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Changes in polymorphonuclear leukocyte elastase concentrations and hemolysis parameters in patients transfused with different blood preparations, and in the blood preparations themselves

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Abstract
Purpose. Massive blood transfusion induces hemolysis and increases polymorphonuclear leukocyte elastase (PMNE) concentration. The purpose of this study was to compare hemolysis and PMNE concentrations in massive blood transfusions with three different preparations.
Methods. In an in vitro study, eight 2-day-old packs of concentrated red blood cells in mannitol, adenine, glucose, phosphate, and citrate solution (MAP-CRC); concentrated red blood cells in citrate, phosphate, and glucose solution (CPD-CRC); or whole blood in citrate, phosphate, and glucose solution (WB) were stored at 4°C. Supernatant concentrations of total and free hemoglobin, total haptoglobin, and PMNE were measured. In an in vivo study, 24 surgical patients with expected bleeding exceeding 3000 ml were transfused with CPD-CRC or MAP-CRC with fresh frozen plasma, or with WB. Platelet count, prothrombin time, activated partial thromboplastin time, serum total and free hemoglobin, and total haptoglobin and plasma PMNE concentrations were measured.
Results. In the in vitro study, total and free hemoglobin concentrations were significantly higher in CPD-CRC than in the other two preparations. Total haptoglobin concentration was highest in the order of WB > MAP-CRC > CPD-CRC. The PMNE concentration was significantly higher in WB than in the other two preparations. In the in vivo study, at 3000-ml transfusion, total and free hemoglobin concentrations were significantly lower and activated partial thromboplastin time was longer in the patients with MAP-CRC compared with values in the other two groups. The PMNE concentration was significantly higher in the order of the WB > CPD-CRC > MAP-CRC groups.
Conclusion. During the storage of MAP-CRC, CPD-CRC, and WB, CPD-CRC had the greatest hemolysis and WB had the highest concentration of PMNE. Patients who received massive blood transfusion of MAP-CRC had the least hemolysis and the lowest concentration of PMNE.

Key words Blood transfusion · Hemolysis · In vitro study · In vivo study

Introduction

Blood transfusion entails the risk of various complications. One of these complications is possible renal damage caused by massive blood transfusion [1]. Stored blood preparations include free hemoglobin and fragile red blood cells that are easily damaged to produce free hemoglobin [2]. Free hemoglobin may induce renal dysfunction due to renal tubular damage caused by hemoglobin mass formation [3] or the inhibition of renal blood flow [4], or renal tubular damage may be caused by heme [5].

In a previous study, in patients who received a transfusion of stored whole blood (WB), free hemoglobin was detected after 2000-ml transfusion [6]. In addition, hemolysis in stored blood preparations increases with the duration of storage, and hemolysis is greater in concentrated red blood cells (CRC) in mannitol, adenine, glucose, phosphate, and citrate solution (MAP-CRC) than in WB [7–9].

Polymorphonuclear leukocyte elastase (PMNE) is released from neutrophils by inflammatory or other stimuli, and PMNE can induce tissue injury [10]. PMNE also induces clotting-factor depletion [11]. Therefore, PMNE may have some roles in coagulation disorders, i.e., bleeding tendency. The transfusion of CRC in citrate, phosphate, and glucose solution (CPD-CRC) increased PMNE levels in patients [7]. In addition, PMNE in stored blood preparations (e.g., MAP-CRC) increases with the number of storage days [12]. Therefore, blood transfusion entails a risk of tissue

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injury and coagulation disorder that may be induced by PMNE.

Considered in light of our previous studies [7–9] of three different stored blood preparations, MAP-CRC, CPD-CRC, and WB, we hypothesized that hemolysis might be greatest in MAP-CRC, and that the PMNE concentration might be greatest in WB, because WB has the highest plasma component, including white blood cells. Therefore, MAP-CRC or WB might induce the greatest hemolysis in patients receiving these transfusions. If this was to be true, the use of MAP-CRC might have to be abandoned and we should return to CPD-CRC. The present study was performed to compare hemolysis, PMNE concentration, and bleeding tendency in patients who received the above three different kinds of stored blood preparations (in vivo study); we also compared these parameters in the different stored blood preparations themselves (in vitro study).

Patients, materials, and methods

After obtaining approval from the research committee of Kagawa Central Hospital (in vivo study) and the Japan Red Cross Society (Takamatsu and Tokyo, Japan; in vitro study), and informed consent from patients (in vivo study), the in vitro and in vivo studies were performed separately. The in vivo studies using WB and CPD-CRC were done simultaneously, and that using MAP-CRC was performed 1 year later. The in vitro studies using WB and CPD-CRC were performed simultaneously and that using MAP-CRC was done 2 years later, because MAP-CRC took the place of CPD-CRC in Japan after the first stage of the study had been performed. These studies were done 13 to 15 years ago; therefore, none of the preparations was irradiated and no filters to remove white blood cells were used at transfusion.

In vitro study

Eight 2-day-old packs (each 200 ml) of MAP-CRC, CPD-CRC, or WB obtained from the Japan Red Cross Society (Takamatsu and Tokyo, Japan) were stored at 4°C in a refrigerator.

Blood samples (5 ml) were drawn from each pack at 2 days (when we got the products from the Japan Red Cross Society), and at 5, 9, 13, 17, and 21 days after the production of each pack and immediately centrifuged at 3000 *g* for 10 min. The supernatant was separated and stored at –40°C until measurement.

The supernatant concentrations of total and free hemoglobin, total haptoglobin, and PMNE were measured. The total hemoglobin concentration was measured by the tetramethyl benzidine method (detection limit, 50 $mg \cdot l^{-1}$). The free hemoglobin concentration was measured by an enzyme-linked immunosorbent assay (detection limit, 50 $mg \cdot l^{-1}$). The total haptoglobin concentration was measured by immunonephelometry (detection limit, 50 $mg \cdot l^{-1}$). These measurements were done at the laboratory of Green Cross (Osaka, Japan). The free haptoglobin concentration could not be measured directly and was calculated by determining the total haptoglobin concentration and the total hemoglobin concentration according to the method of Oomura et al. [13], as follows: when the total haptoglobin concentration is more than 1.42 × the total hemoglobin concentration, the free haptoglobin concentration = the total haptoglobin concentration –1.42 × the total hemoglobin concentration. When the total haptoglobin concentration is 1.42 × the total hemoglobin concentration or less, the free hemoglobin concentration = 0. The PMNE concentration was measured as the concentration of a PMNE complex with α_1-antitrypsin, using an enzyme-linked immunosorbent assay with α_1-antitrypsin antibody. The assay was done at Kitasato Biochemical Laboratory (Kanagawa, Japan; normal range, 12–165 $\mu g \cdot l^{-1}$) [14].

In vivo study

Patients (aged 35 to 75 years) scheduled for extended abdominal surgery, with an expected bleeding level of more than 3000 ml were enrolled, until 24 patients (8 patients for each of CPD-CRC, WB, and MAP-CRC) completed the study. Patients who had a bleeding tendency, liver cirrhosis, or severe anemia before surgery were excluded. Patient selection was not done randomly, but depended on which blood preparations were prepared before surgery by the surgeons.

After premedication with intramuscular atropine 0.2 to 0.5 mg and hydroxyzine 25 to 50 mg, an epidural catheter was inserted into an appropriate interspinal space and anesthesia was induced with thiopental, midazolam, fentanyl, and vecuronium; then the patients were orotracheally intubated. A radial artery was cannulated to draw blood. Anesthesia was maintained with epidural lidocaine and sevoflurane with nitrous oxide in oxygen. Blood transfusion was started when blood loss reached more than 1000 ml, hematocrit decreased to less than 25%, or hemoglobin decreased to less than 80 $g \cdot l^{-1}$. In the CPD-CRC and MAP-CRC groups, fresh frozen plasma was also transfused, starting when the total volume of transfused blood reached 1000 ml, while in the WB group, only WB was transfused during the study. All blood preparations were transfused through a warm bath, heated to 40°C. Arterial blood (8 ml) was drawn before the start of surgery, and after 1000-, 2000-, 2600-, and 3000-ml transfusions were completed. Plate-

let count, prothrombin time, activated partial thromboplastin time, serum total and free hemoglobin and total haptoglobin concentrations, and plasma PMNE concentrations were measured.

Statistical analysis

The χ^2 test and factorial analysis of variance (ANOVA) were used to analyze demographic data. Two-way repeated-measures ANOVA was used for the measured parameters, and the Student Neuman Keuls test was done as a post-hoc test when appropriate. A P value of less than 0.05 was considered to be statistically significant.

Results

In vitro study

In CPD-CRC, total and free hemoglobin concentrations increased as the number of storage days increased.

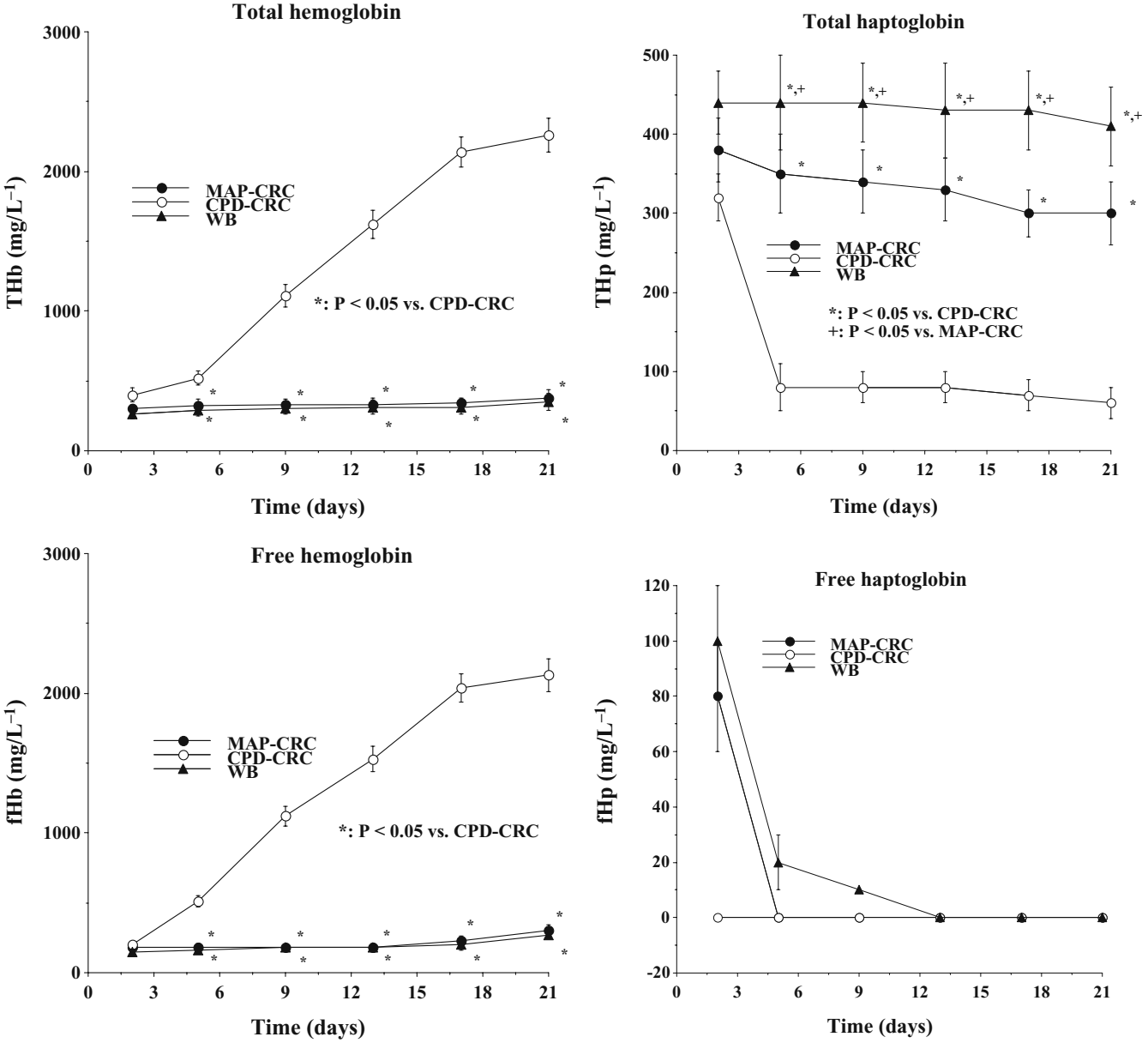

Fig. 1. Changes brought about by storage in total hemoglobin (*THb*), free hemoglobin (*fHb*), total haptoglobin (*THp*), and free haptoglobin (*fHp*) concentrations in stored blood preparations. Data values are shown as means ± SD. *MAP-CRC*, Concentrated red blood cells in MAP (mannitol, adenine, glucose, phosphate, and citrate) solution; *CPD-CRC*, concentrated red blood cells in CPD (citrate, phosphate, and glucose) solution; *WB*, whole blood

Total and free hemoglobin concentrations were significantly higher in CPD-CRC than in MAP-CRC and WB (Fig. 1). Total haptoglobin concentration decreased depending on number of the storage days in CPD-CRC, and it was highest in the order of WB > MAP-CRC > CPD-CRC (Fig. 1). Free haptoglobin was not detected in CPD-CRC at any time point and it was not detected at 13 days of storage in WB. In MAP-CRC, free haptoglobin was not detected at 5 days (Fig. 1). The PMNE concentration increased with the number of storage days increase in all three preparations, with significantly higher values in WB (Fig. 2).

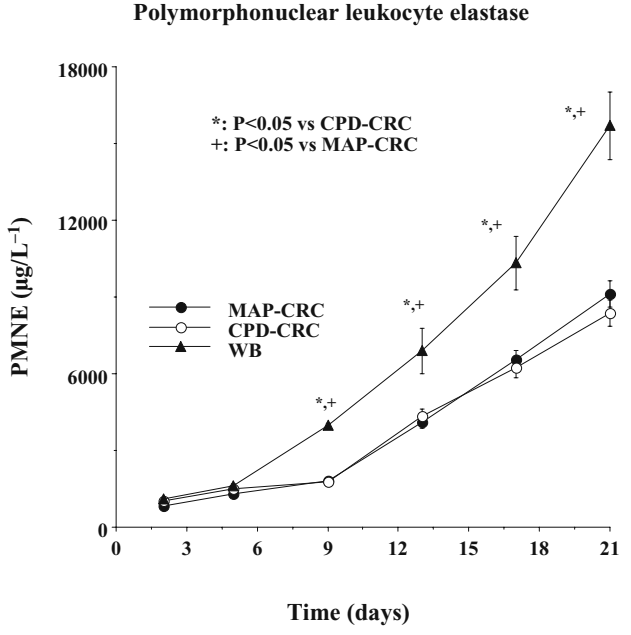

Fig. 2. Changes brought about by storage in polymorphonuclear leukocyte elastase (*PMNE*) in stored blood preparations. Data values are shown as means ± SD. *MAP-CRC*, concentrated red blood cells in MAP (mannitol, adenine, glucose, phosphate, and citrate) solution; *CPD-CRC*, concentrated red blood cells in CPD (citrate, phosphate, and glucose) solution; *WB*, whole blood

In vivo study

Forty-two patients were enrolled in the study, but 18 patients were excluded (9 patients received transfusion volumes of less than 3000 ml, 6 patients received both CPD-CRC and WB, and 3 patients received both MAP-CRC and WB). No differences in the demographic data were seen among the three groups (Table 1).

Total and free hemoglobin concentrations increased in accordance with increasing transfusion volume, with significantly lower values being seen in the MAP-CRC group than in the CPD-CRC and WB groups at 3000-ml transfusion (Fig. 3). Total and free haptoglobin concentrations decreased in accordance with increasing transfusion volume, without any differences among the groups (Fig. 3). The PMNE concentration increased in accordance with increased transfusion volumes, with significantly higher values, in the order of the WB > CPD-CRC > MAP-CRC groups after 2000-ml transfusion (Fig. 4). Platelet counts and prothrombin time did not change significantly and were not different among the three groups (Fig. 5). Activated partial thromboplastin time was significantly longer in the MAP-CRC group than in other two groups after 3000-ml transfusion (Fig. 5).

Discussion

The findings of the present study are that, in the in vitro study, during storage, hemolysis significantly progressed in CPD-CRC, with a lower haptoglobin concentration compared to those in MAP-CRC and WB. PMNE increased during storage, and the increase was greatest in WB. In the in vivo study, in the transfused patients, hemolysis increased in accordance with increasing transfusion volumes, and the increase was smallest in patients who received MAP-CRC. The PMNE concentrations in the transfused patients increased as the trans-

Table 1. Demographic data

	MAP-CRC	CPD-CRC	WB
Age (years)	54 ± 17	58 ± 14	57 ± 11
Male/Female	4/4	5/3	3/5
Body weight (kg)	60 ± 13	59 ± 11	58 ± 12
Height (cm)	162 ± 15	160 ± 18	164 ± 16
Duration of surgery (min)	356 ± 89	387 ± 91	329 ± 82
Transfusion volume (ml)	3898 ± 924	3965 ± 857	4258 ± 923
Crystalloid infusion volume (ml)	5782 ± 1054	6217 ± 935	6542 ± 1058
Mean number of storage days of transfused blood (days)	11.3 ± 4.6	12.5 ± 5.8	10.5 ± 6.1
Transfused fresh frozen plasma volume (ml)	1435 ± 332	1525 ± 419	0

Values are means ± SD
The MAP-CRC group received concentrated red blood cells in MAP solution, the CPD-CRC group received concentrated red blood cells in CPD solution, and the WB group received whole blood

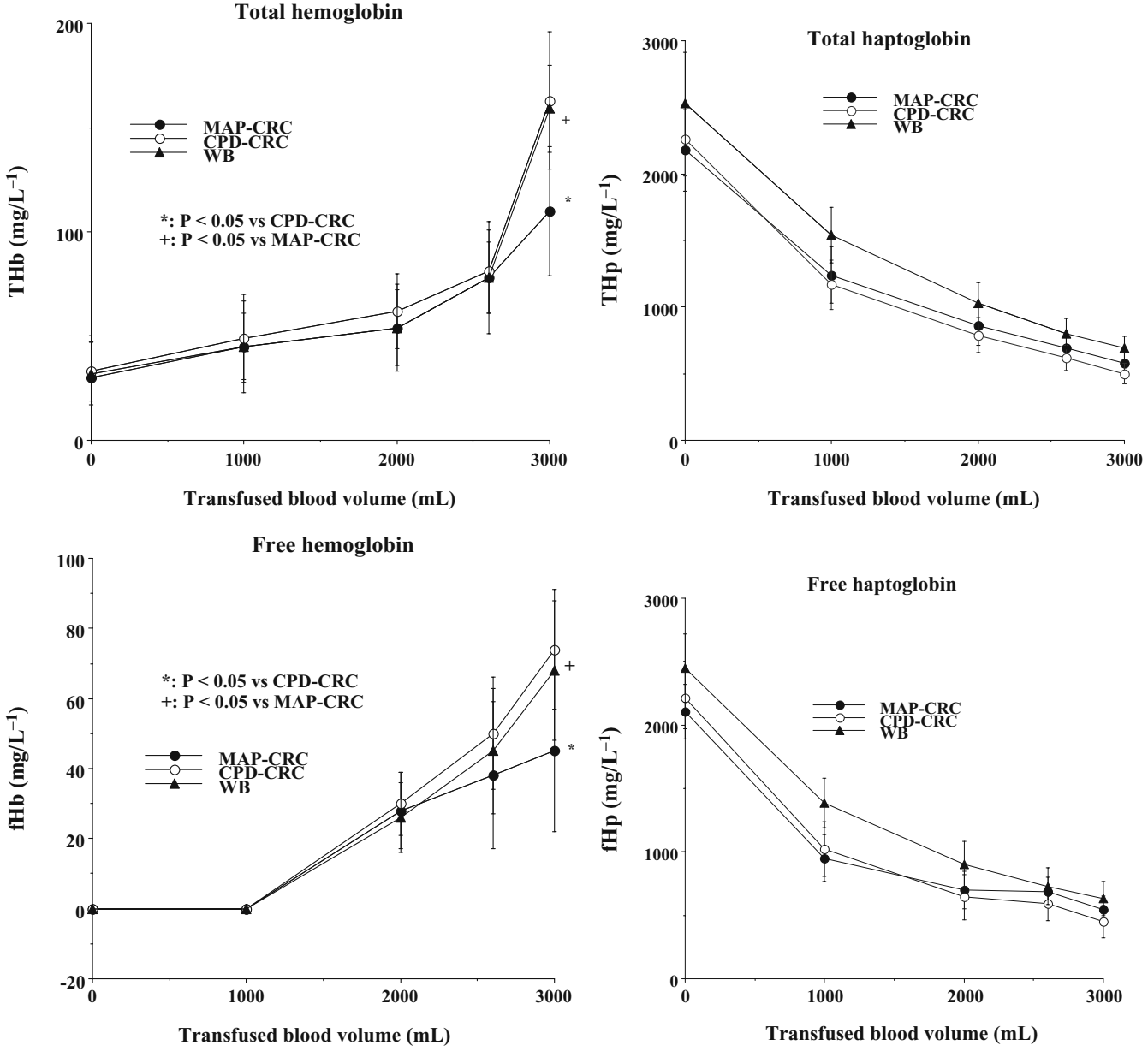

Fig. 3. Changes in total hemoglobin (*THb*), free hemoglobin (*fHb*), total haptoglobin (*THp*), and free haptoglobin (*fHp*) concentrations in patients. Data values are shown as means ± SD. *MAP-CRC*, concentrated red blood cells in MAP (mannitol, adenine, glucose, phosphate, and citrate) solution; *CPD-CRC*, concentrated red blood cells in CPD (citrate, phosphate, and glucose) solution; *WB*, whole blood

fusion volume increased, and the increase was greatest in patients transfused with WB and smallest in patients transfused with MAP-CRC. Activated partial thromboplastin time was significantly longer in patients transfused with 3000 ml MAP-CRC.

In regard to the limitations of our study, we performed the MAP-CRC study at a different period from the study using the other two preparations. All the studies should have been done at the same time. However, after CPD-CRC was no longer used, being replaced by MAP-CRC, it became difficult to get not only CPD-CRC but also WB. Therefore, only the study using MAP-CRC was performed later.

In the in vivo study, because we did not check the number of storage days at each measurement point, at the 1000-, 2000-, and 2600-ml transfusions, the different numbers of storage days of the blood preparations may have had some effects on the results, but at 3000-ml transfusion, the mean number of storage days of transfused blood was not different among the three groups.

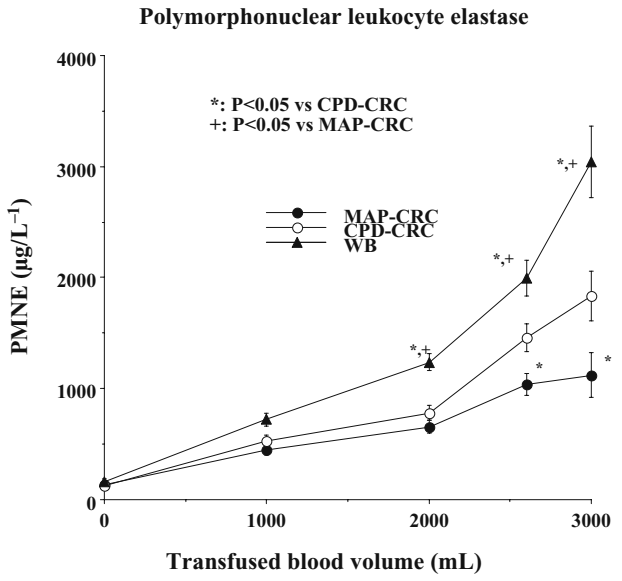

Fig. 4. Changes in polymorphonuclear leukocyte elastase (*PMNE*) in patients. Data values are shown as means ± SD. *MAP-CRC*, concentrated red blood cells in MAP (mannitol, adenine, glucose, phosphate, and citrate) solution; *CPD-CRC*, concentrated red blood cells in CPD (citrate, phosphate, and glucose) solution; *WB*, whole blood

Therefore, the results may be comparable with the same number of storage days.

The free hemoglobin concentration was used to indicate hemolysis [15]. In the stored blood preparations used in the present study, total and free hemoglobin concentrations were slightly higher in MAP-CRC than in WB, findings which were almost consistent with those in our previous study [8]. Total and free hemoglobin concentrations in CPD-CRC were significantly higher than those in MAP-CRC and WB. In the present study, CPD-CRC had the lowest haptoglobin concentrations. This may have been due to a greater consumption of haptoglobin by binding with free hemoglobin, which was present at a higher concentration in CPD-CRC than in MAP-CRC and WB.

In a previous study, when more than 2600 ml of blood preparations with a mean number of storage days of more than 12.5 was transfused, total haptoglobin decreased to less than 1000 $mg \cdot l^{-1}$ and free hemoglobin increased in the patients, however, the kinds of stored blood preparations transfused were not controlled [16]. In another study, free hemoglobin was detected in patients after 2000-ml WB transfusion, when the total haptoglobin concentration decreased to 1000 $mg \cdot l^{-1}$ during surgery for trauma [6]. These findings are almost consistent with the present results. The normal haptoglobin level in adult humans is 300–2000 $mg \cdot l^{-1}$ [15], which is the same as the control value in the present study. Each molecule of haptoglobin can bind two hemoglobin dimers or approximately 1 gram per liter of plasma. Thus, about 3 g of free hemoglobin could be transfused to an adult patient without the occurrence of hemolysis. According to this theory, the free hemoglobin concentrations in the transfused patients in the present study were higher than the expected concentration, and still free haptoglobin existed. The transfusion speed may have been too fast for haptoglobin to have bound with the free hemoglobin. In the present study, the total and free hemoglobin concentrations in patients who received 3000-ml transfusions were significantly lower with MAP-CRC than with WB or CPD-CRC. The CPD-CRC preparation had the largest amount of free hemoglobin; therefore, the amount of free hemoglobin was highest in the patients who received CPD-CRC. The free hemoglobin concentration in patients who received WB was higher than that in patients who received MAP-CRC, although both the WB and MAP-CRC preparations had the same level of free hemoglobin. Therefore, there may have been some other causes of the WB-induced hemolysis in the patients.

In previous studies, PMNE increased during storage in WB [17] and in MAP-CRC [12] and these findings are consistent with the results of the present study. The neutrophil count was reported to be lower in the order of MAP-CRC < CPD-CRC < WB [8, 18], though it was not measured in the present study. PMNE is released from neutrophils, and therefore the PMNE concentration was highest in WB. We investigated the role of PMNE in hemolysis in stored blood preparations, but it has been suggested that PMNE might not be involved in the mechanisms of hemolysis in MAP-CRC [12]. This was also suggested from the present results, in which, although WB had the highest PMNE concentration, it had the lowest free hemoglobin concentration. The transfusion of CPD-CRC was shown to increase PMNE in patients beyond the level of transfused PMNE [7]. This was also shown in the present results, where the PMNE concentration in patients transfused with CPD-CRC was significantly higher than that in patients transfused with MAP-CRC, while the PMNE concentration in the stored blood preparations was almost the same in CPD-CRC and MAP-CRC. The highest PMNE concentration in the patients who received WB may have been, in part, due to the infused PMNE from WB. The PMNE concentration in our patients was higher in the order of those who received WB > CPD-CRC > MAP-CRC, while the free hemoglobin concentration was higher in the order of the patients who received CPD-CRC = WB > MAP-CRC. Therefore, PMNE may not be the main cause of the induction of hemolysis in transfused patients. There is no definite criterion for the PMNE concentration above which organ dysfunction is induced. We did not

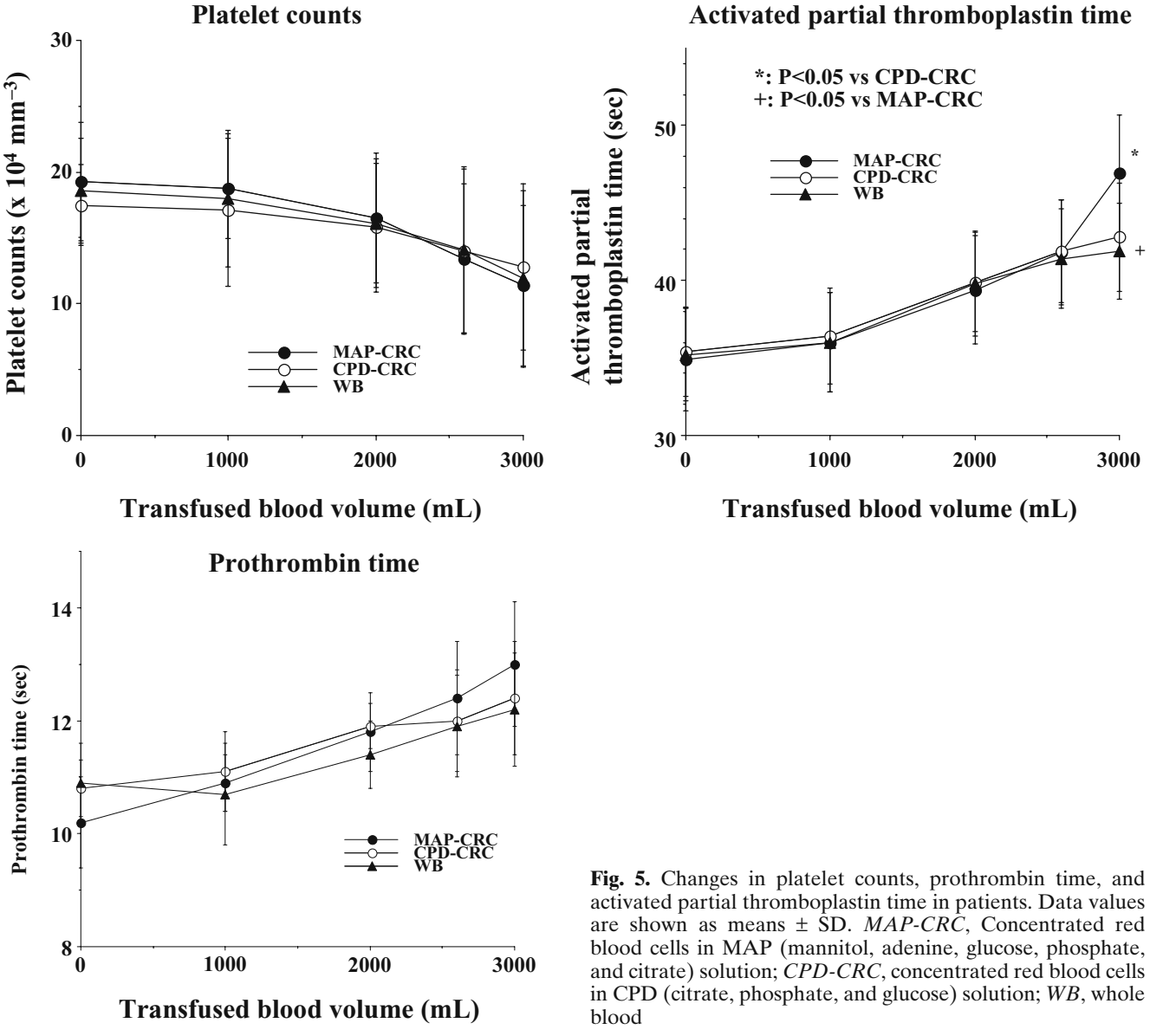

Fig. 5. Changes in platelet counts, prothrombin time, and activated partial thromboplastin time in patients. Data values are shown as means ± SD. *MAP-CRC*, Concentrated red blood cells in MAP (mannitol, adenine, glucose, phosphate, and citrate) solution; *CPD-CRC*, concentrated red blood cells in CPD (citrate, phosphate, and glucose) solution; *WB*, whole blood

study any side effects in patients transfused in the present study because many other factors may affect organ function and outcome, and such factors would have been difficult to equalize; also, we could not control the total volume of blood transfusion during the perioperative period. However, we did not experience any patients who needed intensive care for any organ dysfunction in the present study.

When massive bleeding occurs, bleeding tendency is usually seen because of a loss of coagulation factors. PMNE also induces clotting-factor depletion [11]. Therefore, patients transfused with WB may have the highest bleeding tendency. However, the activated partial thromboplastin time in our study was significantly higher in the patients who received MAP-CRC than in the patients who received CPD-CRC or WB at 3000-ml transfusion. The reason for this is not apparent from the present study, and no clinically apparent differences were observed in bleeding among the patients in our three groups. Clinical coagulopathy arising from dilution does not usually occur until replacement exceeds one blood volume or when prothrombin time or activated partial thromboplastin time exceeds 1.5 to 1.8 times control values [19–22]. Therefore, none of the patients in the present study did not show any clinically apparent bleeding tendency. The transfused fresh frozen plasma may have had some role in inhibiting bleeding tendency in the patients in our study who received MAP-CRC and CPD-CRC. However, it was impossible to transfuse only MAP-CRC or CPD-CRC in a massive transfusion. Therefore, even though fresh frozen plasma was transfused in patients with MAP-CRC or CPD-

CRC, the results showing here are useful, being clinically relevant.

In conclusion, during the storage of MAP-CRC, CPD-CRC, and WB preparations, CPD-CRC showed the greatest hemolysis and WB had the highest concentration of PMNE. Patients who received MAP-CRC had the least hemolysis and the lowest concentration of PMNE, but they may have had coagulopathy.

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