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ORIGINAL ARTICLE

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The influence of time of storage, temperature of storage, platelet number in platelet-rich plasma, packed cell, mean platelet volume, hemoglobin concentration, age, and sex on platelet aggregation test

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Abstract The influences of time of storage of platelet-rich plasma (PRP), temperature of storage of PRP, platelet number in PRP, mean platelet volume in whole blood, sex, age, hemoglobin concentration, and different forms of PRP storage on platelet aggregation (PAG) tests, performed with epinephrine, collagen, arachidonate, and ristocetin by a four-channel aggregation profiler (Platelet Aggregation Profiler, Model PAP-4, Bio/Data Corporation, Hatboro, PA 19040, U.S.A.), were evaluated in four groups of subjects (52 men, 22 women, age range 20–85 years, hemoglobin concentration range 8.4–16.8 g/dl). The PRP was stored with or without packed cells, at room temperature or at 4° C, for 0–6 h. The ideal platelet number of PRP for performing the PAG test fell between 150 and 500×10^9/l. If the number was less than 150×10^9/l, the result of PAG should be meaningless. No significant change was noted for up to 6 h when the PRP was stored either at room temperature or at 4° C. Hemoglobin concentration and mean platelet volume did not affect the PAG. However, there was significant but weak correlation ($p = 0.0125$, $r = 0.3696$) between age and PAG when using arachidonic acid as the agonist. Men had significantly increased PAG when collagen and ristocetin were used as the agonists. The PRP was stored best at room temperature, without packed cells. In conclusion, to obtain the best result from a PAG test, the PRP should be kept without packed cells at room temperature for no longer than 6 h, and the platelet number should fall between 150 and 500×10^9/l.

Key words Temperature · Platelet aggregation test · Packed cell · Platelet number · Platelet size

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Introduction

Platelet aggregation (PAG) tests are among the most important tests for evaluating qualitative platelet disorders. Two methods have been developed: The first was introduced by Born in 1962 [1], based on increased light transmission of platelet-rich plasma (PRP) caused by the formation of aggregates and the decreased volume occupied by the platelets in a photoelectric cell [2]. The second technique was introduced by Cardinal and Flower [3], using "electrical aggregometry" in whole blood, as well as single cell counting to study platelet behavior. However, both methods have the problem of standardizing the condition of the specimens. Many laboratory variables can affect PAG, such as venipuncture technique, centrifugation technique, platelet concentration, time after venipuncture, and temperature at which PRP is stored [4, 5]. Biological variations, such as day-to-day variability in the aggregation of normal platelets [5] and variability in the response of platelets from normal individuals to a threshold level of agonist [6], can affect PAG. In addition, albumin, glucose and bilirubin [7–10] have also been reported to influence PAG. As so many conditions can affect PAG, correct interpretation of the results of PAG can be made only when we have adequate knowledge of all the factors involved. The British Society for Hematology – BCSH Haemostasis and Thrombosis Task Force – suggested the following guidelines for performing PAG with PRP [11]: (a) PRP should be stored at room temperature in a capped tube; (b) the final platelet count should be 200×10^9/l; (c) aggregation should be performed within 2 h. However, for practical reasons, the tests cannot always be performed within 2 h or the platelet count of PRP may be higher or lower than 200×10^9/l. The present study was therefore planned to determine the influence of the platelet number in PRP, mean platelet volume (MPV) in whole blood, sample storage time, temperature of storage, storage method, hemoglobin concentration, age, and sex on the PAG.

Materials and methods

Sample preparations

After clean venipuncture with a plastic syringe, blood was collected into the anticoagulant (1/10 volume of 3.8% sodium citrate). PRP and platelet-poor plasma (PPP) were prepared by centrifugation at room temperature at $150 \times g$ for 6 min and $3000 \times g$ for 10 min, respectively. For storage of PRP, a paraffin stopper was capped on the test tubes and they were kept at room temperature, excepting the special conditions mentioned in the fourth group of subject.

Platelet aggregation tests

PAG tests were performed using a four-channel aggregation profiler set at 37° C with a stirring speed of 1000 rpm (Platelet Aggregation Profiler, Model PAP-4, Bio/Data Corporation, Hatboro, PA 19040, U.S.A.). It was a self-contained instrument, having four independently operated aggregation channels. When aggregation patterns were generated, they were simultaneously and automatically stored in memory and could be recalled either singularly or in any combination as required. PRP was added to a glass cuvette (at a final volume of 0.5 ml, with PRP 0.45 ml and reagent 0.05 ml) and warmed to 37° C for 3–4 min. Four reagents were added to the PRP to perform the test with the final concentrations in the PRP given: (a) collagen (Bio/Data, lyophilized soluble calf skin collagen), 0.19 mg/ml; (b) ristocetin (Bio/Data), 1.5 mg/ml; (c) arachidonic acid (Bio/Data), 500 μg/ml; and (d) epinephrine (Bio/Data), 100 μM. The extent of aggregation was calculated from the maximum change in light transmission. Test results were expressed as arbitrary units between 0% (PRP) and 100% (PPP), according to the manufacturer's instructions for the aggregometer in use.

Study projects and sample proceeding

Four groups of people were included in our study after giving their signed consent. The first group included 33 subjects (24 men, 9 women, mean age 58.0±19.0 years, range 21–85 years) with different platelet counts. They included 22 normal healthy controls and 11 patients; i.e., iron-deficiency anemia with reactive thrombocytosis (platelet $>450 \times 10^9$/l), or thrombocytopenia (platelets $<150 \times 10^9$/l) due to vitamin B_{12} deficiency or causes other than myelodysplastic syndrome or idiopathic thrombocytopenic purpura. They all had normal PAG. None had had hyperlipidemia or taken aspirin or any nonsteroidal anti-inflammatory drugs for at least 14 days before the study. PRP and PPP were obtained from these subjects. Platelet numbers in PRP and whole blood MPV were determined by an automated hematology analyzer (Sysmex M2000, Toa Medical Electronics Co., Ltd., Kobe, Japan). The PRP was then diluted by PPP into five concentrations, if possible, with platelet counts of (a) $>500 \times 10^9$/l, (b) 200×10^9/l, (c) $150–200 \times 10^9$/l, (d) $100–150 \times 10^9$/l, and (e) $50–100 \times 10^9$/l. Four reagents were used to test the response of PAG in the PRP of different platelet concentrations to compare their results.

The second group included 27 normal healthy subjects (18 men, 9 women, mean age 33.6±20.5 years, range 21–76 years) with a mean platelet count of $255 \pm 78 \times 10^9$/l. No aspirin or any nonsteroidal anti-inflammatory drug had been taken by these subjects for at least 14 days before sampling. After sampling, PRP was obtained and stored as described above, and PAG tests were performed without dilution of PRP at 37° C with four reagents immediately (indicated as 0 h, but actually about 1 h after sampling, due to the time consumed in the process), 2, 4, and 6 h after blood had been drawn. The mean platelet count in PRPs was $395 \pm 146 \times 10^9$/l. The results obtained from different storage time of PRP were compared.

The third group included subjects who had platelet count of $150–400 \times 10^9$/l in the above two groups, with a total of 51 subjects (34 were male, mean age 44.7±23.0 years). In the first group, we used the PAG results of the undiluted PRP, and in the second group, we used the 0-h PRP as the basis for calculating the correlations of PRP with age, hemoglobin concentration, sex, and whole blood MPV (determined as described before) in order to determine the influence of these factors on PAG.

The fourth group included 14 normal healthy subjects (10 men, 4 women, mean age 28.1±7.9 years, range 20–42 years). All subjects were drug free during the 2 weeks preceding the experiment. PRP was obtained as described above and stored in three ways: (a) at room temperature without packed cells, (b) at 4° C without packed cells, or (c) at 4° C with packed cells, i.e., after centrifugation; the PRP was not separated from the packed cells until the tests were performed. (In the latter two conditions, the centrifugation temperature was also 4° C). Collagen was used to perform the PAG tests at 37° C 0, 2, 4, and 6 h after drawing of blood, and the results were compared with one another.

Statistical analysis

In the first group, the ANOVA test or Kruskal-Wallis test was used to compare the differences in five subgroups of different platelet concentrations, depending on whether the distribution was normal or not. In the second group, the ANOVA test was used to compare the difference of PAG at different time after drawing of blood. In the third group, the Pearson correlation matrix was used to assess the relationship between parameters. In the fourth group, the paired *t*–test (in normal distribution) or the Wilcoxon matched-paired signed-rank test (in non-normal distribution) was used to test the difference of three storage methods at different periods.

Results

The influence of platelet count in PRP on PAG

Table 1 shows the PAG results in five PRPs with different platelet counts stimulated by arachidonic acid, collagen, epinephrine, and ristocetin. When arachidonic acid or epinephrine was used as the agonist, the optimal platelet count in PRP was $150–500 \times 10^9$/l. When the platelet count was lower than 150×10^9/l, PAG was markedly decreased. On the other hand, when collagen or ristocetin was used as the agonist, the range of the platelet count in PRP was wider, from 100×10^9/l to 500×10^9/l, without affecting the results.

The influence of storage time on PAG

Table 2 shows the PAG results performed at room temperature and at different times stimulated by arachidonic acid, collagen, epinephrine and ristocetin. No significant change was found up to 6 h after sampling. In other words, the storage time, counted up to 6 h, did not affect the results of PAG, regardless of which agonist was used.

Table 1 Platelet aggregation in PRP with different platelet counts stimulated by arachidonic acid (AA), collagen (CN), epinephrine (EP), and ristocetin (RN) in 33 subjects

Platelet count range ($\times 10^9$/l)	Platelet aggregation Mean ± SD (%) (Number observed)			
	AA	CN	EP	RN
(A) >500	60.8 ± 16.0 (6)	61.5 ± 12.9 (6)	59.8 ± 16.9 (4)	63.7 ± 14.3 (7)[e]
(B) 200–500	66.8 ± 8.9 (26)	71.3 ± 11.3 (29)	68.1 ± 7.7 (16)	75.3 ± 11.9 (30)
(C) 150–200	63.0 ± 14.8 (23)	73.0 ± 12.4 (25)	54.9 ± 27.4 (14)	80.0 ± 7.5 (26)
(D) 100–150	37.2 ± 23.6 (28)[a]	68.6 ± 13.4 (32)	36.7 ± 28.0 (18)[d]	74.2 ± 15.0 (33)
(E) 50–100	21.8 ± 24.3 (24)[a, b]	59.3 ± 21.9 (26)[c]	25.9 ± 25.4 (15)[d]	68.2 ± 18.8 (27)[e]

[a] $p = 0.0001$, compared with (B) and (C), ANOVA test
[b] $p = 0.0001$, compared with (A) and (D), ANOVA test
[c] $p = 0.0105$, compared with (B) and (C), ANOVA test
[d] $p = 0.0002$, when compared with (B), Kruskal-Wallis test
[e] $p = 0.0118$, compared with (C), ANOVA test

Table 2 PAG results after different PRP storage times at room temperature in 27 normal healthy subjects, with arachidonic acid (AA), collagen (CN), epinephrine (EP), and ristocetin (RN)

	Mean ± SD				p values[b]
	0 h[a]	2 h	4 h	6 h	
AA	65.0 ± 11.7	66.4 ± 12.7	61.7 ± 14.1	60.0 ± 15.7	0.3339
CN	74.4 ± 11.7	71.4 ± 12.4	75.3 ± 9.8	76.9 ± 10.8	0.3263
EP	68.7 ± 11.5	70.2 ± 11.6	71.1 ± 12.6	67.8 ± 14.2	0.7932
RN	75.2 ± 10.3	73.6 ± 9.7	73.3 ± 12.6	73.4 ± 10.1	0.9098

[a] About 1 h after blood collection (see text)
[b] ANOVA test

Table 3 Correlation between PRP and age, hemoglobin concentration, or MPV in 51 subjects with normal platelet counts (*Hb* Hemoglobin)

Items (range)	PRP stimulated by			
	Arachidonate	Collagen	Epinephrine	Ristocetin
Age (21–85 years)	0.3696*	0.2581	0.2817	0.2799
Hb (8.4–16.8 g/dl)	0.1005	0.2488	0.2119	0.2049
MPV (7.5–11.4 fl)	0.1906	0.1286	0.1204	0.0551
Male[a]	0.2294	0.3692*	0.3017	0.2835*

* $p < 0.05$
[a] Degree of influence by male sex

The influence of age, sex, hemoglobin concentration and MPV on PAG

Fifty-one subjects (34 male) were included for analysis of the influence of age, sex, hemoglobin concentration, and MPV on PAG. Their mean age was 44.7 ± 23.0 years, with a range of 21–85 years. There was no significant correlation between PAG and hemoglobin concentration or MPV. A weak but significant correlation was noted between age and PAG when arachidonic acid was used as the agonist ($r = 0.3696$, $p = 0.0125$). Male sex positively and significantly influenced the PAG when collagen and ristocetin were used as the agonists ($r = 0.3692$, $p = 0.0090$ and $r = 2835$, $p = 0.0460$, respectively; Table 3).

The influence of temperature of storage and sample storage method on PAG

Table 4 shows the PAG results at different storage times and temperatures, and with different storage methods when stimulated by collagen. There was no significant difference between the PRP stored separately at room temperature and at 4° C, whether PAG was performed at 0, 2, 4, or 6 h after sampling. However, when the PRP was stored at 4° C, with or without packed cells, a significant difference at 0 and 4 h was noted ($p = 0.0313$ and $p = 0.0016$, respectively).

Table 4 PAG results at different storage temperatures and storage methods, with collagen, in 14 normal healthy subjects (*RT* Room temperature)

Time (h)	Mean ± SD (range) (%)		
	RT (without RBC)	4° C (without RBC)	4° C (with RBC)
0	71.6 ± 9.8 (50–89)	69.5 ± 19.7 (36–93)	73.9 ± 18.1 (41–94)*
2	72.6 ± 12.7 (40–89)	72.6 ± 16.9 (37–93)	75.4 ± 11.7 (50–93)
4	82.1 ± 8.8 (57–94)	75.3 ± 13.9 (45–96)	84.4 ± 9.5 (67–97)**
6	87.2 ± 65.0 (68–96)	82.1 ± 10.0 (51–91)	85.9 ± 7.0 (70–95)

* $p = 0.0313$, compared with 4° C without RBC, Wilcoxon matched-paired signed-rank test
** $p = 0.0016$, compared with 4° C without RBC, Wilcoxon matched-paired signed-rank test

Discussion

PAG tests are used in the diagnosis of qualitative platelet disorders, but correct interpretation of the data obtained requires standardized testing conditions [12]. As strict standardization is usually difficult and impractical [13], the clinical usage of PAG tests is discounted. On the other hand, although we cannot control all the conditions, we can at least control some conditions or know their possible influence so that interpretation will make more sense. Our present study demonstrates several practical conditions which might affect the PAG.

The concentrations of the agonist were rather high; this kind of test would be performed as a first step to look for major functional defects in platelet responses. Although it is suggested that the PAG test be stored for not more than 2 h at room temperature [11], the samples might possibly be kept at 4° C by an incautious technician or the test performed after 2 h due to unavoidable reasons, and, as our previous study showed that the suitable storage condition for PPP used to determine activated partial thromboplastin time (APTT) and prothrombin time (PT) should be with packed cells at 4° C [14], whether this kind of storage disturbs the PAG results deserves our investigation. The present study thus gave us the answers for the above conditions when we performed PAG.

In the first study group, platelet number in the PRP was found to be a major factor affecting the PAG. The optimal platelet count in the PRP without affecting the result was $150–500 \times 10^9$/l, whether arachidonic acid, collagen, epinephrine, or ristocetin was used. Although all four agonists gave lower values when the platelet count was $>500 \times 10^9$/l in PRP (up to $1{,}115 \times 10^9$/l in our study) in comparison with the platelet count between $150–500 \times 10^9$/l in PRP, only ristocetin gave a significantly lower value. When the platelet count was $<100 \times 10^9$/l, the PAG tests gave significantly lower values whether arachidonic acid, collagen, epinephrine, or ristocetin was used. When the platelet count was $100–150 \times 10^9$/l, only collagen and ristocetin gave the satisfactory results. Thus, when the patient has an initially high platelet count, we should either avoid ristocetin as agonist or dilute the PRP with PPP to have the platelet count around $150–500 \times 10^9$/l before performing PAG tests. When the patient has a low platelet count (with $100–150 \times 10^9$/l in PRP), collagen or ristocetin can be used as agonists without affecting the results.

In the second study group, we found that storage for up to 6 h, did not significantly affect the result at room temperature, whether we used arachidonic acid, collagen, epinephrine, or ristocetin. The result was surprising, whether it is due to the high concentration of agonists is not known, but in our study, it seemed to be true in nearly every sample tested. Our study demonstrated that although the best time to perform PAG is within 2 h [11], if the sample process time were delayed for unpredictable reasons, the results of PAG tests are still reliable with up to 6 h of sample storage. Hardeman et al. reported [13, 15] that the labile but strong plasmatic inhibitors would greatly affect the results of PAG if it were performed within 1 h after venipuncture. As in our study PAG was performed 1 h after venipuncture, the influence of these inhibitors may have been lost in the process, to finally give similar results throughout the 6-h period.

In the third study group, we found that the hemoglobin concentration (in our study, 8.4–16.8 g/dl) and MPV (7.5–11.4 fl) did not affect PAG, in spite of the wide range. Men had significantly increased PAG when collagen and ristocetin were used as agonists ($r = 0.3692$, $p = 0.009$ and $r = 0.2835$, $p = 0.046$, respectively), the reason for such a correlation is not very clear, as collagen is a strong agonist, and ristocetin-induced PAG usually refers to the activity of von Willebrand's factor, which has recently been recognized as a risk factor of coronary artery disease [16]. Whether this implies a different prevalence of coronary artery disease between men and women deserves further investigation. Age did not affect PAG, except when arachidonic acid was used as agonist ($r = 0.3696$ and $p = 0.0125$). In the present study, we did not use ADP as an agonist, but it is generally agreed that platelet reactivity to ADP increases with age. Thus, age and sex, but not hemoglobin or MPV, should be considered as factors which can affect the PAG.

In the fourth study group, we found that the temperature for sample storage, i.e., either room temperature or 4° C, did not affect the PAG. Although it is generally accepted that PRP should be stored at room temperature [11], PAG can still be performed if it has been stored at 4° C. This may well occur, as most of the laboratory reagents are kept at 4° C. On the other hand, the method of storage will affect the PAG. When the PRP was stored with packed cells, PAG was found significantly higher at 0 and 4 h compared with when PRP was stored without packed cells at 4° C ($p = 0.0313$ and $p = 0.0016$, respectively). It is not certain whether this is related to the release of adenine nucleotides from the lysed cells [17]. Nevertheless, we suggest that PRP be stored without packed cells to avoid their influence on the PAG. This finding is rather interesting, as in our previous study on performing APTT and PT we found that the most suitable condition for storing the plasma, the PPP in this case, was with packed cells at 4° C [14]. Thus, before performing clotting tests (with PPP) and PAG tests (with PRP), the samples should be stored in different ways. This should be noted by newcomers.

In conclusion, when high concentrations of collagen, epinephrine, arachidonic acid, and ristocetin are used to perform PAG, valuable data about platelet function (in order to rule out a major platelet defect, although ADP, one of the main agonists, was not studied) can be obtained with the Platelet Aggregation Profiler (Model PAP-4, Bio/Data) even with a platelet count in PRP as low as 150×10^9/l, and even when PRP has been stored for up to 6 h after blood collection.

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