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Platelet Separation From Whole Blood in an Aqueous Two-Phase System With Water-Soluble Polymers

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Abstract. Platelet-rich plasma (PRP) stimulates tissue healing and centrifugation is the only method for PRP preparation. The purpose of the present study was to develop a method to separate platelets without centrifugation. We used 16 polymers of different chemical characteristics and mixed each polymer with fresh whole blood containing anticoagulant citrate-dextrose. Then, we observed blood cell separation. Focusing on the effective polymers and comparing our method with the conventional centrifugation method, we examined platelet recovery rate and P-selectin expression, which represents platelet activation. Poly-L-glutamic acid (PGA) and poly-L-aspartic acid (PAA) separated platelets efficiently; however, these polymers activated platelets. On the contrary, poly (2-methacryloyloxyethyl phosphorylcholine-co-*n*-butyl methacrylate) (PMB), which has phosphorylcholine residues mimicking a cellular membrane molecule, separated platelets moderately and the PMB-separated platelets were nearly inactive compared to the separation with the ordinary centrifugation and PGA. Conclusively, the present experiments demonstrated that without centrifugation platelets can be separated from whole blood with some water-soluble polymers, such as PGA, PAA, and PMB, and that PMB has an advantage not to activate platelets.

Keywords: Platelet-rich plasma, separation, aqueous two-phase system, polymer, P-selectin

Introduction

Platelets contain various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), and vascular endothelial growth factor (VEGF) (1 – 3). When platelets are activated, they release these growth factors, which subsequently play important roles in wound healing processes (4, 5). Thus, it is reasonable to use platelets as a wound healing stimulator. Clinically platelet-rich plasma (PRP) has been prepared from patient's blood and applied to the surgical wound of the same patient. Effectiveness of PRP in dental, orthopedic, and cosmetic surgical treatments has been reported (6 – 13). Notably, since PRP is autologous, there is no possibility of infectious transmission via PRP, which is also an advantage of PRP.

Although PRP is clinically effective, the procedure of centrifugation and transferring the supernatant is required in PRP preparation. Kits for easy PRP preparation has been developed and available (14 – 16); however, centrifugation is still necessary. In the current PRP preparation, purchasing a cyclone separation, an additional staff for PRP preparation during the surgery, and the risk of an accident in handling needles in the preparation procedure are inevitable, which makes it difficult to use PRP in general practice, especially in a small clinic. The purpose of the present study was to develop a method to separate platelets without centrifugation. Here we report several water-soluble polymers, which efficiently separate platelets from whole blood without centrifugation.

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Materials and Methods

Polymers and platelet separation

Firstly, effect of various polymers on platelet separation was examined and we used sixteen different polymers (Table 1). Three milligrams of each polymer was dissolved in 0.15 ml of 3.8% anticoagulant citrate-dextrose (ACD) (Sigma-Aldrich, St. Louis, MO, USA) in a 1.5 ml tube. Then, 1.35 ml of fresh blood was added and mixed gently. The tubes were allowed to stand for 30 min at room temperature. The samples in the tubes were visually observed and pictures were taken every 10 min. Secondly, in the similar manner, effects of different molecular weights of poly-L-glutamic acid (PGA) (Sigma-Aldrich) were examined on platelet separation. Three milligrams of each PGA were added to the blood. The molecular weights of PGA were 1500 – 3000 (PGA1), 10,900 (PGA2), 31,700 (PGA3), 50,300 (PGA4), and 84,600 (PGA5). Thirdly, different amounts (1 – 5 mg) of PGA5 were added to the blood to determine dose-dependency.

Conventional platelet preparation was also carried out as previously reported (17). At first, 8.5 ml of blood was mixed with 1.5 ml of ACD and then centrifuged at 3600 rpm (1200G) for 15 min. The upper layer was discarded, and then the lower layer, which contains red blood cells and platelets, was centrifuged again at 2400 rpm (570G) for 10 min. Finally, 1 ml of the supernatant was collected as the platelet-rich solution.

Platelet counting, recovery rate, morphology, and P-selectin expression

Blood cells including platelets in the sample were counted with a fully automatic hematology analyzer (Celltac α, MEC-6318; Nihon Kohden Co., Tokyo). Platelet recovery rate is the ratio of the total platelet number in the final sample to the platelet number in the initially-collected whole blood. The cell morphology was also examined with a phase contrast microscope (IX70; Olympus Co., Tokyo).

The expression of P-selectin was examined after the platelet separation with PGA, poly (2-methacryloyloxyethyl phosphorylcholine-co-*n*-butyl methacrylate) (PMB) (NOF Co., Tokyo) and the conventional method. Briefly, immediately after the separation of platelets, 5 μl of each sample was incubated with 20 μl of phycoerythrin (PE) conjugated anti P-selectin monoclonal antibody (CD62P PE) (Becton Dickinson, San Jose, CA, USA) and 20 μl of CD41-FITC (Immunotech, Marseille, France) at room temperature. After 15 min, all samples were mixed with 1% paraformaldehyde (CellFIX; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Platelets incubated with PE-coupled Mouse IgG (Platelet control γ1 PE) (Becton Dickinson Immunocytometry Systems) served as controls. Then, the P-selectin expression on the platelet surface was

Table 1. The polymers used in the present study and their classification based on blood cells separation

Group	Polymer	Molecular weight	Platelet recovery rate (%)	
			Upper layer	Lower layer
1	Poly-L-glutamic acid sodium salt (PGA)	84,600	88	12
	Poly-L-aspartic acid sodium salt (PAA)	35,400	97	3
2	Poly-L-lysine hydrobromide	84,000	0	—
	Alginic acid sodium salt	—	10	90
	Carboxylmethylcellulose sodium salt (CMC)	180,000	6	94
3	Single strand DNA	—	79	21
	Poly (2-methacryloyloxethyl phosphorylcholine-co-*n*-butyl methacrylate) (PMB)	10,000	53	47
4	Hyaluronic acid sodium salt	10,700	10	90
5	Poly-L-asparagine (from Sigma-Aldrich)	10,700	—[a)]	—[b)]
	Dextran	60,000 – 90,000		
	Poly (ethylene glycol) (PEG)	10,000		
	Poly (acrylic acid) (from Wako)	2,000		
	Double strand DNA	—		
	Poly-L-histidine (from Sigma-Aldrich)	20,000		
6	Poly-L-arginine hydrochloride	141,400	Gel formation	
	Glycol chitosan (glycol chitin, deacetylated)	—		

—[a)]: unable to count cells; —[b)]: no measurement.

analyzed by a fluorescence activated cell sorter (FACS) (BD FACSCalibur, Becton Dickinson). All samples were identified by a forward versus side scatter plot set to a logarithmic scale, and 10,000 events were evaluated. The fluorescence of stained platelets was analyzed with the Cell Quest software to obtain both the percentage of positively stained cells and the mean particle fluorescence intensity (18, 19).

Statistical analyses

Numerical data were presented as the mean ± S.E.M. Differences between the groups were examined with Tukey and Student's paired *t*-tests.

Results

The polymer used in these experiments and their effects on platelet separation are summarized in Table 1 and Fig. 1. The polymers were divided into 6 groups in terms of separation speed and platelet localization. When poly-L-arginine or chitosan (both, Sigma-Aldrich) was used (Group 6), two-phase separation did not occur and gel formation was observed. In the other polymer groups two-phase separation was observed; however, separation speed and platelet localization were different. When PGA or poly-L-aspartic acid (PAA) (Sigma-Aldrich) was used, the separation speed was fast and most platelets localized in the upper layer. After the separation with PGA, the platelet increase in the upper layer was evident under a phase contrast microscope, which was comparable to the platelet-rich solution after the conventional separation (Fig. 2). Although poly-L-lysine (Sigma-Aldrich), alginic acid (Sigma-Aldrich), and carboxylmethylcellulose (CMC) (Dai-ichi Kogyo Seiyaku Co., Ltd., Tokyo) also induced quick two-phase separation, platelets localized with red blood cells in the lower layer. In the medium speed separation groups, single strand DNA (kindly supplied by Nippon Chemical Feed Co., Ltd., Hakodate) and PMB of group 3 separated platelets in the upper layer, whereas hyaluronic acid (Wako Pure Chemical Industries, Ltd., Osaka) of group 4 distributed platelets in the lower layer with red blood cells. Polymers, which showed neither gel formation nor distinct blood separation even after 30 min were classified into group 5.

Effect of PGA molecular weight on the separation are demonstrated in Fig. 3. The separation speed and platelet recovery rate were extremely high when PGA4 or PGA5 was used. Then, we used PGA5 and examined the effect of the amount of PGA5 on the separation. As shown in Fig. 4, both the separation speed and platelet recovery rate were high when the amount of PGA5 in the tube was more than 2 mg.

When 3 mg of PGA or PMB was used, blood cell separation occurred within 30 or 40 min, respectively. Thirty minutes after the incubation, the platelet recovery rate of PGA separation was significantly higher than those of PMB separation and also the conventional

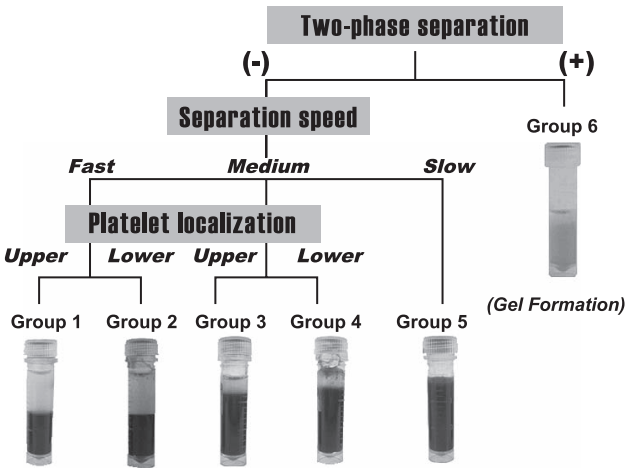

Fig. 1. Classification of the polymers. Each polymer was mixed with whole blood and classified into 6 groups.

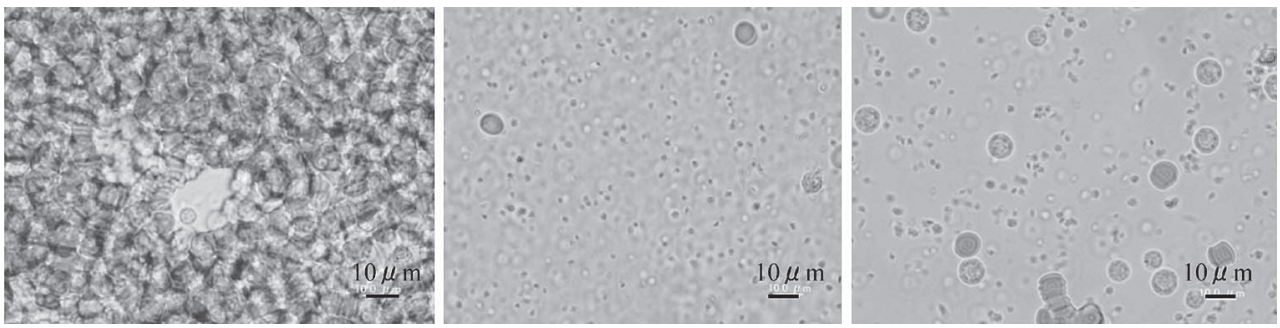

Fig. 2. Images of phase contrast microscope. Whole blood (A), platelet-rich plasma (B), and upper layer of two-phase separation with poly-L-glutamic acid (PGA) (C).

method (Fig. 5A). The platelet recovery rate of PMB was comparable to the one of the conventional method. The result of P-selectin expression after the separations are presented in Fig. 5B. PMB separation demonstrated low P-selectin expression compared to the conventional method, whereas PGA separation caused extremely high P-selectin expression.

Discussion

In oral surgery, PRP has been clinically used either as PRP itself or as a mixture combined with autogenous bone or bone substitute (6 – 10). PRP has been also applied in orthopedic and cosmetic surgeries (11 – 13). PRP preparation kits have been developed and available (14 – 16), which makes PRP preparation simple to some degree. However, centrifugation is still required in PRP preparation, and the cyclone separator and PRP preparation kits are both expensive (13). In the present study we separated platelets from a small amount of blood without centrifugation.

The addition of a polymer to the solution separates some of the molecules in the solution in the upper or lower layer, which is called an "aqueous-two phase system" (20 – 22). We speculated that we could use the "aqueous-two phase system" to prepare platelets from whole blood. Furthermore, for clinical application, we looked for polymers to precipitate red blood cells in a short time. It has been reported that when polymers, such as dextran and polyethylene glycol (PEG) are mixed with blood, "rouleaux" of red blood cells occurs, subsequently accelerating precipitation of red blood cells (20, 23 – 25). However, dextran and PEG (both, Sigma-Aldrich) did not precipitate red blood cells without centrifugation and these polymers were classified in Group 5 in the present study.

Baumler et al. proposed two models to explain rouleaux of erythrocytes with some polymers (26), although the exact mechanism of rouleaux is not clear. One is the "bridging model" in which polymer molecules attach to erythrocyte cell surfaces forming bridges between erythrocytes. Another is the "depletion

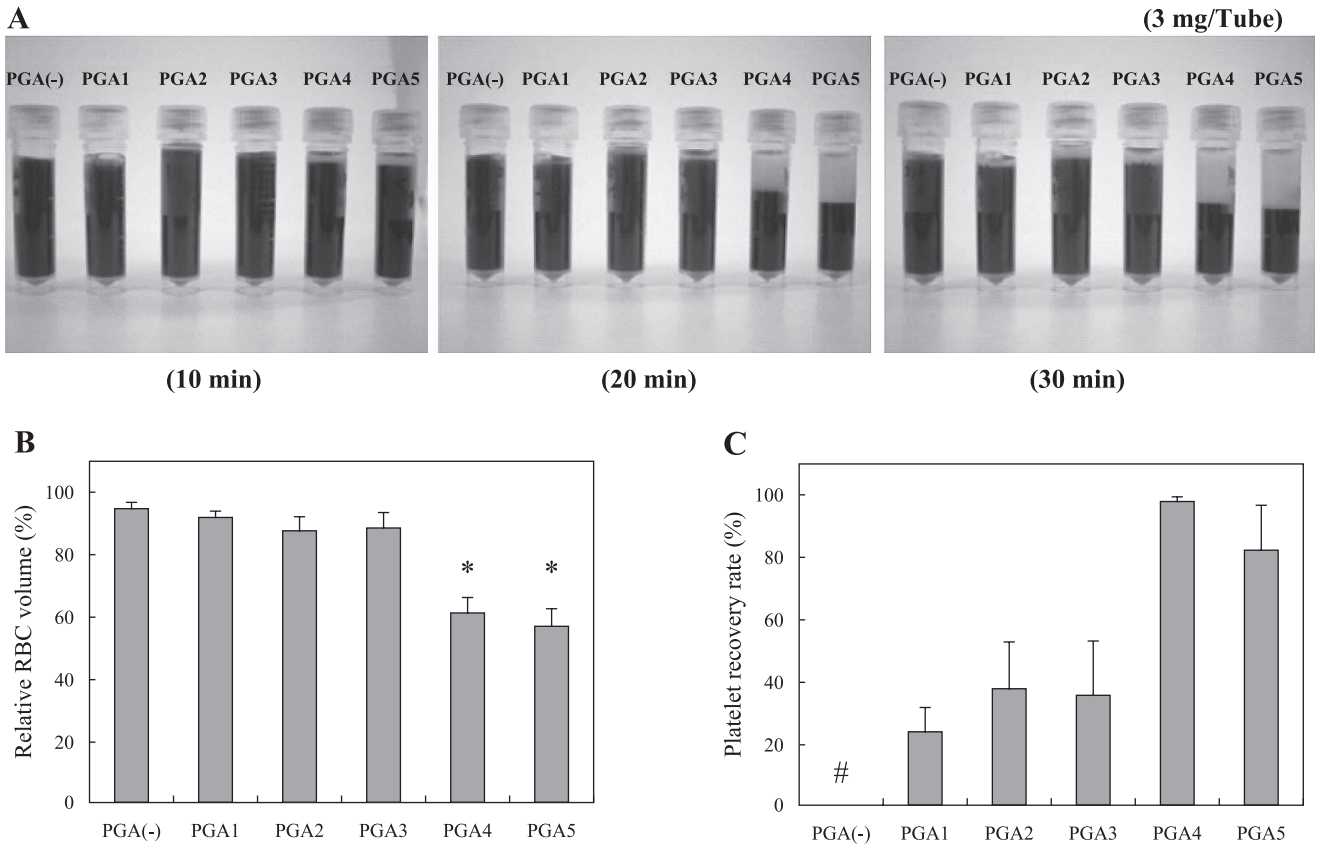

Fig. 3. Effects of molecular weight of PGA on blood cell separation. Three milligrams of PGA of different molecular weight was mixed with 0.15 ml of ACD and 1.35 ml of whole blood. The molecular weight of PGA used in this experiment was 1,500 – 3,000 (PGA1), 10,900 (PGA2), 31,700 (PGA3), 50,300 (PGA4), and 84,600 (PGA5). Visual images are presented in panel (A), red blood cell (RBC) volume in the tube after 30 min and platelet recovery rate are presented in panel (B) and (C), respectively. *: Significantly different from other groups, $P<0.01$. #: Not measured.

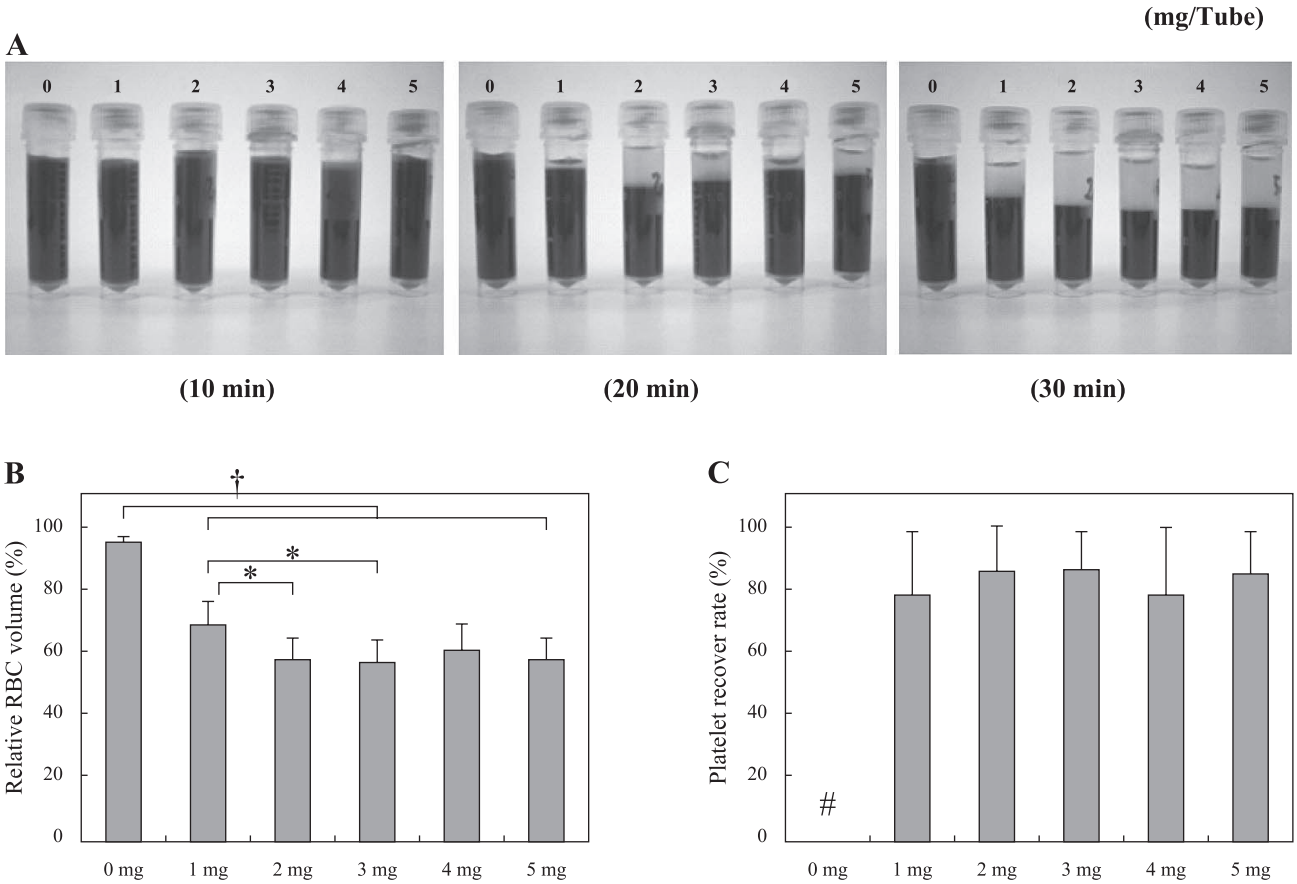

Fig. 4. Effect of PGA amount on blood cell separation. Different amount of PGA, molecular weight of 84,600, was mixed with 0.15 ml of ACD and 1.35 ml of whole blood. Visual images are presented in panel (A), red blood cell (RBC) volume in the tube after 30 min and platelet recovery rate are presented in panel (B) and (C), respectively. *: Significantly different from the 1-mg group, $P<0.05$. †: Significantly different from other groups, $P<0.01$. #: Not measured.

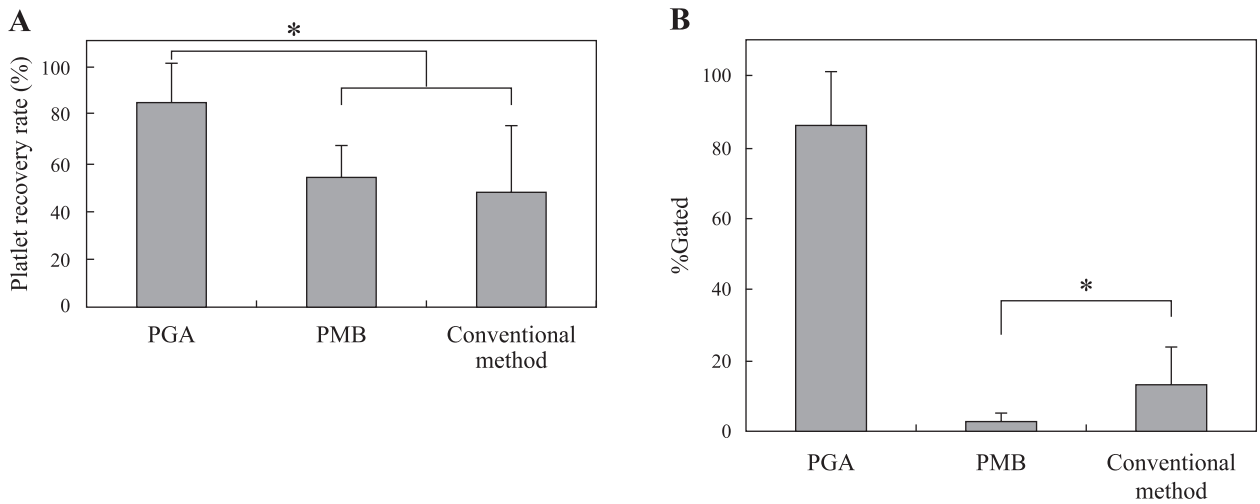

Fig. 5. Comparison of two-phase separation of two polymers (PGA and PMB) and the conventional method. Platelet recovery rate (A) and P-selectin expression (B). *: $P<0.05$.

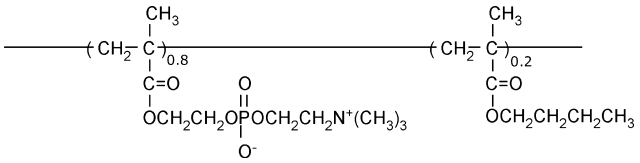

Fig. 6. Molecular structure of PMB.

model", in which depletion of macromolecules from the interface leads to connect erythrocytes when the cells come into close proximity due to the osmotic pressure difference between the pressure in the surrounding phase and in the gap of erythrocytes. These two models might partially explain the separations that we observed in the present study.

The polymers of Group 1 and Group 2 in the present study were able to precipitate red blood cells within 30 or 40 min and the volume of the precipitates were approximately similar to hematocrit. Especially, poly-L-lysine precipitated all blood cells at the fastest speed and they aggregated in the lower layer. In poly-L-lysine sedimentation, the upper layer did not contain any cells. The aggregate of the cells became round and small in the tube several hours after the treatment. The surfaces of the cells are negatively charged, whereas poly-L-lysine is a cationic polymer. Thus, it is likely that poly-L-lysine bridged all the blood cells subsequently precipitating all of them (27).

PGA and PAA in the group 1 and alginic acid (Sigma-Aldrich) and CMC in the group 2 are anionic polymers having carboxyl residues in the molecules. These polymers precipitated red blood cell and the sedimentation speed followed the one of poly-L-lysine. Interestingly, when PGA or PAA was mixed with whole blood, most platelets were located in the upper layer. On the contrary, the platelets were located in the lower layer when alginic acid or CMC was used. We were not able to elucidate the mechanism of these separations; however, it is obvious that these polymers interacted with some molecules around them or with blood cells, subsequently separating each cell population in the different layers.

Considering the clinical application of PRP, it is important that the polymer for the separation should be bio-inert to blood cells and safe in the body. Thus we selected PGA because PGA has been already used as a pharmaceutical conjugate (28). We also selected PMB because PMB does not activate platelets when PMB contacts blood (29, 30). This unique nature of PMB is due to phosphorylcholine structure in its molecule (Fig. 6) (31). We compared the platelet separations in PGA, PMB, and conventional centrifugation methods: We examined platelet recovery and platelet activation in these separations.

In most of the previous reports on PRP by other investigators, the degree of platelet activation has not been evaluated, although final platelet concentration has been examined. When platelets are activated, growth factors in platelets are released. When platelet activation occurs during PRP preparation, growth factors will be diluted before PRP application. Theoretically, if platelets are not activated, growth factors remain in these cells and we could apply these growth factors to the surgical site. Thus, it is obvious that less activation of platelets during PRP preparation is ideal.

High separation speed and high recovery rate (32) were observed in PGA separation: however, most platelets, more than 90%, were activated in this procedure. In PMB separation platelet activation was extremely low, although separation speed and recovery rate were lower than those in PGA separation. It has been reported that P-selectin expression level in the conventional method is approximately 20% (33), which is comparable to the value obtained in the present study. It is clear that the centrifugation separation procedure activates platelets to some degree. The present results demonstrated that intact platelets are available by the PMB separation.

In the present study, utilizing some polymers such as PGA or PMB, we were able to prepare platelets and plasma easily in a short time without centrifugation. In this method, we do not need to transfer the supernatant to another tube for centrifugation, which is required in most of the PRP preparation kits. Thus, we can avoid accidents in handling needles during the preparation procedure. Especially using PMB, we can prepare intact inactivated platelets compared to the conventional centrifugation procedure. PMB is a bio-inert material as we have already reported (29, 30, 34 – 38). Thus, although further studies are required, our separation method with PMB could be applied for PRP preparation during various surgical operations.

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