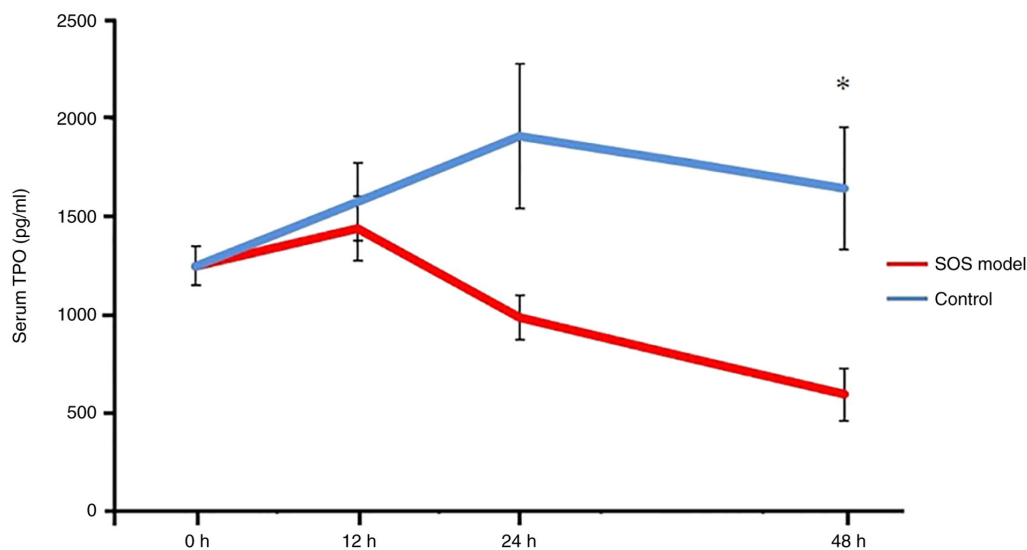


Figure 6. Serum TPO protein expression levels. Blood samples were collected at 0, 12, 24 and 48 h after MCT or PBS administration. TPO levels significantly decreased 48 h after MCT administration in the SOS model group as compared with that in the control group. * $P < 0.05$ vs. control. Data were analyzed by two-way ANOVA and Tukey's test. Error bars indicate standard error. SOS, sinusoidal obstruction syndrome; MCT, monocrotaline; TPO, thrombopoietin.

substance exchange due to capillary vascularization of the sinusoids. This situation, known as sinusoidal capillarization, can result in the deposition of a dense extracellular matrix in the space of Disse, the formation of a basement membrane and the disappearance of small pores in the endothelial cells (25-27). Sinusoidal capillarization in SOS model rats was previously reported by immunostaining with CD34, which was suppressed by PDE III inhibitors (12,13). Plasminogen activator inhibitor-1 and transforming growth factor- β released by EPA-activated platelets have been shown to promote hepatic fibrosis but may also contribute to sinusoidal capillarization (2,3,12,13). In the present study, TPO secretion may be impaired by capillary angiogenesis, but at an earlier stage, EPA aggregation may cause substance exchange failure. In the future, further investigations, such as confirmation of platelet localization in hepatocytes by immunostaining and TPO accumulation by electron microscopy, are required. In addition, although the model in the present study was a model of acute-phase SOS, further investigation is needed for the chronic phase with fibrosis.

In conclusion, the present study analyzed the involvement of TPO in platelet homeostasis and revealed that serum TPO levels were significantly reduced in a mouse model of SOS compared with those in a control group at 48 h; therefore, TPO may be used as a biomarker for future clinical studies.

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Availability of data and materials

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

Authors' contributions

HY, HidT, YY and TO designed this study. SM taught the experimental methods of RT-qPCR and ELISA. HY performed the experiments. SM, MO, YO, SN, IM, HirT and TM contributed to the data analysis. All authors have read and approved the final manuscript. HY and HT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All institutional and national guidelines for the care and use of laboratory animals were followed. Animals were treated in accordance with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University (approval no. 183934).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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