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**Platelet and Leukocyte Activation in Salvaged Blood and the Effect of Its
Reinfusion on the Circulating Blood**

*M. de Jong MD**; *M. Ray PhD[†]*; *S. Crawford FRACS**; *S.L. Whitehouse PhD[‡]*; and *R.W. Crawford DPhil (OXON)[‡]*

From the * Orthopaedic Department; [†]Northside Pathology, Queensland Health Pathology Service; and [‡] the Orthopaedic Research Unit, Queensland University of Technology, The Prince Charles Hospital, Rode Road, Chermside Queensland, Australia.

Correspondence to: R. Crawford, DPhil, Prince Charles Hospital, Rode Road, Chermside Q 4032, Australia. Phone: 61-7-3350 8481; Fax: 61-7-3350 8043; Email: r.crawford@qut.edu.au.

Postoperative wound drainage reinfusion reduces the frequency of homologous blood transfusion. The salvaged blood is depleted of coagulation factors but may contain platelets and leukocytes which are activated, and therefore potentially procoagulant. We ascertained the degree of activation of platelets and leukocytes in salvaged blood and asked whether their infusion produced any measurable effect on patients' coagulation system. We prospectively randomized 24 patients who had total knee arthroplasties to reinfusion of salvaged autologous blood (n = 12) or a standard drain with no reinfusion (n = 12). Analysis of the salvaged blood showed marked activation of platelets as shown by their expression of P-selectin, CD40 ligand, and Factor V/Va, and as increased numbers of platelet-derived microparticles. After reinfusion there was no measurable effect on activation markers of circulating platelets or leukocytes but there was a decrease in platelet count in the reinfused group compared with the control group. Levels of prothrombin fragment F 1+2 (suggesting thrombin formation) increased in the reinfused group compared with control group, possibly indicating activation of coagulation systemically. The platelets and leukocytes in salvaged blood are markedly activated and their reinfusion causes a decrease in platelet count in the recipient and a possible increase in thrombin generation potentially favoring thrombosis.

Level of Evidence: Level II Therapeutic study. See the Guidelines for Authors for a complete description of levels of evidence.

Blood transfusions are common after joint replacement surgery, although the incidence has decreased because of measures such as transfusion protocols, normovolemic hemodilution, preoperative erythropoietin, and antifibrinolytic drugs.³ Postoperative wound drainage reinfusion reduces the frequency of homologous blood transfusion between 67% and 91%.^{10,13,16,17} Reinfused drainage contains low levels of clotting factors such as Factor VIII and fibrinogen.^{4-6,8} One study examined the effect of reinfusion of postoperative wound drainage blood on leukocyte-derived and platelet-derived bioactive substances and on blood coagulation and concluded “reinfusion of drainage blood did not change the coagulative capacity of the patients.”⁸

Platelet activation has been associated with the development of venous thrombosis. Radiographically confirmed deep venous thrombosis (DVT) in patients after total knee arthroplasty (TKA) is associated with an increased platelet activation marker, P-selectin.¹⁹

We ascertained whether platelets and leukocytes had increased activation in salvaged blood and whether their infusion produced any measurable effect on patients’ coagulation system.

MATERIALS AND METHODS

We prospectively enrolled 24 patients with osteoarthritis having primary TKAs in a prospective, consecutive, systematically allocated study. The patients were divided into two groups: a treatment group and a control group. Patients experience a decrease in platelets of approximately 30% immediately after surgery.¹⁸ However, an increase

in the amount of this decrease attributable to reinfusion may indicate an increase in clotting potential. To detect a substantial difference between the two groups in the decrease in platelet count of 50% with 80% power, 11 patients were required in each group. To allow for dropout or testing anomalies, a sample size of 12 patients was deemed necessary. The treatment group (12 patients) received autologous salvaged blood from a reinfusion drain (CBCII ConstaVac, Stryker Instruments, Kalamazoo, MI). A control group (12 patients) had TKA with a standard drain (Bellovac, Astra, Molndal, Sweden). The patients were matched for age and gender. Demographic data between the groups did not differ (Table 1). Intake of any antiplatelet drugs was discontinued at least 7 days before surgery. A tourniquet was used routinely and was released after wound closure and application of pressure dressings.

The CBCII ConstaVac is a closed blood recovery system used to collect, filter, and reinfuse autologous blood. The blood first passes through a 240- μ m filter before entering an 800-mL plastic container. This first filter removes particles in the blood such as bone fragments, clotted blood, and fat particles. No anticoagulant is used. During reinfusion, the blood passes through a 40- μ m pore filter before being returned to the patient. This filter removes additional debris while allowing platelets, leukocytes, and red cells to pass. The last 100 mL of shed blood remained in the container and was not reinfused. The transfusion of shed blood collected from the wound postoperatively was started 4 hours after initiating collection.^{6,15}

Systemic blood samples from the patients in the treatment group were taken after surgery on three occasions; 1 hour before salvaged blood reinfusion (A), 1 hour after completion of reinfusion (B), and the following day. The reinfusion was initiated 4

hours after surgery and was completed within $\frac{1}{2}$ to 1 hour. A separate sample was acquired from the salvaged blood, after it had passed the final filter, at the time of reinfusion.

The blood samples of the control group were taken at 3 (A) and 5.5 (B) hours postoperatively and the following day to correspond to the time samples were taken from the treatment group. To prevent activation of blood during the drawing process, approximately 5 mL of blood was collected in a plain tube to clear the line. This specimen was discarded. Blood (2.7mL) was drawn into Vacutainer tubes (Becton Dickinson, NJ) containing 0.109 mol/L buffered trisodium citrate. For measurements of patients' full blood count, venous blood was collected in a Vacuette tube (Greiner bio-one, Kremsmünster, Austria) containing EDTA K2. Citrated blood samples from each patient at times A and B and their salvage at the completion of reinfusion were collected. These were immediately centrifuged at 3000 g for 10 minutes and the plasma stored in liquid nitrogen until testing.

The European Working Group on Clinical Cell Analysis recommends using fixation after staining or counting cells promptly.¹⁴ The latter option has been used. The citrated blood was diluted 1:6 in Dulbecco's phosphate buffered saline (PBS) (CSL, Parkville, Victoria, Australia) containing 2% fetal bovine serum and 0.1% sodium azide. Platelets were selected by forward scatter and side scatter and by positive staining with CD42a (BD Biosciences, San Jose, CA) that targets GP IX and GP 1b-IX-V common to all platelets. Platelet-derived microparticles were selected similarly, their size being less than 0.3 μm as indicated by the side-scatter value. This value was established by calibration with 0.2 μm , 0.5 μm , and 0.71 μm polymer microspheres

(Duke Scientific Corporation, Palo Alto, CA). Platelet activation markers included activated GPIIb/IIIa (PAC1) (BD Biosciences), P-selectin (CD62P) (BD Biosciences), factor V/Va (American Diagnostica Inc, Greenwich, CT), and CD40 ligand (CD154) (BD Biosciences).¹⁵ Monocytes, neutrophils, and lymphocytes were selected by forward scatter and side scatter plus positive staining with CD14 (BD Biosciences) for the monocytes or CD45 (BD Biosciences) for the neutrophils. Leukocyte activation markers were platelet-monocyte aggregates, and platelet-neutrophil aggregates, using CD42a to identify adherent platelets. Other leukocyte markers were MAC-1 (CD11b), tissue factor (American Diagnostica Inc), and P-selectin glycoprotein ligand-1 (CD 162) (BD Biosciences).

The flow cytometer used was a BD Biosciences FACScan with a 488-nm argon-ion laser performing 3-color analysis. The CELLQuest program was used to analyze the data. Daily quality control of the flow cytometer was performed with CaliBRITE 3 beads (BD Biosciences).

A prothrombin fragment F 1+2 immunoassay was performed using the Enzygnost kit (Dade-Behring, Marburg, GmbH, Germany). The frozen plasma samples were thawed at 37°C for 5 minutes before testing. Full blood counts were performed on a Coulter Gen S System 2 analyzer (Coulter Corporation, Miami, FL).

Data were not normally distributed (Kolmogorov-Smirnov test for normality), therefore nonparametric tests were applied. Values are expressed as medians (interquartile ranges). For comparison of paired data the Wilcoxon signed-rank test was used. The Mann-Whitney U test was used for analyses of data in independent

groups. Probability was considered significant when $p < 0.05$. A Bonferroni correction was applied where appropriate.

RESULTS

P-selectin, Factor V/Va, CD40 ligand, and platelet-derived microparticles were increased in salvaged blood compared to the patients' blood 1 hour before reinfusion (Fig 1). The platelet-leukocyte activation markers; platelet-monocyte aggregates, and platelet-neutrophil aggregates in the salvaged blood of the treatment group were greater than in systemic blood at time- A ($p = 0.005$ and 0.003 , respectively).

Monocyte expression of MAC-1, tissue factor, and P-selectin glycoprotein ligand-1 in the salvaged blood of the treatment group were greater than in the patients' circulation before reinfusion ($p = 0.005$, 0.003 , and 0.008 , respectively). Neutrophil expression of MAC-1, tissue factor, and P-selectin glycoprotein ligand-1 were similarly greater in the salvaged blood ($p = 0.003$, 0.006 , and 0.008 , respectively). One hour after infusion (B), the patients in the reinfused group had a greater increase ($p = 0.002$) in prothrombin fragment F 1+2 than the control group (Table 2). The salvaged blood had high levels of prothrombin fragment F 1+2 (median of 1060 [963-1347]) nmol/L.

One hour after reinfusion, the median platelet count decreased from preinfusion levels ($p = 0.004$) whereas the control group showed no change ($p = 0.168$) (Table 3). On the first postoperative day, the median platelet count of the reinfused group showed an additional and greater decrease from preinfusion levels ($p = 0.002$) than the decrease in the control group ($p = 0.019$). The reinfusion of autologous salvaged blood did not affect platelet activation or leukocyte markers in the patients' circulation.

In the patients who had reinfusion, the salvaged blood had a lower hemoglobin, hematocrit, platelet count, leukocyte count, neutrophil count, and monocyte count than the systemic blood (Table 4). Reinfusion produced an increase ($p = 0.01$) in the patients' median (interquartile range) hemoglobin from a level before reinfusion of 114 g/L (104–131 g/L) to 119 g/L (104–131 g/L) 1 hour after the end of reinfusion. The median hemoglobin levels in the control group at the same times were unchanged, being 111 g/L (102–117 g/L) and 112 g/L (99–120 g/L) at the times corresponding to before and after reinfusion, respectively.

DISCUSSION

Autologous salvaged blood differs from circulating blood regarding platelet and leukocyte activation and full blood count. The reinfusion of autologous salvaged blood to patients having a TKA does not lead to increased activation of circulating platelets and leukocytes but may cause activation of coagulation in the recipient. We therefore assessed hematologic laboratory parameters in patients having TKAs and receiving autologous reinfusion.

The major limitation of this study was that the small number of patients recruited prevented us from making clinically significant findings due to the low incidence of DVT and PE. However, we did not intend to ascertain potential clinical thrombotic events (DVT or pulmonary embolism) or to compare transfusion rates.

In spite of the small numbers we were able to demonstrate a significant effect of reinfusion on the physiological factors in the salvaged blood.

. An additional limitation was the difficulty in interpreting the importance of the increase in prothrombin fragment F 1+2 after reinfusion in relation to its indication of the patients' endogenous thrombin generation due to the high levels of prothrombin fragment F 1+2 in the salvage.

In accordance with previous research,⁴ shed blood had lower hemoglobin, hematocrit, leukocytes, and platelets than circulating blood.

Our data shows the degree of activation of platelets and formation of platelet-leukocyte aggregates in blood salvaged after TKA is increased compared with these variables in patients' circulation 1 hour before reinfusion. Increased platelet and leukocyte activation markers in the circulation are associated with systemic thrombosis. Yang et al¹⁹ reported an increased platelet activation marker, P-selectin, after TKA is associated with increased radiographically confirmed DVT. Activation of platelets causes their shedding of vesicles, termed platelet-derived microparticles. Procoagulant platelet-derived microparticles may have an important role in the assembly of the components of the coagulation system.¹² Our study showed substantial amounts of microparticles are added to the patients' circulation with the salvaged blood. Platelet activation also causes a change in the glycoprotein IIb/IIIa receptor complex on the platelet, making it receptive to fibrinogen that provides a ligand for platelet-to-platelet aggregation.⁹ Platelet adhesion to the endothelium or immobilized fibrinogen via GP IIb/IIIa up-regulates the CD40 ligand, making it part of the signaling system in the inflammatory response.⁷

Increases in levels of platelet-monocyte aggregates have been associated with acute myocardial infarction.¹¹ Increased levels of platelet-derived microparticles are linked to the development of cerebrovascular events.⁸ Blood draining from the surgical site and into the drain bottle may have the thrombotic process initiated with platelets being activated by exposure to thrombin and collagen. The activated platelets release the procoagulant contents of their alpha granules, thereby exposing P-selectin on the alpha granule membrane, which binds to P-selectin glycoprotein ligand-1 on leukocytes forming platelet-leukocyte aggregates. Platelet activation also releases platelet-derived microparticles that provide a platform for the coagulation cascade binding factors such as factor V/Va.¹ Tissue factor is a potent procoagulant and P-selectin promotes its production from monocytes.² Our data show evidence of all these processes in the drainage blood.

Reinfusion of activated blood did not lead to activation of the patients' circulating platelets and leukocytes. This may be explained by the small amount of reinfused blood compared with the total volume of blood in the patients' circulation. In addition, the concentration of platelets and, to a lesser extent, the leukocytes in the salvaged blood are low compared with those in the patients' circulation.

Our data suggest tissue factor expressed on the monocytes and neutrophils was introduced to the patients' circulation with the salvage blood. Tissue factor initiates the extrinsic coagulation pathway, with eventual conversion of prothrombin to the enzyme thrombin that changes fibrinogen to the fibrin clot. This conversion releases prothrombin fragment F 1+2 so plasma levels provide a measure of clot formation. Before reinfusion, the treated and control groups showed evidence of increased

postoperative thrombin generation, the prothrombin fragment F 1+2 levels being 2 to 21 times the upper limit of the established reference range of 0.4 to 1.1 nmol/L. This is most likely a product of the surgery. Prothrombin fragment F 1+2 has a half-life of 1.5 hours and would have been cleared to a degree 1 hour after the end of reinfusion. Also, it was diluted approximately 1:10 in the patients' circulation. One hour after the end of reinfusion, when compared with the control group, there was an increase in prothrombin fragment F 1+2 levels in the circulation of the treated group. Although this increase may be partly explained by prothrombin fragment F 1+2 being reinfused with the salvaged blood rather than being generated endogenously, the levels in systemic blood for individuals did not correlate with the F1+2 levels in the salvaged blood they received, suggesting reinfusion may have caused additional thrombin formation.

Reinfusion led to a 14.2% decrease in the platelet count 1 hour after the reinfusion as compared to the 2.5% decrease observed in the control group. This greater loss of platelets may be attributable to consumption brought about by the initiation of coagulation by reinfusion.

An increased activation and coagulability of the blood in drainage bags was observed in previous research⁸, although we used parameters not studied previously. We are unaware of previous reports of a systemic effect of reinfusion of autologous drainage blood. In particular, the rate of thrombotic complications, including DVT and pulmonary embolisms, in patients having TKAs where autologous reinfusion has been used, has not been reported with this or any other drainage system. Though we have

shown systemic effects on coagulation factors, we have not been able to determine if these changes may lead to a clinically relevant increase in DVT or PE.

The reinfusion of autologous salvaged blood to patients having a TKA does not lead to increased activation of circulating platelets and leukocytes but as noted earlier may activate coagulation in the recipient as suggested by a decrease in platelet count and increased levels of prothrombin fragment F 1+2. Although there are measurable systemic effects we do not know whether these effects lead to increased adverse clinical events. These theoretical clinical risks of reinfusion need to be weighed against the known risks of higher allogenic transfusion rates.

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Legends

Fig 1. Measurements of platelet activation markers by flow cytometry reveal strongly significant increases in platelet activation in salvaged blood as shown by platelet expression of P-selectin, Factor V/Va, Cd40 ligand, and increased numbers of platelet-derived microparticles. The columns represent the median values and the error bars represent the 25th and 75th percentiles; p is the significance of the difference between circulating and salvaged blood.

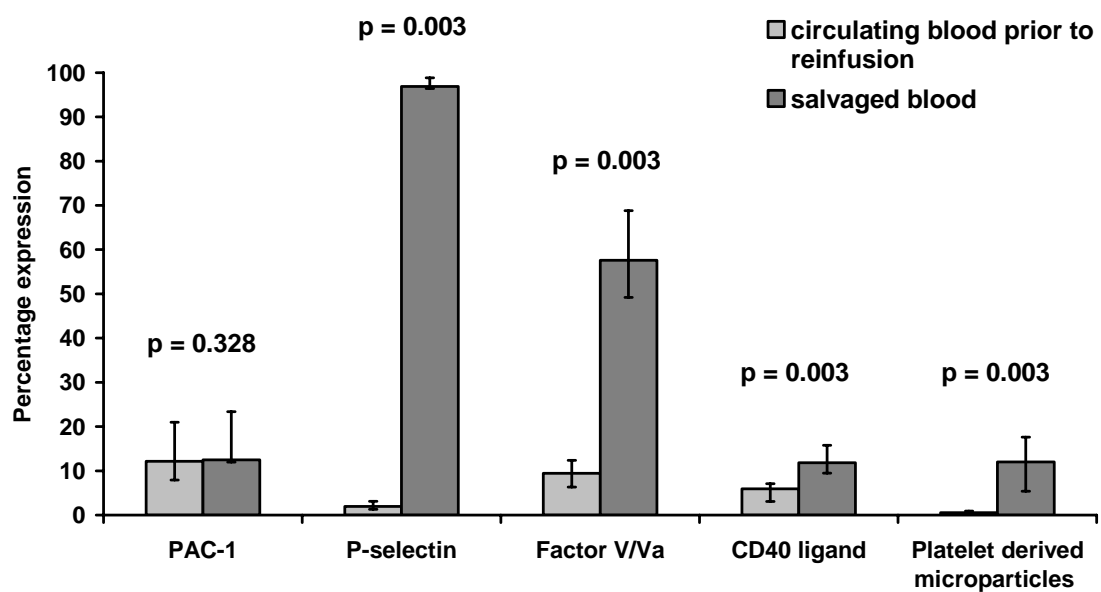


TABLE 1. Demographic Data

Parameter Measured	Treatment Group (n = 12)	Control Group (n = 12)
Female	7	9
Age (years)	72 (65–74)	74 (65–82)
Weight (kg)	84 (76–94)	82(68–89)
Preoperative hemoglobin (g/L)	138 (115–148)	133 (122–136) *
Preoperative hematocrit	0.41 (0.36–0.44)	0.38 (0.36–0.42) *
Preoperative platelet count (x10 ⁹ /L)	254 (213–276)	271 (220–286) *
Preoperative white blood cell count (x10 ⁹ /L)	6.9 (5.8–8.6)	7.8 (6.4–8.8) *
Preoperative APTT ¹ (sec)	28 (26–31)	25 (23–28)
Operation time (min)	89 (79–115)	86 (76–103)
Blood loss (mL)	800 (490–1058)	600 (445–806)
Reinfused blood (mL)	475 (400–600)	–
Hospital stay (days)	7.5 (6–10)	7 (6–9)

Data are expressed as median (interquartile ranges); * n = 11; APTT = activated partial thromboplastin time

TABLE 2. Prothrombin Fragment F 1+2 Levels Measured One Hour Before and One Hour After the Completion of Reinfusion . The Control Group Measurements Were Made at Corresponding Time Points Postoperatively.

Parameter Measured	<u>One Hour Before</u>		<u>One Hour After</u>		p Value
	Reinfused	Control	Reinfused	Control	
Prothrombin fragment F 1+2 (nmol/L)	4.6 (2.6–9.2)	3.3 (2.6–4.4)	24.3 (21.4–38.5)	3.7 (2.9–5.0)	0.002

Data are expressed as median (interquartile ranges). p = significance of the difference of the change in level from before reinfusion to after reinfusion time points between reinfusion and control groups.

TABLE 3. Patients' Platelet Count and Haemoglobin One Hour Before and One Hour After the Completion of Reinfusion and the Next Day. The Control Group Measurements Were Made at Corresponding Time Points Postoperatively.

Parameter Measured	<u>One Hour Before</u>		<u>One Hour After</u>		<u>Next Day</u>	
	Reinfused	Control	Reinfused	Control	Reinfused	Control
Platelets ($\times 10^9/L$)	211 (184-230)	236 (175-281)	181 (159-218)	230 (168-259)	163 (156-198)	212 (175-224)
Hemoglobin (g/L)	114 (104-131)	111 (102-117)	119 (110-131)	112 (98-120)	105 (96-118)	96 (89-106)

Data are expressed as medians (inter-quartile ranges).

TABLE 4. Full Blood Count of the Patient Before Reinfusion and of the Salvaged Blood

Parameter Measured	Patients Before Reinfusion (n = 12)	Patients' Salvaged Blood (n = 12)	p Value
Haemoglobin (g/L)	114 (104–131)	89 (63–111)	0.003*
Hematocrit	0.34(0.31–0.39)	0.26 (0.18–0.32)	0.003*
Platelet count ($\times 10^9/L$)	211 (184-230)	31 (21-49)	0.002*
Leucocyte count ($\times 10^9/L$)	12.5 (10.5–14.1)	4.7 (2.5-6.3)	0.002*
Neutrophils count ($\times 10^9/L$)	10.90 (9.47–13.12)	2.90 (1.95–4.52)	0.002*
Monocytes count ($\times 10^9/L$)	0.30 (0.23–0.65)	0.10 (0.00–0.2)	0.018

Data are expressed as medians (interquartile ranges); p=significance of the difference between patients circulating blood and their salvaged blood; *significant after Bonferroni's correction ($p < 0.007$)