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Haptoglobin Reduces Inflammatory Cytokine INF-γ and Facilitates Clot Formation in Acute Severe Burn Rat Model

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Haptoglobin exerts renal protective function by scavenging free hemoglobin from the urine and blood stream in patients with hemolytic disorders. Recent studies elucidate the relationships between haptoglobin and inflammation. In addition, coagulopathy is often induced by systemic inflammation characterized by the presence of vascular endothelial damage. We hypothesize that haptoglobin might have an anti-inflammatory effect and affect hypercoagulability using rat burn model. Thirty anesthetized rats of six-weeks of age received over 30% full-thickness scald burn on the dorsal skin surface. All rats were injected with either haptoglobin (Hpt) or normal saline (NS) intraperitoneally. The rats were divided into three groups: 1) control group (NS 20 mL/kg); 2) low concentration of Hpt group, L-Hpt, (Hpt 4 mL (80 U)/kg+NS 16 mL/kg); and 3) high concentration of Hpt group, H-Hpt, (Hpt 20 mL (400 U)/kg). While under anesthesia, all rats were euthanized by exsanguination at 6 hours (N=5) and 24 hours (N=5). Inflammatory and anti-inflammatory cytokines were measured and whole-blood viscoelastic tests were performed by thromboelastometry (ROTEM). Haptoglobin significantly reduced free hemoglobin 24 hours after the injury. Improvement of hematuria was confirmed in the H-Hpt group. There were no differences in thrombin-antithrombin complex and plasmin-α2 plasmin inhibitor complex. The haptoglobin tended to decrease interferon-gamma (IFN-γ) in H-Hpt group. ROTEM findings of the L-Hpt group showed significantly higher clot firmness and shorter time to maximum clot formation velocity than the control group. Haptoglobin reduced INF-γ, and accelerated speed of clot formation in acute phase of severe burn. (J Nippon Med Sch 2017; 84: 64–72)

Key words: haptoglobin, severe burn, IFN-γ, clot formation, thromboelastometry

Introduction

Severe burn injury induces systemic inflammation and results in intravascular hemolysis. Free hemoglobin (Hb), which is released into the blood stream, binds to haptoglobin, a hemoglobin scavenger, and is eventually degraded in the liver[1,2]. Although massive hemolysis accelerates the consumption of haptoglobin, unbound free Hb goes through the renal glomerulus and is reabsorbed in the renal tubular epithelial cells where free radicals can damage epithelial cells[3].

In Japan, haptoglobin (Japan Blood Products Organization, Tokyo, Japan) has been approved for hemoglobinemia and hemoglobinuria following profound hemolysis in patients with severe burn, a massive transfusion, or open-heart surgery using extracorporeal circulation. One of the main effects of haptoglobin is to protect renal function by means of scavenging free hemoglobin from the urine and blood stream[4]. Recent studies show that

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haptoglobin has anti-inflammatory effects when hepatocytes are stimulated by various kinds of inflammation[5–8]. Supplementation of haptoglobin prevents infection related acute lung injury, and improves organ dysfunction and clinical outcome in animals and human[9–11]. Furthermore, a closed bi-directional relationship between inflammation and coagulation is known in various severely ill patients[12,13]. Hypercoagulability evoked by severe thermal injury is often related to a disseminated intravascular coagulation (DIC) and multiple organ failure[14,15]. However, it is unclear that the impact of haptoglobin on the association between inflammation and coagulation in acute phase of severe burn. In this study, we hypothesized that haptoglobin might affect the burn related inflammation and hypercoagulability using rat severe burn model.

Materials and Methods

Animals

All protocols are approved by the Institutional Ethics Committee for animal experiments of Saga University (No.240340). Six-week-old male Sprague-Dawley rats (Kyudo. Co., Ltd., Saga, Japan) weighing 222.4 to 249.2 g were used in this study. Animals were allowed to acclimate to their surroundings for 5 days. Commercial rat chow and tap water were available freely throughout the experimental periods.

Rat Burn Injury Model and Study Design

The burn model in this study produced full-thickness skin scald on about 30% of the total body surface area under inhalation anesthesia with isoflurane[16]. Thirty rats were assigned to normal saline (NS) group (n=10), low concentration of haptoglobin (L-Hpt) group (n=10), and high concentration of haptoglobin (H-Hpt) group (n=10). All rats were intraperitoneally injected either with 20 mL/kg of normal saline (NS) or 80 units (4 mL)/kg of haptoglobin and 16 mL/kg of normal saline (L-Hpt), 400 units (20 mL)/kg of haptoglobin (H-Hpt), respectively, at the same time of the burn injury. The 80 units/kg of haptoglobin is a recommended dose for clinical use in case of massive hematuria, and 400 units/kg was used as a high dose according to the drug information company provided[17]. We selected an intraperitoneal route to administrate every drug because it was more simple and faster than the intravenous and oral routes. All injured rats were observed in their cages until recovering from anesthesia. Local anesthesia (2% lidocaine jelly) was applied to the burn wound, in addition to gauze and bandages. All animals were euthanized by exsanguination under deep anesthesia at 6 hours (n=5 per group) and 24 hours (n=5 per group) after the burn injury.

Evaluation of Hematuria, Hemolysis, and Hypercoagulability

Urine samples were obtained by bladder puncture immediately after exsanguination. We determined the presence of hematuria by Uro-Paper III (EIKEN Chemical CO., LTD., Tokyo, Japan). The degree of hematuria was interpreted as "none (-)," "mild (+/- or +)," and "severe (++ or +++)," respectively. We also checked the level of free Hb (colorimetric methods; BML, Inc., Tokyo, Japan) in the plasma to evaluate severe burn-induced hemolysis. Plasma thrombin-antithrombin complex (TAT; enzyme immunoassays; BML, Inc., Tokyo, Japan) and plasmin-α2 plasmin inhibitor complex (PIC; latex agglutination; BML, Inc., Tokyo, Japan) values were measured in order to confirm hypercoagulability and hyperfibrinolysis in the severe burn model.

Bio-Plex® Cytokines Array Assay

To evaluate the activated induction of inflammatory cytokines, plasma samples were tested for multiple cytokines using the Bio-Plex Pro Rat Cytokine Th1/Th2 Assay (Bio-Rad Laboratories, California, USA). The cytokines tested include interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). The values below the detection limits were defined as zero.

Thromboelastometry (ROTEM®) Analysis

Whole blood viscoelastic tests were performed using ROTEM (Tem International GmbH, Munich, Germany) within 90 minutes of blood sampling. We evaluated the extrinsic coagulation pathway, which was mildly activated with a tissue factor (EXTEM). Parameters analyzed in the EXTEM include the clotting time (CT), the amplitude at 10 min (A10), 20 min (A20) after the CT, maximum clot firmness (MCF), clot formation time (CFT), the alpha angle (α), the maximum Lysis (ML), and the time to maximum clot formation velocity (MAXV-t). The ROTEM was run more than 60 minutes at 37℃.

Statistical Analysis

To evaluate the statistical differences of every parameter between the groups, non-parametric statistics were performed. The p values were derived from the Mann-Whitney U test and Kruskal Wallis test for continuous variables after Bonferroni correction for multiple comparisons. A Chi-square test for categorical variables was also used. Values of $p<0.05$ were considered significant. The statistical analysis was performed using IBM SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Of the 30 rats, one rat of the NS group died 30 minutes after the burn injury even though all procedures were performed in the same manner as in the other rats. The cause of death was considered to be due to an acute circulatory collapse. The other rats survived throughout the study.

Hemolysis and Hematuria in Severe Burn Injury

Twenty-eight rats had severe macrohematuria six hours after the injury and one rat in the H-Hpt group showed mild macrohematuria. There was no statistical significance. Interestingly, the macrohematuria was significantly improved in the H-Hpt group 24 hours after injury ($p<0.05$, **Table 1**). Furthermore, haptoglobin significantly decreased free Hb values at 24 hours after the injury, as compared with values at six hours of the injury ($p<0.01$). However, there was no concentration-related effect of haptoglobin (**Fig. 1**).

Table 1 The degree of hematuria after the administration of haptoglobin or normal saline in the severe burn model

		NS	L-Hpt	H-Hpt	p value
6 hours	none	0	0	0	0.343
	mild	0	0	1	
	severe	5	5	4	
24 hours	none	0	0	0	0.027
	mild	1	0	4	
	severe	3	5	1	

Abbreviations: NS, normal saline; L-Hpt, low concentration of haptoglobin; H-Hpt, high concentration of haptoglobin.

Plasma TAT and PIC Values in Severe Burn Injury

TAT is known as a parameter of hypercoagulability, especially in the case of severe burn injury. Although there were no significant statistical differences, the groups administered with haptoglobin showed smaller variations of TAT value compared with that in the NS group (**Fig. 1**). PIC, an indicator of fibrinolysis, fell below the measurable limits in all rats (<0.2 μg/mL, data not shown).

Cytokine Analysis by BioPlex

Results of cytokine measurements by BioPlex revealed that every cytokine showed a characteristic pattern reflecting systemic inflammation induced by severe thermal stress, although there were a few which had statistical significance (**Fig. 2**, **Table 2**). Most of the cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α) six hours after injury in the NS group tended to increase at 24 hours after injury, whereas the others (IL-2, IL-4, and IL-5) did not. On the other hand, all cytokines except IL-6 and TNF-α in the L-Hpt group decreased during the same time period. The H-Hpt group showed the same tendency except IL-6 (**Table 2**). Especially in the L-Hpt group, there were significant decreases in IL-5 ($p<0.05$) and IL-10 ($p<0.01$) and a significant increase in IL-6 ($p<0.05$) 24 hours after the injury when compared with the values at six hours (**Fig. 2**). Furthermore, although no significant differences in the level of cytokines were confirmed at six hours of injury in all groups, haptoglobin tended to decrease IFN-γ at 24 hours after injury in the high group compared with the NS group ($p=0.062$)

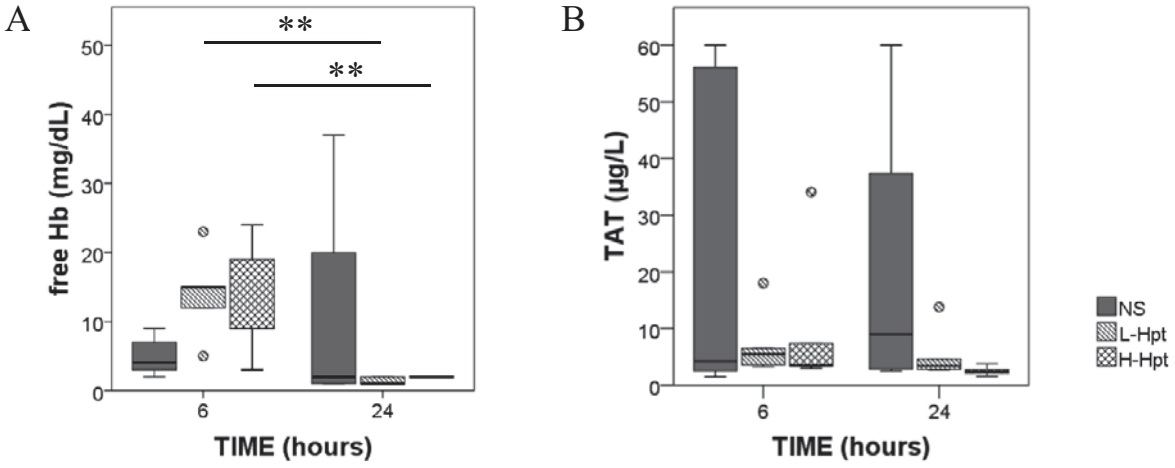

Fig. 1 Free Hb (A) and TAT values (B) in a severe burn model.
Haptoglobin significantly decreased free Hb after 24 hours compared with six hours without concentration dependency. In addition, haptoglobin administration groups showed smaller variations of TAT value compared with NS group. However, there was no statistical significance within each group.
Hb, hemoglobin; TAT, thrombin-antithrombin complex; NS, normal saline; L-Hpt, low concentration of haptoglobin; H-Hpt, high concentration of haptoglobin. **, $p<0.01$.

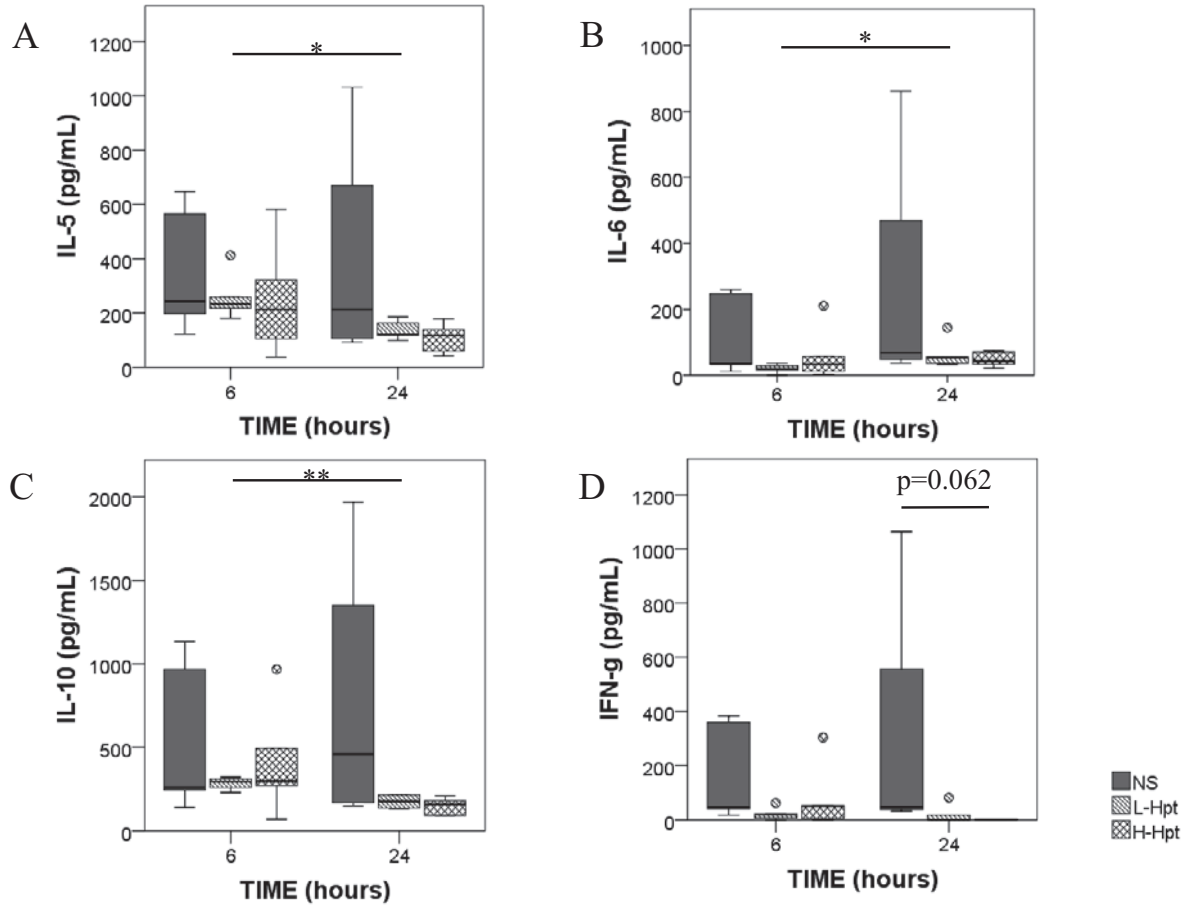

Fig. 2 The effect of haptoglobin on inflammatory cytokines in a severe burn model.
In the L-Hpt group, IL-5 (A), IL-6 (B) and IL-10 (C) showed significant differences after 24 hours of thermal injury compared with six hours. On the other hand, haptoglobin treatment tended to decrease IFN-γ (D) after 24 hours of the injury compared with NS group.
IL, interleukin; IFN-g, interferon gamma; NS, normal saline; L-Hpt, low concentration of haptoglobin; H-Hpt, high concentration of haptoglobin. *, $p<0.05$; **, $p<0.01$.

Table 2 The effects of various concentrations of haptoglobin on inflammatory cytokines by Bio-Plex analysis

	NS			L-Hpt			H-Hpt		
	median after 6 hours (pg/mL)	median after 24 hours (pg/mL)	rate of change (%)	median after 6 hours (pg/mL)	median after 24 hours (pg/mL)	rate of change (%)	median after 6 hours (pg/mL)	median after 24 hours (pg/mL)	rate of change (%)
IL-1a	51.46	61.50	119.51	40.39	20.63	51.08	26.26	0.00	0.00
IL-1b	430.10	532.22	123.74	420.95	243.28	57.79	434.69	177.88	40.92
IL-2	193.12	164.17	85.01	185.69	63.19	34.03	178.14	41.49	23.29
IL-4	19.42	0.00	0.00	9.97	0.00	0.00	0.00	0.00	
IL-5	243.88	213.35	87.48	232.87	120.27	51.65	213.43	116.86	54.75
IL-6	34.83	68.17	195.72	19.66	53.77	273.50	33.00	43.24	131.03
IL-10	259.43	459.50	177.12	294.94	177.27	60.10	297.71	156.81	52.67
IL-12	66.91	78.33	117.06	14.23	12.31	86.51	44.83	8.37	18.67
IL-13	109.97	125.89	114.47	106.04	49.99	47.14	109.97	38.71	35.20
IFN-g	45.95	46.64	101.49	19.28	0.00	0.00	50.16	1.83	3.65
TNF-a	28.08	36.16	128.77	18.12	19.56	107.95	29.35	659.00	22.45

Abbreviations: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; NS, normal saline; L-Hpt, low concentration of haptoglobin; H-Hpt, high concentration of haptoglobin.

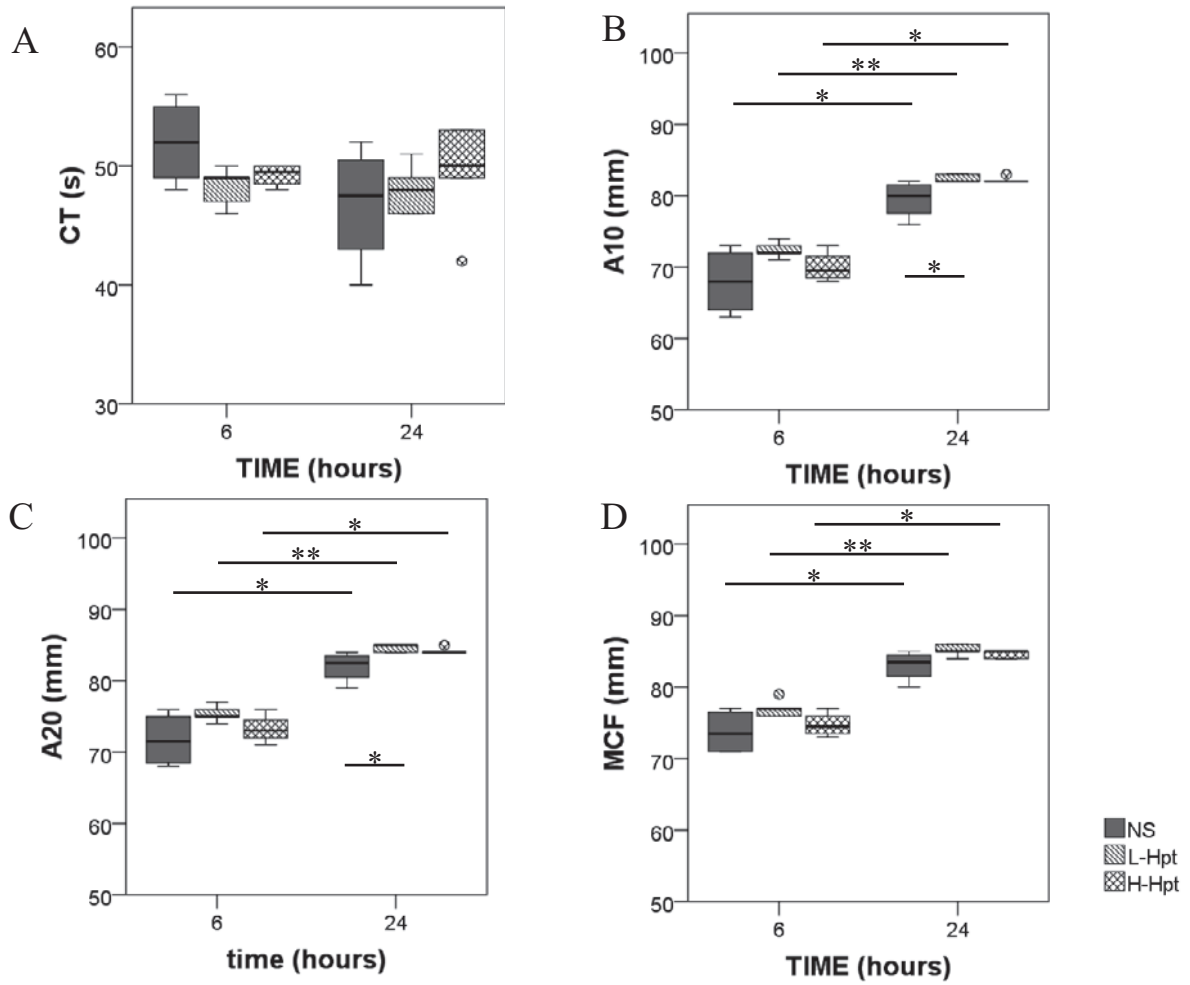

Fig. 3 The effect of haptoglobin on the initiation of coagulation and strength of clot in a rat severe burn model.
A10 (B), A20 (C) and MCF (D) of all groups significantly increased at 24 hours post injury compared with 6 hours, although no statistical difference was confirmed in the CT (A). Moreover, haptoglobin significantly increased A10 and A20 at 24 hours after the injury compared with NS group.
CT, clotting time; A, amplitude; MCF, maximum clot firmness; NS, normal saline; L-Hpt, low concentration of haptoglobin; H-Hpt, high concentration of haptoglobin. *, $p<0.05$; **, $p<0.01$.

Burn Induced Hypercoagulability by ROTEM

The thromboelastometric analysis showed that A10, A20 and MCF in all groups were significantly higher at 24 hours after burn injury than those at six hours (NS; $p<0.05$, L-Hpt; $p<0.01$, H-Hpt; $p<0.05$, **Fig. 3**). Moreover, statistical differences were observed in the A10 between the L-Hpt group and NS group at 24 hours after injury ($p<0.05$, **Fig. 4**). There were also significant differences in the A20 between the L-Hpt group and NS group at 24 hours ($p<0.01$). The CFT of the L-Hpt and H-Hpt groups was shorter at 24 hours after the injury compared with six hours after injury ($p<0.05$). Additionally, the α angle in the L-Hpt group significantly increased at 24 hours, when compared to six hours after injury ($p<0.05$). MAXV-t, which indicates the time to maximum velocity of clot formation, in the haptoglobin administration groups was significantly shorter at 24 hours than six hours (L-Hpt; $p<0.05$, H-Hpt; $p<0.01$). Furthermore, the MAXV-t of the L-Hpt group at 24 hours after injury was significantly shorter than in the NS group ($p<0.01$). There were no significant differences in CT and ML.

Discussion

We demonstrated that intraperitoneal administration of haptoglobin reduced INF-γ and accelerated the speed of clot formation using severe burn injury model that presents macro hematuria.

In the present study, all animals showed apparent macro hematuria immediately after burn injury. This hematuria was improved by high concentration of hapto-

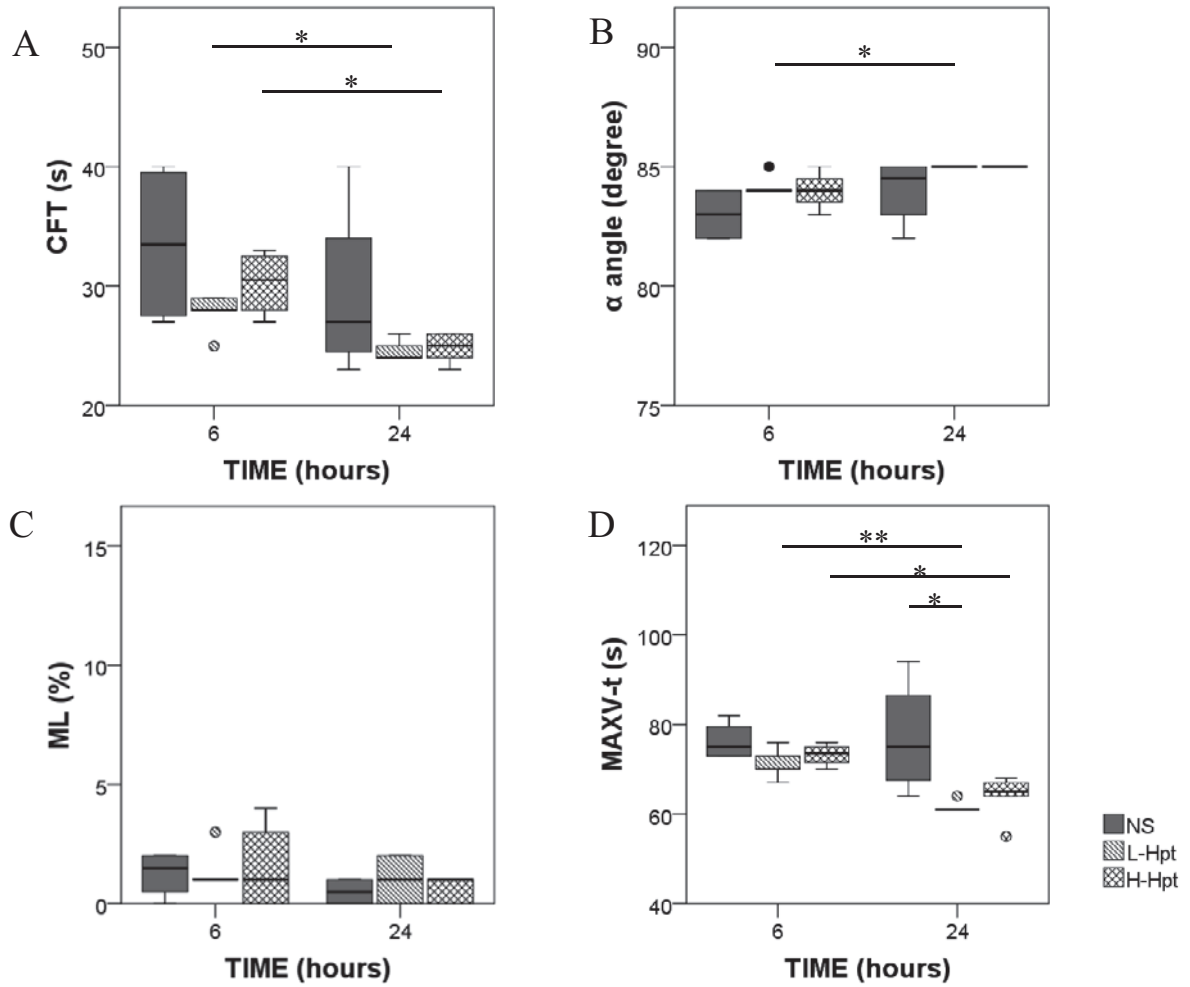

Fig. 4 The effect of haptoglobin on accelerated clot formation and fibrinolysis in a rat severe burn model.
Haptoglobin significantly shortened CFT (A) and MAXV-t (D) and elevated α angle (B) 24 hours post injury compared with 6 hours. Moreover, L-Hpt significantly shortened MAXV-t at 24 hours after the injury compared with NS group. No statistical difference was confirmed in the ML (C).
CFT, clot formation time; ML, maximum lysis; MAXV-t, time to maximum clot formation velocity; NS, normal saline; L-Hpt, low concentration of haptoglobin; H-Hpt, high concentration of haptoglobin. *, $p<0.05$; **, $p<0.01$.

globin injected intraperitoneally at 24 hours after the burn injury. On the other hand, low concentration of haptoglobin could not subside hematuria adequately. It might be expected that the intraperitoneal administration haptoglobin does not reach as high blood concentrations as intravenous injection to relieve the hematuria, but a clear explanation is not apparent.

Haptoglobin is an acute phase protein and its production in the hepatocytes is stimulated by IL-6 in patients with severe burn injury[5,6]. Although there is little evidence to date about other functions that haptoglobin regulates, recent studies implicate that it may possess an anti-inflammatory property[7,8]. The mechanisms in response to acute inflammation are related to suppression of proliferation of T cells and their cytokine production[8]. In addition, the Hb-Hp complex binds to its receptor, CD163, which is located on the surface of macrophages, and then it is degraded to heme and globin. This induces an anti-inflammatory effect triggered by heme decomposition and an anti-oxidant effect by produced bilirubin[2,18]. It has been published that the haptoglobin knockout mouse is more susceptible to endotoxins, and its mortality rate is worse than the wild type[19].

Serum IFN-γ levels are increased after burn injury of small animals due to elevated IFN-γ production by Th1 cells which are stimulated with potent immunomodulatory signals from hyperactive macrophages[20,21]. Although we did not set the control group (no burn or treatment), the serum levels of IFN-γ increased as the size of burn injury increased[21]. In fact, our severe burn model also

showed an elevation of IFN-γ in the NS group. The effects of the immunomodulation of IFN-γ on the clinical course of severe burn remain controversial, but several in vivo and in vitro experiments indicate that the production of IFN-γ is adversely associated with the wound healing process and clinical outcome[20,22–24]. For example, increased IFN-γ prolongs wound healing and suppresses collagen synthesis by fibroblasts[23]. Additionally, accelerated wound healing after severe scald burns[23] and excisions[24] was observed in the IFN-γ knockout mouse compared with the wild type. Importantly, this elevation of IFN-γ was inhibited by the intraperitoneal injection of haptoglobin at 24 hours after thermal injury. We conclude that the haptoglobin might possess an anti-inflammatory effect after severe burn injury by potently suppressing IFN-γ production.

One more interesting observation in our data was the changes of variation in the data of every cytokine in each group (**Fig. 2**). The greater variations in the level of all cytokines were found in the NS group compared with the haptoglobin groups. Unfortunately, the values of cytokines in the NS group varied more than in the haptoglobin group, which may have contributed to the inability of achieving statistical significance during data analysis. In addition, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-12 and IL-13 of the L-Hpt group and all cytokines in the H-Hpt group had smaller variations after 24 hours compared to six hours (data not shown). The median value of most of the cytokines, except for IL-6, in the haptoglobin groups decreased when comparing the values at six hours with those at 24 hours after injury. These data suggest that haptoglobin tends to dampen most of the cytokines induced by severe burn injury (**Table 2**). Interestingly, haptoglobin did not inhibit IL-6 during this period. We presume that the haptoglobin concentration in the blood stream was not sufficient enough to inhibit IL-6 because it is known to be produced by various cells and keeps increasing for the first five days in severe burn patients[25,26]. Others reported that IL-6, which is released by LPS-stimulated peripheral blood mononuclear cells, is not affected by haptoglobin[19].

Severe injury damages vascular endothelium, which triggers systemic inflammation and inflammation-induced coagulopathy[27]. Similar damage responses were observed in patients with severe burn[28]. Hypercoagulability is a characteristic of activated extrinsic coagulation pathway in the damaged intravascular cells, which is correlated with the severity and range of burn[29–32]. Unfortunately, we could not find articles concerning association between IFN-γ and acceleration of clot formation in burn patients. However, upon a sequence of inflammation cascade, INF-γ produced by activated helper T cells contributes to the inflammatory cells-mediated tissue factor production, which induced extrinsic hypercoagulation[33,34]. In the present study, ROTEM analysis revealed that haptoglobin increased amplitude of clotting and promoted clot formation in the early phase of blood clotting. Haptoglobin did not affect the level of TAT/PIC, despite smaller variations of TAT value was confirmed in the groups administered with haptoglobin compared with that in the NS group. Furthermore, there was no pathological evidence of clot formation in liver, lung, and kidneys (data not shown). These findings suggest that ROTEM analysis was sensitive enough to detect minimum degree of hypercoagulability induced by haptoglobin within 24 hours after the burn injury. Indeed, ROTEM, which reflects coagulation cascade and its interaction with activated platelet, is more physiologically than conventional coagulation testing[35]. However, it is still very difficult to interpret data between ROTEM, so called point-of-care test using whole blood sample, and conventional coagulation test such as the TAT and PIC. Our study focused on the micro thrombi formation in the early phase of burn injury, which is essential for protecting the integrity of microcirculation around the burn site by preventing bleeding at wounded vessels[29,31]. Although the present study focused on the acute phase only, hypercoagulability caused by haptoglobin may play a vital role in maintaining the microvascular patency.

The present study has several drawbacks. Small sample size is one of them. We could follow up in the only acute phase of severe burn injury because of technical issues. We also cannot overlook the fact that injection method of haptoglobin is different from clinical situation. Future researches using IV injection of haptoglobin in same rat model will be needed to translate into clinical setting. We did not measure platelet counts and fibrinogen that influence the amplitude of clotting and CFT in EXTEM test[36]. However, there was no data to support the relationship between haptoglobin administration and platelet/fibrinogen values. Moreover, we could not measure other hematological parameters including chemistry and standard coagulation tests due to severe burn induced hypovolemia.

Burn-induced sepsis results in a much poorer outcome due to multiple organ failure caused by both overwhelming coagulation and inflammation[29,37,38]. Therefore, the normalization of coagulation system and improvement of

systemic inflammation are essential to treat severe burn. We would like to emphasize that IFN-γ and most of the cytokines regulated by haptoglobin prevent life-threatening systemic inflammatory responses, and cytokine storm during the early phase of severe burn. It should also be noted that the haptoglobin-related hypercoagulability is associated with a potential benefit of keeping microcirculation in the burn wound site. Finally, our data suggested that haptoglobin might be a novel therapeutic drug that modulates the inflammation and coagulation status in the early phase of severe burn.

In conclusion, the intraperitoneal administration of haptoglobin exerted an anti-inflammatory effect and promoted clot formation of extrinsic coagulation cascade in rat severe burn model.

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Conflict of Interest: The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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