

ORIGINAL PAPER  
ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

## Reversal of platelet aggregation by supplementation with amikacin *in vitro*

**OBJECTIVE** One of the major causes of pseudothrombocytopenia is platelet clumping due to faulty phlebotomy technique. Platelet clumps caused by ethylenediaminetetraacetic acid (EDTA) pseudothrombocytopenia have been reported to be dissolved by aminoglycosides. This study investigated whether platelet clumps caused by flawed phlebotomy practice can be dissolved *in vitro* by the addition of amikacin. **METHOD** In 66 blood samples, the platelets were counted using a hematology analyzer. Platelet clumping was then provoked by the addition of calcium chloride (CaCl<sub>2</sub>) and confirmed by microscopic observation of blood smears. To each sample, 20 mg/mL of amikacin was added, and the platelets were recounted after 2 and 6 hours. The platelet count of each sample was also estimated by microscopic observation of blood smears. **RESULTS** Two hours after supplementation with amikacin, the platelet count had increased significantly ( $p < 0.001$ ) compared to that immediately after the formation of clumps. Six hours after the addition of amikacin a further significant increase in platelet count was observed ( $p < 0.001$ ). In 90% of the blood samples, the platelet increase was greater than 50% of the initial value. The rise was gradual and ranged from 23 to 100%, with a mean of 86%. Microscopic observation of blood smears revealed partial or complete dissolution of platelet aggregates. **CONCLUSIONS** The addition of amikacin to an EDTA blood sample can achieve the partial or complete dissolution of platelet clumps caused by faulty phlebotomy practice, enabling a better approximate estimate of the real platelet count.

One of the biggest problems of performing whole blood cell counts using hematology analyzers is the erroneous platelet count that occurs when the platelets aggregate and form platelet clumps. Because of their size, platelet aggregates cannot be measured in the platelet population and they may interfere with red and white blood cell counts or be included in the cluster of "non-white cells".<sup>1</sup>

Platelets usually aggregate *in vitro* due to faulty phlebotomy technique, including too tight tourniquet, delay in blood drawing or transferring the sample into ethylenediaminetetraacetic acid (EDTA) tubes, and insufficient tube agitation. Other reasons for platelet clumping include

the presence of cold agglutinins or EDTA dependent autoantibodies.<sup>2</sup>

If platelet clumps are not detected, they lead to underestimation of the platelet count, resulting in false diagnosis, unnecessary investigations, patient anxiety, and even unnecessary platelet transfusion.

Indications of platelet aggregation in the whole blood count include an abnormal platelet histogram pattern and flags for platelet clumps and giant platelets. In some analyzers, the white and red cell counts or the red cell parameters may also be flagged. The presence of clumps is confirmed by observing the whole blood smear. In addition, platelets

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Αναστροφή της συσσώρευσης αιμοπεταλίων μέσω προσθήκης αμικασίνης *in vitro*

Περίληψη στο τέλος του άρθρου

### Key words

Amikacin  
Phlebotomy  
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cannot be enumerated accurately in the whole blood smear or in a Neubauer counting chamber using a phase contrast microscope because of the uneven distribution and the dissimilar sizes of the clumps.

Recent studies have focused on the prevention of platelet clump formation or the dissolution of platelet clumps *in vivo* to avoid thromboembolic episodes,<sup>3,4</sup> and laboratory studies on the prevention of platelet aggregation or the dissolution of platelet clumps using an aggregometer.<sup>5-8</sup> According to one study, the addition of amikacin to platelet-rich plasma prevented platelet aggregation induced by adenosine diphosphate (ADP).<sup>9</sup> Various methods have been proposed for the dissolution of the platelet clumps in EDTA pseudothrombocytopenia, including the addition of aminoglycosides.<sup>10</sup> Amikacin, specifically, has been demonstrated to dissolve platelet clumps in EDTA pseudothrombocytopenia.<sup>11</sup>

How platelet aggregation caused by erroneous phlebotomy technique can be reversed in clinical practice remains to be discovered.

The aim of this study was to investigate whether platelet clumps caused by faulty venepuncture practice can be dissolved by the addition of amikacin to the EDTA tube, in order to avoid a second phlebotomy.

## MATERIAL AND METHOD

### Participants

A total of 66 healthy individuals aged 20–30 years were involved in this study. The participants responded to an open announcement for subject recruitment and were asked to give signed consent for their participation in the study. The full medical history of each participant was recorded to exclude individuals with thrombocytopenia or those receiving antiplatelet medication. In addition, the platelet count of each participant was within the normal range.

### Blood sampling

Blood samples were collected from each participant in a 3-mL BD vacutainer tube (Becton Dickinson, Crowley, UK) containing 5.4 mg of dipotassium EDTA (K<sub>2</sub>EDTA) anticoagulant. The blood samples were analyzed using a CELL-DYN 1700 hematology analyzer to determine the initial platelet count. The samples were processed in their fresh state.

### Platelet aggregation after blood withdrawal

Platelet aggregation occurs in K<sub>2</sub>EDTA tubes after faulty phlebotomy technique, due to platelet activation. Platelet aggregation was provoked technically in all the study samples with a normal

platelet count, in order to imitate platelet clumping caused by faulty phlebotomy technique, by adding the platelet activator calcium chloride (CaCl<sub>2</sub>) to the EDTA tubes. The required quantity of CaCl<sub>2</sub> varied among the samples. To achieve platelet clumping, each sample was divided into three aliquots and diluted as follows: (a) 500 µL of the blood sample and 50 µL of CaCl<sub>2</sub>, (b) 500 µL of the blood sample and 55 µL of CaCl<sub>2</sub>, and (c) 500 µL of the blood sample and 60 µL of CaCl<sub>2</sub>.

All the diluted specimens were incubated at room temperature for 40 minutes, and the platelets were recounted using the hematology analyzer. Samples that presented clots were rejected. The platelet count was corrected for the dilution effect of CaCl<sub>2</sub>. In dilutions with a platelet count below 100×10<sup>9</sup>/L, the presence of platelet clumps was confirmed by microscopic observation of blood smears. Only one dilution of each sample presented platelet clumps, and this dilution was processed for aggregate dissolution.

### Supplementation with amikacin

Amikacin was added to each diluted sample at a concentration of 20 mg/mL. Platelets were recounted after 2 and 6 hours of incubation at room temperature. The platelet count was corrected for the dilution effect of amikacin. In addition, the platelet count of each sample was estimated by microscopic observation of blood smears.

### Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS), version 21.0 software for Windows. The normality of continuous data was determined. For comparisons among groups, the paired-samples t-test and the Wilcoxon signed ranks test were used for parametric and nonparametric tests, respectively. To study the possibility of gradual alteration of the parameters, a one-way ANOVA test was used. A two-tailed p value of <0.05 was considered statistically significant for all comparisons.

## RESULTS

The effect of amikacin on platelet clumps was studied 2 and 6 hours after supplementation to the K<sub>2</sub>EDTA blood samples.

Two hours after supplementation with amikacin, the platelet counts were increased significantly ( $p < 0.001$ ) compared to those after the formation of clumps (tab. 1). Microscopic examination of the blood smears indicated partial dissolution of the platelet clumps. No significant change was observed in the platelet histogram patterns of the amikacin-supplemented samples compared to those of the samples examined after the formation of clumps (fig. 1).

Six hours after the supplementation with amikacin, the

**Table 1.** Comparison of platelet count increases after supplementation with amikacin (n=66).

	Mean difference	95% CI of the difference		p value
		Lower	Upper	
Pair 1	-59.46154	-67.91612	-51.00696	<0.001
Pair 2	-101.44615	-113.48955	-89.40276	<0.001
Pair 3	-41.98462	-47.78675	-36.18248	<0.001

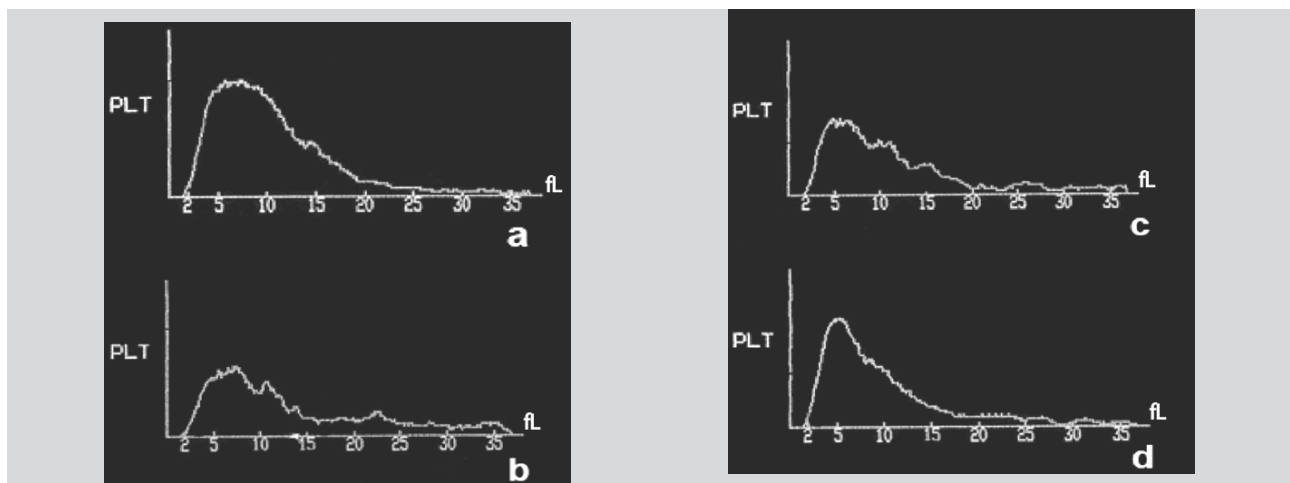
Pair 1: Platelet counts after the formation of platelet clumps – 2 hours after the addition of amikacin. Pair 2: Platelet counts after the formation of platelet clumps – 6 hours after the addition of amikacin. Pair 3: Platelet counts 2 hours after the addition of amikacin – 6 hours after the addition of amikacin.  
CI: Confidence interval

platelets were recounted using the hematology analyzer, and the platelet count was compared to that after the formation of clumps. A statistically significant increase in the platelet count was observed 6 hours after the addition of amikacin ( $p < 0.001$ ) of more than 50% of the initial value in

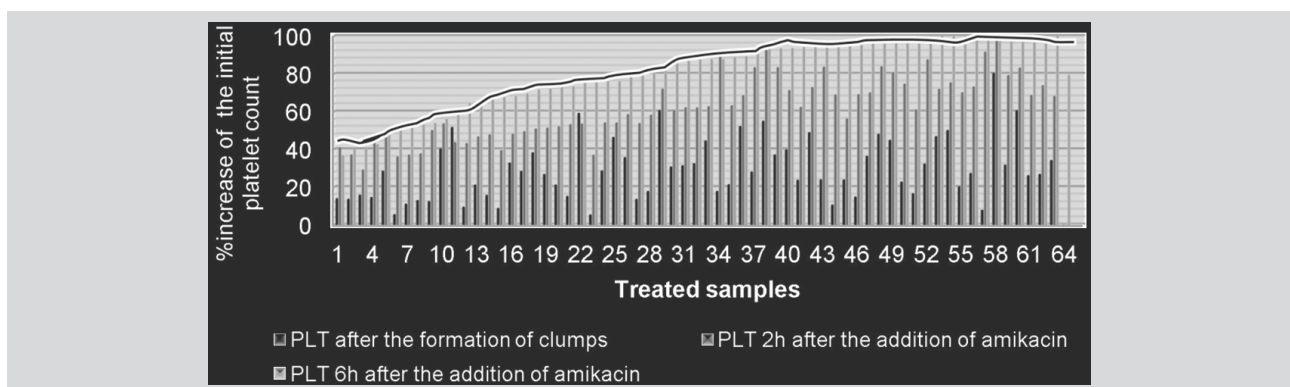
90% of the samples. Partial or complete dissolution of the aggregates was observed on microscopic examination of blood smears. The rise in platelet count was gradual and statistically significant ( $p < 0.001$ ), ranging from 23% to 100%, with a mean of 86% (fig. 2). In addition, a significant change in the platelet histogram patterns was observed in the amikacin-supplemented samples in comparison to those after the formation of clumps (fig. 1). Microscopic observation of blood smears confirmed the partial or complete dissolution of the platelet clumps (fig. 3).

## DISCUSSION

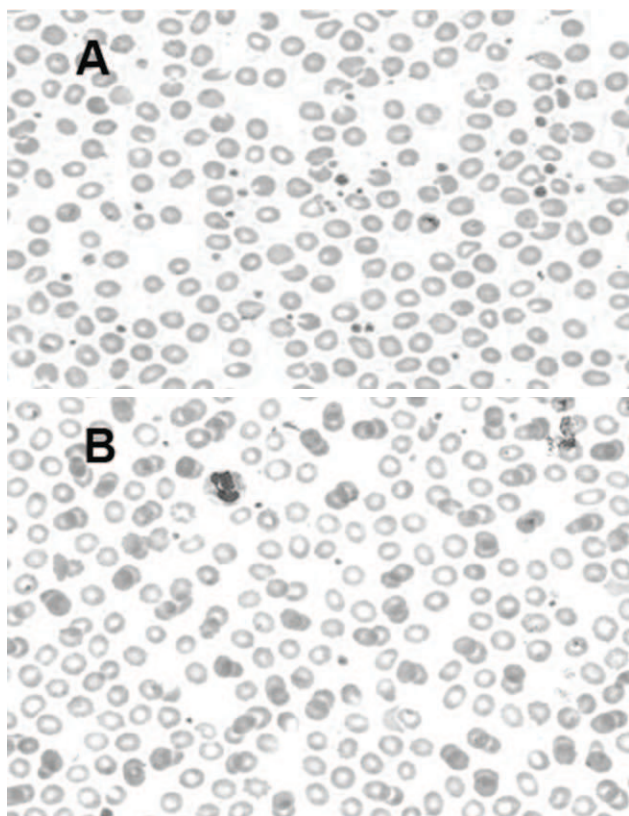
The major cause of platelet count errors in hematology analyzers is platelet aggregation in EDTA tubes after blood withdrawal. Causes of this phenomenon are EDTA-induced pseudothrombocytopenia, the presence of cold agglutinins and erroneous phlebotomy technique.



**Figure 1.** Platelet histogram patterns in one of the 66 samples: (a) Untreated, (b) after the formation of platelet clumps, (c) 2 hours after amikacin supplementation, (d) 6 hours after amikacin supplementation.



**Figure 2.** The increase in platelet count 2 hours and 6 hours after the addition of amikacin.



**Figure 3.** Microscopic examination of blood samples 6 hours after supplementation with amikacin showing: (A) Partial and (B) complete dissolution of platelet clumps.

In the case of EDTA-dependent autoantibodies or cold agglutinins, various methods have been proposed to overcome this artifact and achieve accurate platelet counts, such as warming the sample to 37 °C or recollecting a sample using sodium citrate or heparin as an anticoagulant. Other remedies include additional sampling adding  $\beta$ -hydroxyethyltheophylline,  $\text{CaCl}_2$ /heparin, a magnesium additive, ammonium oxalate, sodium fluoride, trisodium citrate, pyridoxal 5'-phosphate and Tris (CPT), antiplatelet agents or potassium azide.<sup>12,13</sup>

Faulty phlebotomy technique is the most common cause of this artifact. For the purposes of this study, as it was impossible to induce platelet clumping during phlebotomy without clot formation, the formation of platelet clumps was achieved by the addition of  $\text{CaCl}_2$  to the EDTA

tube after phlebotomy and subsequent incubation of the samples at room temperature for 40 minutes. Previous studies reported the effect of aminoglycosides on the dissolution of platelet clumps. The addition of aminoglycosides before or after blood withdrawal prevents EDTA-dependent pseudothrombocytopenia.<sup>10</sup> Presupplementation of the EDTA anticoagulant with kanamycin has also been shown to prevent EDTA-induced pseudothrombocytopenia,<sup>14,15</sup> and the addition of kanamycin to blood samples containing EDTA can dissociate EDTA-dependent platelet clumps.<sup>16</sup> The differences from other reports of the present findings may be attributed to the structure of the antibiotic used and to a different mechanism of platelet aggregation. Regarding the effect of amikacin, it is documented that the presupplementation of EDTA tubes with amikacin prevented platelet clumping in a patient with multianticoagulant-dependent pseudothrombocytopenia.<sup>17</sup> In that patient, amikacin addition within one hour of blood sample withdrawal increased the platelet count by approximately 100% compared to that made immediately after blood sampling.

In the present study, the increase in the platelet count observed after amikacin supplementation was time-dependent. Partial platelet clump dissolution and increase in the platelet count were evident no sooner than 2 hours after the addition of amikacin. The increase in the platelet count was even greater 6 hours after amikacin supplementation. Both studies demonstrated the effect of amikacin on platelet clump dissociation, but a difference was reported in the time required for the dissolution of aggregates. Since the antibiotic used was the same in both studies, the possible reasons for this difference are the dissimilar sizes of the samples studied and the different mechanisms of platelet clumping. It appears that the effect of amikacin on platelet clumps varies according to the underlying mechanism of clump formation. The mechanism of action of amikacin in platelet clump dissociation remains to be elucidated.

In conclusion, amikacin supplementation of EDTA tubes at a concentration of 20 mg/mL may dissolve platelet clumps caused by faulty phlebotomy technique, enabling an approximate real platelet count in only a few hours without the need for a second phlebotomy and associated patient mismanagement.



## ΠΕΡΙΛΗΨΗ

**Αναστροφή της συσσώρευσης αιμοπεταλίων μέσω προσθήκης αμικασίνης *in vitro***

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**ΣΚΟΠΟΣ** Να διερευνηθεί εάν η αμικασίνη μπορεί να διαλύσει τους σωρούς αιμοπεταλίων που προκλήθηκαν από τεχνικό σφάλμα, ώστε να καταστεί δυνατή η καταμέτρησή τους. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Σε 66 δείγματα αίματος μετρήθηκαν τα αιμοπετάλια σε αιματολογικό αναλυτή και κατόπιν προκλήθηκε συσσώρευση αιμοπεταλίων με την προσθήκη CaCl<sub>2</sub>. Μετά τη δημιουργία αιμοπεταλιακών σωρών, η ύπαρξη των οποίων επιβεβαιώθηκε με μικροσκόπηση επιχρίσματος αίματος, επαναμετρήθηκαν τα αιμοπετάλια των δειγμάτων. Στη συνέχεια, σε κάθε δείγμα προστέθηκε αμικασίνη σε αναλογία 20 mg/mL αίματος και επαναμετρήθηκαν τα αιμοπετάλια σε 2 και 6 ώρες, ενώ παράλληλα εκτιμήθηκε ο αριθμός τους από επίχρισμα αίματος. Με το λογισμικό πρόγραμμα Statistical Package for Social Sciences (SPSS), έκδοση 21.0, έγινε σύγκριση των τιμών των αιμοπεταλίων με το One-Way-ANOVA test και το t-paired test. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Στο 90% των δειγμάτων τα αιμοπετάλια αυξήθηκαν >50% της αρχικής τους τιμής και στο επίχρισμα παρατηρήθηκε μερική μέχρι πλήρης διάλυση των σωρών. Στο σύνολο των δειγμάτων η αύξηση ήταν σταδιακή και στατιστικά πολύ σημαντική (p<0,001), ανερχόμενη σε 23–100% (διάμεσος: 86%).

**Λέξεις ευρητηρίου:** Αιμοπετάλια, Αμικασίνη, Αριθμός αιμοπεταλίων, Σωροί αιμοπεταλίων, Φλεβοκέντηση

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