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Role of the Hematocrit in a Rabbit Model of Arterial Thrombosis and Bleeding

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Background: A decrease in hematocrit lengthens bleeding time. The authors studied the role of hematocrit variations in an experimental model of arterial thrombosis and bleeding.

Methods: The Folts model was used in 24 rabbits. After anesthesia was induced and common monitors were positioned, the right common carotid artery was exposed and a 60% stenosis was induced. A compression injury of the artery was then produced, which triggered a series of cyclic episodes of thrombosis and clot lysis (cyclic flow reductions [CFRs]). After count-ing the number of CFRs that occurred in 20 min (CFR1), the animals were assigned randomly to one of three groups (n = 8 in each group): control, hemodilution with rabbit homologous platelet-rich plasma, and hemodilution with gelatin solution and then reinfusion of the shed blood. The effect of hemodilu-tion with replacement by platelet-rich plasma or by colloid was observed by recording the number of CFRs during another 20-min period (CFR2). A third period of observation (CFR3) followed shed blood reinfusion in the gelatin solution group. Ear immersion bleeding time was recorded after each CFR period.

Results: In the two experimental groups, the decrease in hematocrit (from 36 ± 3% to 23 ± 2% and from 38 ± 3% to 23 ± 2%, respectively; mean ± SD) abolished CFRs (from a median of 4 to 0 and 7 to 0, respectively) and significantly lengthened bleeding time (from 76 ± 24 s to 114 ± 36 s and from 84 ± 37 s to 127 ± 29 s, respectively). Blood reinfusion in the group that received the gelatin solution caused CFR to reappear (CFR3 = 4).

Conclusions: Decreases in hematocrit reduced the cyclic arte-rial thrombosis rate and increased the bleeding time in the rabbits in this study. Hematocrit normalization caused throm-bosis to reappear. (Key words: Cyclic flow reductions; hemodi-lution.)

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HEMATOCRIT is known to influence hemostasis and is partially responsible for the increased bleeding time ob-served in severe anemia, especially during chronic renal failure.[1-3] Erythrocytes provide adenosine diphosphate, a powerful platelet aggregation agonist, facilitate platelet accretion to the endothelium, and enhance platelet ac-tivity.[3-8] Using the Baumgartner perfusion chamber model, Escolar *et al.*[4] have shown that low hematocrit markedly decreases platelet endothelium accretion on the rabbit aorta. In the rabbit, Blajchman *et al.*[5] have shown that thrombocytopenia and low hematocrit val-ues both are independent factors that lengthen bleeding time. Cadroy and Hanson[6] have studied the role of he-matocrit in the constitution of the platelet plug in an *in vivo* model. They found that hematocrit variations be-tween 20–55% can inhibit or increase platelet aggrega-tion and thrombus formation, depending on local blood flow conditions. Finally, Santos *et al.*[7,8] have shown that
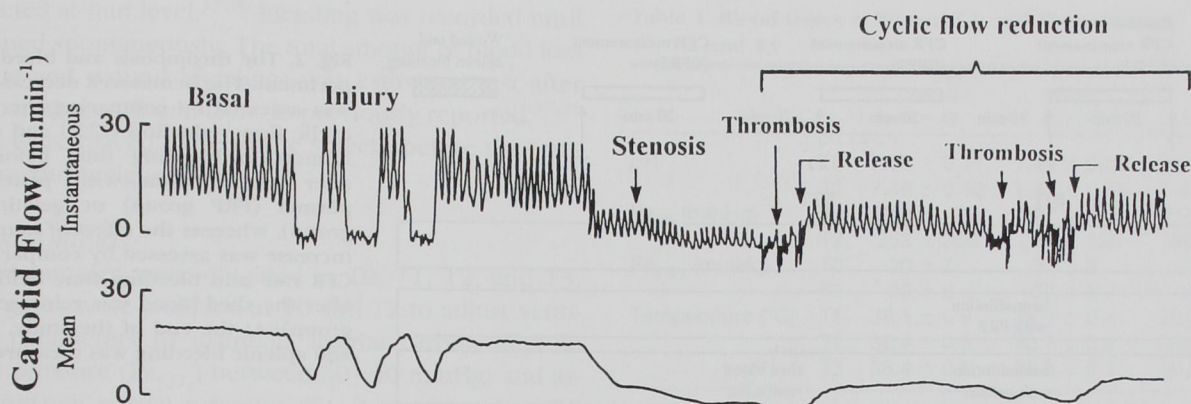
Fig. 1. Arterial flow recordings. The vessel was injured and stenosed, before spontaneous cyclic flow reductions occurred and corresponded to thrombosis, followed by spontaneous embolization (release) and recurrence of all phenomenon. No time scale is provided because the time is shorter on the right portion of the figure to facilitate the reader's understanding of the thrombosis process. However, the injury and stenosis require less than 30 s, and every cyclic flow reduction required approximately 3 or 4 min in the control group.

metabolically intact erythrocytes markedly enhance the platelet release reaction, eicosanoid synthesis, and further platelet recruitment, extending the concept that thrombus formation is a multicellular event.

Interest in animal models of arterial thrombosis[9-12] has increased in recent decades. The Folts model of arterial thrombosis can be reproduced.[13] In this model, a vascular wall injury, associated with stenosis, induces cyclic flow reduction (CFR), which can permanently stop flow. The endothelial damage induces platelet accretion and the formation of a platelet plug, which embolizes and is then formed again in a periodic cycle[14-16] (fig. 1).[17] First described in the canine coronary artery,[14] the Folts model was then applied to other arteries, especially the carotid, and in many different species, such as baboons,[18] pigs,[19-22] and rabbits.[17,23-26]

The aim of our study was to determine how a decrease in hematocrit with a stable platelet count influences the thrombotic process in the platelet-dependent Folts model of arterial thrombosis. We also evaluated the hemorrhagic risk associated with a low hematocrit by measuring the bleeding time from a small ear incision and the blood loss from a transected spleen.

Materials and Methods

Care of the rabbits conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance with the regulations of the official edict of the French Ministry of Agriculture. This study involved two parts: the application of the Folts model of the carotid arteries of the rabbit and a bleeding

model involving wound and spleen sections and ear immersion bleeding time.

Surgical Procedure

This prospective study included 37 male New Zealand rabbits that weighed 2.5 ± 0.2 kg (Elevage des Dombes, Romans, France).

Anesthesia was induced using intravenous sodium pentobarbital (30 mg/kg; Sanofi-Santé Vétérinaire, Libourne, France) and was maintained with sodium pentobarbital (10 mg/kg), as required. Tracheotomy and mechanical ventilation (Harvard Apparatus; Harvard Instruments, Boston, MA) were performed (respiratory rate, 45 cycles/min; tidal volume, 20–30 ml). Body temperature was recorded continuously using a rectal probe and maintained at approximately 38°C using an electric blanket (Homeothermic Blanket Control Unit; Harvard Apparatus) and a warming table (Scientific Research Instruments, Edenbridge, Kent, UK). A femoral artery catheter was placed and blood pressure was recorded continuously. The electric activity of the heart was recorded using five hypodermic electrodes.

The right carotid artery was exposed and isolated over approximately a 2-cm length. A 1.5-mm-diameter precalibrated electromagnetic circular flow probe (Skalar Instruments, Delft, The Netherlands) was placed around the right common carotid artery on the distal part of the exposed segment and connected to a flowmeter (model MDL 1401; Skalar Instruments). Zero calibration was obtained directly by occluding the artery with a cotton-tipped swab.
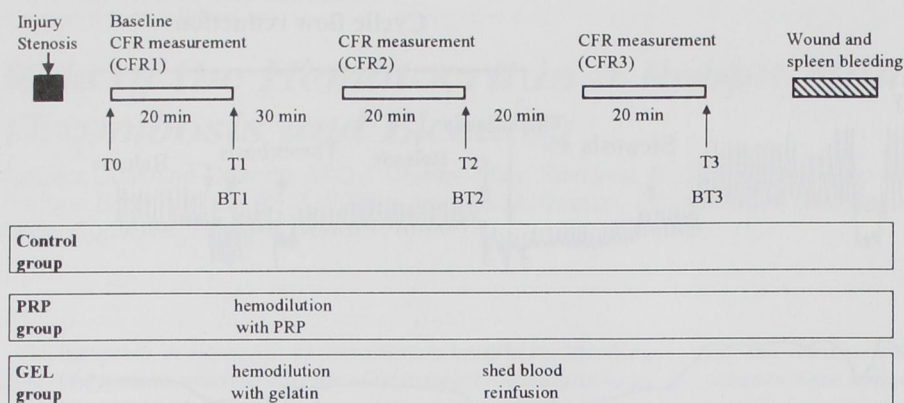
Fig. 2. The thrombosis and bleeding experiment. The hematocrit decrease effect was assessed by comparing the rate of cyclic flow reduction (CFR) and the ear immersion bleeding time before and after hemodilution with platelet-rich plasma (PRP group) or gelatin (GEL group), whereas the effect of hematocrit increase was assessed by comparing the CFR rate and bleeding time before and after the shed blood was reinfused (GEL group). At the end of the study, wound and splenic bleeding was measured.

Thrombosis

After 10 min of stabilization (baseline flow), a 60% stenosis of the right common carotid artery was produced by placing a vascular clamp around the artery in the proximal part of the 2-cm exposed segment. This stenosis was released after 10 min. An arterial injury was induced by three consecutive cross-clampings of the middle of the exposed segment of the artery in less than 10 s with a Mayo-Hegar needle holder forceps (Harvard Instruments) with three ratchet clicks closed, and a 60% stenosis was reapplied below the injury. This triggered a series of CFRs characterized by repetitive decreases in blood flow, followed by an abrupt spontaneous return of flow to the original levels (fig. 1). The return to original levels was facilitated occasionally by gently shaking the artery. Beginning with the first CFR, the thrombosis lysis process was observed for 20 min (baseline, CFR1). The number of CFRs during this 20-min observation period was noted. If no CFR was observed during this period, arterial injury was repeated at the same place, and a new 20-min period of observation was allowed. If no CFR occurred during this period, injury was induced on the controlateral carotid artery. This first period was common to all animals.

After this first 20-min period (CFR1), animals were allocated randomly to the following groups (fig. 2).

Control Group. The CFRs were recorded at two different times (CFR2, CFR3). The periods between CFR1 and CFR2 (30 min) and between CFR2 and CFR3 (20 min) were identical in the three groups.

Platelet-rich Plasma Group. Animals were gradually hemodiluted with platelet-rich plasma (PRP) according with the following formula:

$$\text{volume} = \text{body weight of the animal}$$
$$\times 0.07[2(Ht_i - Ht_f) \div (Ht_i + Ht_f)]$$

where Ht_i is the initial hematocrit and Ht_f is the final hematocrit to be obtained.

Hematocrit was monitored until it reached 23%. The PRP was obtained before each study from two other rabbits. Blood was collected on adenosine citrate dextrose in a double chamber device centrifuged to 280g for 3 min to separate PRP. The platelet count was maintained at more than 200 giga/l. CFR2 and CFR3 evaluated the effect of a low hematocrit on CFR.

The Gelatin Solution Group. A normovolemic hemodilution was performed by infusing a gelatin solution (Plasmion; Rhône Poulenc Rorer, Montrouge, France; GEL group). Blood was collected using a double-chamber device with adenosine citrate dextrose. Rabbit hematocrit was monitored regularly until it reached 23%. After a 30-min period and a second recording of CFR (CFR2), the shed whole blood was reinfused over 20 min. A third recording of CFR (CFR3) enabled an estimation of the effect of erythrocyte restitution on CFR.

Four points of measurements were defined: T0 at the beginning of CFR1, T1 at the end of CFR1, T2 at the end of CFR2, and T3 at the end of CFR3 (fig. 2) All the CFR recordings were reviewed blindly by an independent observer (author P.B.).

Bleeding

The ear immersion bleeding time was measured at the beginning and after each CFR period (fig. 2). The ear was cleaned and shaved on the external side. A small incision (5 mm) was made with a surgical blade (Surgicutt ITC; Ortho-Diagnostics-France, Roissy, France) and the ear was placed in a beaker containing 20 ml saline solution maintained at 38°C. Bleeding time was measured until the trickle caused by the incision stopped, as previously reported.[17,26]

At the end of the experiment, a xyphopubic laparotomy was performed. The spleen was isolated and

transected at mid level.[17,26] Bleeding was recorded until it stopped spontaneously. The total amount of blood loss (splenic and wound bleeding) was estimated just after the laparotomy, 15 min later, as previously reported.[17,26] Swabs were placed close to the spleen, before the transection, and weighed.

Blood Analysis

Blood samples were obtained at T0, T1, T2, and T3. Blood gases were analyzed at T0 and T2 to adjust ventilatory parameters to maintain arterial carbon dioxide partial pressure (Pa_{CO_2}) between 30–40 mmHg, and arterial oxygen partial pressure (Pa_{O_2}) at more than 70 mmHg. Hematocrit (Compur Microspin; Bayer Diagnostics, Domont, France) levels were measured, and arterial blood samples were collected on EDTA acid (Becton Dickinson-France, Le Pont de Claix, France) for platelet counts and on 3.8% trisodium citrated tubes (9:1 vol/vol, Becton Dickinson) for prothrombin time, fibrinogen (Hemolab; Biomérieux, Marcy-l'Etoile, France), and platelet aggregation measurements.

Ex vivo platelet aggregation analysis (Pack 4—Platelet aggregation chromogenic kinetic system; Helena Laboratories, Beaumont, Texas) was performed on PRP and calibrated with platelet-poor plasma. Platelet-rich plasma was obtained by centrifuging whole blood at 200g for 10 min at 37°C. Platelet-poor plasma was prepared from the same blood sample by centrifuging blood at 1,500g for 20 min. Platelets were counted in PRP to check the homogeneity of the samples. Platelet aggregation was induced by 5 mg/ml arachidonic acid (Helena-France, St. Leu, France). The increase in light transmission was recorded for 4 min after the aggregating agent (agonist) was added. Aggregation induced by the agonist in PRP was evaluated by measuring light transmission in stimulated PRP, assuming that light transmission was 100% in platelet-poor plasma and 0% in nonstimulated PRP.

The maximal intensity of platelet aggregation was defined as the maximal increase in light transmission, and the velocity of platelet aggregation (slope of the curve) was defined as the speed of the increase in light transmission, after the aggregating agent was added.

Statistical Analysis

Data are expressed as the mean ± SD, except for discrete variables (such as CFRs and spleen bleeding), which are expressed as medians with ranges. Several mean values were compared using analysis of variance, followed by the Scheffé test. Medians were compared using the Kruskall–Wallis test for independent measures,

Table 1. Blood Gases at T0 and T2, and Temperature T1, T2, and T3

		Control (n = 8)	PRP (n = 8)	GEL (n = 8)
pH	T0	7.56 ± 0.11	7.40 ± 0.08	7.51 ± 0.09
	T2	7.46 ± 0.10	7.43 ± 0.13	7.43 ± 0.08
Pa_{O_2} (mmHg)	T0	218 ± 200	297 ± 214	342 ± 253
	T2	253 ± 266	264 ± 230	293 ± 179
Pa_{CO_2} (mmHg)	T0	30 ± 7	35 ± 5	33 ± 7
	T2	30 ± 8	37 ± 8	37 ± 6
Temperature (°C)	T1	38.1 ± 0.9	38.1 ± 0.4	38.3 ± 0.6
	T2	38.4 ± 0.6	38.1 ± 0.5	38.1 ± 0.3
	T3	38.4 ± 0.5	38.3 ± 0.4	38.2 ± 0.9

Values are mean ± SD. No significant difference was observed between the three groups.

Pa_{O_2} = arterial partial pressure of oxygen; Pa_{CO_2} = arterial partial pressure of carbon dioxide. Four points of measurements were defined: T0 at the beginning of CFR1, T1 at the end of CFR1, T2 at the end of CFR2, and T3 at the end of CFR3.

followed, when significant, by a Mann–Whitney U test with Bonferroni correction, and by the Friedman test for repeated measure and, when significant, by the Wilcoxon rank sum test with Bonferroni correction. All comparisons were two sided. Probability values less than 0.05 were required to reject the null hypothesis. Statistical analysis was performed using a computer and Statview SE Graphics (Abacus Concepts, Berkeley, CA).

Results

Thirteen rabbits among 37 were not included: in five, complete thrombosis developed on both carotid arteries, two were excluded because of severe hypercapnia (≥ 70 mmHg) during the entire procedure, and six had CFR value less than three during the baseline period. Thus, 24 rabbits are included in the final analysis.

No significant difference was observed among the three groups for body weight and blood gas parameters (*pH*, Pa_{CO_2}, Pa_{O_2}) at T0 and T2 (table 1). No significant difference was observed among the groups for arterial blood pressure and heart rate during the experiment (data not shown). Temperatures were recorded continuously during the study, and there were no significant differences among the groups (table 1).

Blood Samples

No significant variation in hematocrit was observed in the control group during the study. In the PRP group, hematocrit decreased significantly as a consequence of hemodilution, and then remained stable until the end of

Table 2. Hematocrit, Platelet Count, Prothrombin Time, Fibrinogen, and Arachidonic Acid Induced Platelet Aggregation Parameters [Maximal Intensity (Max) and Slope of the Curve] at T1, T2, and T3

		Control (n = 8)	PRP (n = 8)	GEL (n = 8)
Hematocrit (%)	T1	37 ± 4	36 ± 3	38 ± 3
	T2	36 ± 4	23 ± 2*	23 ± 2*
	T3	34 ± 3	22 ± 3*	31 ± 3*†
Platelet count	T1	243 ± 54	263 ± 82	317 ± 61
(giga/l)	T2	236 ± 60	236 ± 66	220 ± 66
	T3	211 ± 47	228 ± 65	240 ± 45
Prothrombin time	T1	116 ± 19	117 ± 13	113 ± 13
(% of control)	T2	117 ± 19	114 ± 14	100 ± 8
	T3	108 ± 15	110 ± 107	95 ± 20
Fibrinogen (g/l)	T1	2.1 ± 0.7	2.2 ± 0.6	2.4 ± 0.7
	T2	2.0 ± 0.6	1.9 ± 0.4	1.5 ± 0.4
	T3	1.9 ± 0.7	1.8 ± 0.3	1.5 ± 0.3
Platelet aggregation	T1	55 ± 16	47 ± 25	45 ± 21
(Max (%))	T2	42 ± 23	31 ± 17	17 ± 14*
	T3	36 ± 20	29 ± 16	15 ± 15*
Slope (%/min)	T1	57 ± 20	62 ± 21	57 ± 18
	T2	55 ± 18	43 ± 15	33 ± 20*
	T3	41 ± 17	41 ± 16	36 ± 16

Values are mean ± SD. No significant difference was observed between groups for prothrombin time and fibrinogen.

* $P < 0.05$ *versus* control group at the same time.

† $P < 0.05$ *versus* GEL group at T2.

the study. In the GEL group, a comparable significant decrease in hematocrit was observed during hemodilution. Hematocrit significantly increased from 22 ± 3% to 31 ± 3% ($P < 0.05$) after blood reinfusion (table 2).

No significant difference was observed between the three groups regarding platelet count, prothrombin time, and fibrinogen. In the GEL group, arachidonic acid–induced platelet aggregation was significantly decreased at T2 and T3 for maximal intensity and at T2 for velocity. There was no significant difference in the PRP group for maximal intensity or velocity (table 2).

Thrombosis

In the control group, the number of CFRs was stable during the experiment. In the PRP group, CFRs were absent during the CFR2 and CFR3 periods. In the GEL group, CFRs were also abolished after hemodilution, but a significant reappearance of CFRs was observed after the sampled whole blood was reinfused (table 3).

Table 3. Cyclic Flow Reductions (CFR)

	Control (n = 8)	PRP (n = 8)	GEL (n = 8)
CFR1 (n)	4.5 (3–6)	4.0 (3–10)	7.0 (3–13)*
CFR2 (n)	4.0 (1–5)	0.0 (0–1)*	0.0 (0–0)*
CFR3 (n)	3.5 (0–6)	0.0 (0–1)*	4.0 (2–9)†

Values are median (extremes).

* $P < 0.05$ *versus* control group at the same time.

† $P < 0.05$ *versus* GEL group at T2.

Bleeding

Bleeding time was increased in the PRP and GEL groups at T2 and T3 compared with the control group (50 and 51% in the PRP and GEL groups, respectively). In the GEL group, blood reinfusion did not correct the prolonged bleeding (table 4).

A significant increase in bleeding after splenic section was observed in the GEL group, despite whole blood reinfusion (table 4).

Discussion

In the current study, a low hematocrit value induced a significant antithrombotic effect in a rabbit model of injured carotid arteries. Blood transfusion reversed this effect. Hemodilution prolonged the bleeding time and increased the volume of bleeding. These effects were not corrected by blood reinfusion.

We tried to control most of the parameters that could interfere with the occurrence of CFR. Our model allowed blood gases and hemodynamic and thermic conditions to be maintained. These factors are relevant because hypercapnia and hypoxia partially inhibit platelet aggregation in the rabbit and reduce the formation and the migration of the platelet plug.[27] Important modifications in platelet aggregation during acidosis and hypercapnia have been reported.[28] Continuous recording of

Table 4. Bleeding Time at T1, T2, and T3, and Wound and Splenic Bleeding

		Control (n = 8)	PRP (n = 8)	GEL (n = 8)
Bleeding time (s)	T1	77 ± 25	76 ± 24	84 ± 37
	T2	76 ± 21	114 ± 36*	127 ± 29*
	T3	73 ± 21	118 ± 38*	114 ± 42*
Wound and splenic bleeding (g)		6 (3–16)	9 (4–23)	13 (8–30)*

Values are mean ± SD for the BT; and median (extremes) for wound and splenic bleeding.

* $P < 0.05$ *versus* control group at the same time.

arterial blood pressure provided hemodynamic stability and protection against variations of flow, independent of the intensity of the vessel stenosis. Arterial pressure changes may induce variations in carotid flow signal that can simulate CFR. Finally, temperature was monitored and maintained at a constant level during the study to avoid a platelet dysfunction associated with hypothermia.[29]

To study the thrombotic process *in vivo*, many experimental models of arterial thrombosis have been devised. Folts *et al.*[13-16] and Hill *et al.*[23] designed a quantitative model of platelet thrombus formation that involves stenosis and intimal injury of the coronary artery in the dog and the monkey. They showed that marked stenosis associated with an injury promoted platelet accretion into the injured vessel wall. If the vessel was fixed for ultrastructural examination during the nadir of flow reduction during CFR, the stenotic area of the vessel contained a platelet plug. Because of the high reproducibility of this model in the rabbit, we used it as an experimental model of arterial thrombosis.[17,26] The role of hematocrit in arterial thrombosis had not been studied with this model.

An adequate hematocrit level is necessary to achieve optimum hemostasis. Erythrocytes have an established role in the development of arterial thrombosis formation.[1-8,30-34] Escolar *et al.*[4] studied the influence of erythrocytes and platelet count on the interaction of platelets with the subendothelium using the Baumgartner perfusion technique. They showed that the constitution of a platelet aggregate was impaired when the hematocrit was decreased to 20%, and this was independent of platelet count. It has been suggested that a low hematocrit may influence the bleeding rate from small vessels by reducing viscosity. Transfusion of packed erythrocytes, to restore hematocrit to nearly normal levels, is associated with a reduction in the bleeding rate of patients with severe anemia.[31-33] Hellem *et al.*[32] emphasized the major effect of hematocrit on platelet adhesion compared with the total platelet count. Blajchman *et al.*[5] observed an inverse correlation between the bleeding time and the hematocrit level in normal or thrombocytopenic rabbits. They also noted that a low hematocrit had a greater effect than did thrombocytopenia on bleeding time. Furthermore, the adverse effect of anemia was magnified by thrombocytopenia.

Three hypotheses have been proposed to explain the role of erythrocytes in primary hemostasis: a rheologic effect, the potential release of adenosine diphosphate by erythrocytes, and more recently, the enhancement of platelet reactivity and modulation of eicosanoid production by erythrocytes. Subsequent to a blood vessel injury, platelets adhere to the damaged vessel wall and initiate the hemostatic response. The rheologic effect could be depicted as the platelet margination in the periphery of the vessel because larger erythrocytes occupy the center of the vessel. Therefore, platelets are in a better condition to adhere to the subendothelium.[1,30,33,34] In contrast, Blajchman *et al.*[5] suggest that disruption of erythrocytes by the hemostatic plug formation releases adenosine diphosphate and thus improves platelet aggregation. Furthermore, when local accumulation of adenosine diphosphate is prevented, the effect of erythrocytes on the bleeding time is abolished.

Finally, one mechanism could be the direct activation of platelets by erythrocytes, as described by Santos *et al.*[7,8] This group also has advanced a third mechanism to explain the involvement of erythrocytes in hemostasis and thrombosis. Erythrocytes increase platelet serotonin release despite enzymatic adenosine diphosphate removal. These authors showed that erythrocytes modify the metabolism of platelet arachidonate, the release of eicosapentaenoate, and eicosanoid formation. A dramatic promotion of thromboxane formation has been shown in their model.[7]

Ex vivo, platelet aggregation with arachidonic acid decreased with hematocrit in the GEL group, and remained low after retransfusion, whereas CFRs reappeared significantly. The existence of an adenosine citrate dextrose–induced platelet dysfunction by citrate in the adenosine citrate dextrose may explain these persistently low values, even after blood retransfusion. Hellem[34] showed that platelets are nonadhesive in citrated plasma, and erythrocytes are necessary to transform nonadhesive platelets into adhesive platelets *in vitro*. In our study, platelet aggregation occurred in PRP with no erythrocytes. This could be one hypothesis for our results. However, if this hypothesis can be proposed for T3 in the GEL group and for T2 in the PRP group, the decrease in platelet aggregation at T2 in the GEL group could be explained by an antiplatelet activity of the gelatin solution.[35] Nevertheless, even if the decrease in bleeding time between T2 and T3 in the GEL group was not statistically significant, there was an interesting and logical trend. Furthermore, this impairment in platelet aggregation did not prevent the reappearance of CFR in this platelet-dependent model.

Ear immersion bleeding time previously was described and validated in the rabbit.[17,26] It is the only available test that globally assesses primary hemostasis in the rabbit. Microvascular bleeding, reflected by the splenic and wound bleeding, has been described before.[26] A decrease in hematocrit lengthens bleeding time.[1-5] In our study, we observed lengthened bleeding time in the PRP and GEL groups. These results could be relevant in humans because normovolemic hemodilution techniques are common in the operating room, although their usefulness is controversial.[36] In conclusion, the combination of a bleeding model with the Folts model allowed us to show that a low hematocrit decreases arterial thrombosis and is associated with increased blood loss and prolonged bleeding time.

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