

Research Article

Study of Ethylenediaminetetraacetic acid (EDTA) - Dependent Pseudothrombocytopenia – An Incidental but Important Finding

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Abstract

Objectives: To study EDTA-PTCP for its prevalence, association with different clinical states, medications and duration of anticoagulation of blood.

Methods: In all cases 2 ml of blood sample is collected in K3-EDTA and CPT vials separately and subjected for different tests viz. Peripheral smear examination, manual platelet counts, comparison of platelet counts obtained by automated cell counter at 30 minutes of blood collection from both anticoagulants and with manual counts and analysis of data for any relationship with particular disease or drug intake.

Results: Thus, prevalence of EDTA-PTCP was 0.07% of total haemograms and 4.9% of total thrombocytopenia. The mean manual platelet count in these cases was $222.63 \times 10^9/L$, whereas, in anticoagulated blood, the respective values in EDTA were 171.40 and $226.63 \times 10^9/L$ in CPT. Thus, platelet counts in EDTA anticoagulated blood are much lower than the actual counts by manual method or with CPT as anticoagulant. There was no specific association between EDTA-PTCP and particular disease or medication.

Conclusions: Recognition of EDTA-PTCP will prevent needless evaluations of thrombocytopenia and related therapeutic decisions, hence, importance of this study.

Keywords: Pseudothrombocytopenia, Ethylenediaminetetraacetic acid, CPT (trisodium citrate, pyridoxal 5'-phosphate and Tris), Manual platelet count

1. Introduction

Spurious thrombocytopenia, also called Pseudothrombocytopenia (PTCP), is a relatively uncommon phenomenon caused by *ex vivo* agglutination of platelets¹. As a result of platelet clumping, platelet counts reported by automated blood cell counters may be much lower than the actual count in the blood because these devices cannot differentiate platelet clumps from individual cells².

Mant *et al*³ and Manthorpe *et al*⁴ first studied the frequency of the phenomenon and reported figures of 0.3% and 1.2 % respectively, which referred however to a small case series. EDTA- dependent Pseudothrombocytopenia (EDTA-PTCP) occurs in 0.2% of asymptomatic individuals, but the incidence may be as great as 1.9% in hospitalized patients⁵.

Onder *et al*⁶ observed PTCP due to platelet clumping, when blood was anticoagulated with EDTA, and not with other anticoagulants. The phenomenon of PTCP is complex and is influenced by various factors including immunological (antiplatelet antibodies), chemical (anticoagulants), and physical (temperature) mechanisms^{5,7-8}. The presence of certain anticoagulants (especially EDTA), prolonged time intervals between blood drawn and assays, and low temperature, are the factors that enhance the occurrence of PTCP⁹⁻¹².

EDTA-Dependent Pseudothrombocytopenia (EDTA-PTCP) is due to presence of agglutinating anti-platelet antibodies that react with platelets in blood anti-coagulated with EDTA resulting in underestimation of platelet counts by electronic counting machines⁵. The manual platelet counts, performed at the microscope using counting chambers, still remains as a 'gold standard' for confirmation of platelet count in EDTA-PTCP cases^{8,13}.

EDTA – PTCP has been reported both in apparently normal adults and children and in association with a variety of specific disease (e.g., autoimmune diseases), situations of possible sensitization (i.e., pregnancy or transfusions), or the use of specific drugs; in addition, PTCP has not been associated with hemorrhagic diathesis or platelet dysfunction^{8,9}.

Awareness of this phenomenon is important because Pseudothrombocytopenia may lead to the erroneous diagnosis of thrombocytopenia, with resultant unnecessary and costly additional laboratory testing, inappropriate treatment with delay of surgery and unwarranted exposure to transfusion-related complications; all being the potential outcomes for an individual with this form of in vitro artifact; hence the purpose of this study^{8,14,15}.

2. Material and Methods

This cross sectional study was carried out in the Hematology division of the Department of Pathology, over a period of 2 years from May 2011 to May 2013, in a medical institute in central India. During routine reporting of complete blood counts of patients who were admitted in the hospital and those seen on outpatient basis, the cases in which the automated counter report showed thrombocytopenia with platelet counts less than 130×10^9 /litre and simultaneous peripheral blood film examination showed platelets in fair number, either diffusely distributed or in clumps or aggregates and appeared to be within normal limits were considered as pseudothrombocytopenia and were included in the study. The cases showing marginal decrease in platelet counts and the cases with known cause for thrombocytopenia were excluded.

In all cases 2 ml of blood sample is collected in K3-EDTA and CPT (trisodium citrate, pyridoxal 5'-phosphate and Tris) vials separately and subjected for following tests –

1) Peripheral blood smears were prepared, air-dried, labelled and stained with Leishman's stain. The smears are examined with light microscope (Olympus CH30) using oil immersion lens (100x) with (10x objectives) for evaluating platelet count, morphology and platelet clump counts.

2) Manual platelet counts are performed with light microscope using improved Neubauer's chamber. An arithmetically convenient procedure is to count five groups of 16 small squares in the central area (0.02ml).

$$\text{Platelet count per litre} = \frac{\text{Number of cells counted} \times \text{Dilution} \times 10^6}{\text{Volume counted (ml)}}$$

To ensure a coefficient of variation of 8-10 %, the total number of platelet count should always exceed 200.

3) The automated platelet count was obtained by doing complete blood count on automated 3 part differential haematology analyser at 30 minutes of blood collection.

- Platelet counts obtained by manual method; by automated counter at 30 minutes using two different anticoagulants were compared. These platelet counts were also compared with the initial platelet counts on which pseudothrombocytopenia was suspected.
- The manual platelet count is considered 'gold standard' for this comparison^{8,16}.
- Clinical records of the patients were reviewed for any particular disease or drug intake associated with EDTA-PTCP.
- **Microscopic evaluation** of the peripheral blood smear for counting of the platelet clumps is done according to the size of the clumps. The area selected for microscopic examination is the body part of the film near the tail end where RBCs are in one layer and their margins are just touching each other. The clump counting is done as under -

- a) Small clumps (Platelet numbers: <5 platelets in clumps),
- b) Medium clumps (Platelet numbers: 5-10 platelets in clumps),
- c) Large sized clumps (Platelet numbers: >10 platelets in clumps).

The number of clumps of different sizes were counted in 20 oil immersion (1000x magnification) fields and their number was correlated with the initial platelet count on which the pseudothrombocytopenia was suspected.

The finding observed is recorded and the data is analyzed statistically using z-test and test statistics. The software used in the analysis is SPSS 17.0 version and graph pad prism 5.0. The p-value of less than 0.05 is considered as statistically significant.

3. Results

Total 122523 haemograms were done during the study period of which 1906 cases showed thrombocytopenia. Amongst these, the pseudothrombocytopenia was suspected in 103 cases and was found to be correct in 94 cases, thus accounting for the incidence of EDTA-PTCP in 0.07% cases of total haemograms and 4.9 % cases of total thrombocytopenia.

There were 43 males and 60 females with M: F ratio of 1:1.3. The age of the patients varied from 3-85 years with the mean age of 36.78 ± 20.33 years. The distribution of the cases showed that there were maximum 30 cases of inflammatory disorders, followed by 20 obstetrics and gynaecological cases, 14 surgical cases and 8 cases of bone pathology. PTCP was observed in variety of disorders; though there were less number of cases (table 1). The 4 cases shown as miscellaneous did not have any pathology and were the normal healthy persons came for routine check up.

Table 1: Showing distribution of patients with EDTA – Dependent Pseudothrombocytopenia (n=103).

Sr. No	Disease conditions	No. of cases (=n)
1	Inflammatory	30
2	Obstetric and Gynaecological	20
3	Surgical	14
4	Bone	8
5	Respiratory	6
6	Central nervous system	5
7	Neoplastic	4
8	Cardiovascular	3
9	Liver	3
10	Kidney	2
11	Auto-immune	2
12	Metabolic	2
13	Miscellaneous	4
	Total	103

The mean initial platelet count, when the pseudothrombocytopenia was suspected, was found to be $103.67 \pm 25.34 \times 10^9/l$. The mean manual platelet count with fresh blood sample was $222.63 \pm 85.22 \times 10^9/l$. The mean platelet counts in EDTA and CPT anticoagulated blood were 171.40 ± 78.10 and $226.63 \pm 93.25 \times 10^9/l$ respectively, between 0 to 30 minutes of blood collection, as shown in table 2.

Table 2: Showing comparison of initial platelet count suspicious of pseudothrombocytopenia with manual platelet count and platelet count in EDTA and CPT anticoagulated blood at 0-30 minutes.

Number of cases	Mean initial platelet count ($\times 10^9/l$)	Mean manual platelet count ($\times 10^9/l$)	Mean automated platelet count ($\times 10^9/l$) at 0-30 min	
			EDTA	CPT
103	103.67 ± 25.34	222.63 ± 85.22	171.40 ± 78.10	226.63 ± 93.25
Initial Platelet Count		z-value	8.37	12.91
		p-value	0.000, S, $p < 0.05$	0.000 S, $p < 0.05$
Manual Platelet Count		z-value	4.49	0.32
		p-value	0.000, S, $p < 0.05$	0.74 NS, $p > 0.05$
Initial Vs Manual Platelet Count		z-value	13.57	
		p-value	0.000, S, $p < 0.05$	

S: Significant; NS: Not significant

Thus, between 0-30 minutes of blood collection, the mean manual platelet count and mean automated platelet counts in both the anticoagulants did not show thrombocytopenia. However, the mean platelet count in EDTA anticoagulated blood was much lower than that in the manual count or in the CPT anticoagulated blood; and the difference was statistically significant using z-test to compare the means ($z=8.37$; $p=0.000$) between 0-30 minutes of blood collection.

The mean manual platelet count and mean platelet count in CPT anticoagulated blood were found to be comparable.

Significant difference was found between initial platelet count and platelet count in EDTA anticoagulated blood between 0-30 minutes ($z = 8.37$; $p = 0.000$); between initial platelet count and platelet count in CPT anticoagulated blood at 0-30 minutes ($z = 12.91$; $p=0.000$); and between initial platelet count and manual platelet count ($z = 13.57$; $p = 0.000$).

Similarly, a significant difference was found between mean manual platelet count and mean platelet count in EDTA anticoagulated blood at 0-30 minutes ($z = 4.49$; $p = 0.000$). But, no statistically significant difference was found between mean manual platelet count and mean platelet count in CPT anticoagulated blood at 0-30 minutes ($z = 0.32$; $p=0.74$) respectively.

Thus, the platelet counts in EDTA anticoagulated blood on suspicion of thrombocytopenia, as well as even after collection of fresh blood sample from same patient in EDTA and the counts at 0-30 minutes showed significantly lower counts than that observed in CPT anticoagulated blood at the parallel time intervals and than that in the manual platelet counts. These findings suggested that the low platelet counts were probably because of the effect of EDTA anticoagulant on platelets. Thus, the CPT anticoagulant was found to be a better anticoagulant for getting correct platelet counts in cases of EDTA- dependent pseudothrombocytopenia.

As the EDTA- dependent Pseudothrombocytopenia shows clumping of platelets observed in peripheral blood films, the present study made an attempt for counting the platelet clumps in the peripheral blood films of these patients according to the size of the clumps in relation to the platelet count. The findings are shown in table 3 and figure 1 and 2.

Table 3: Showing platelet clump counting in correlation with initial platelet count in cases of Pseudothrombocytopenia.

Platelet count range x $10^9/l$	Total no. of cases	Small clumps (<5 platelets)	Medium clumps (5-10 platelets)	Large clumps (>10 platelets)
0-50	6	94	42	20
51-70	8	92	62	54
71-90	12	128	58	76
91-110	22	370	163	94
111-130	55	910	216	167
Total	103	1594	541	411
Correlation 'r'		0.11	-0.17	-0.01
p-value		0.23 NS, $p>0.05$	0.14 NS, $p>0.05$	0.93 NS, $p>0.05$
S: Significant; NS: Not significant				

There was gradual increase in the number of cases as well as number of small, medium, and large clumps with gradual increase in the platelet counts. Group of small clumps showed positive correlation to increase in platelet count with correlation coefficient 'r' of 0.11. However, medium and large clump groups showed negative correlation with correlation coefficient 'r' of -0.17 and -0.01. But, statistically no significant difference was found between the platelet clump counts in comparison with the initial platelet count in cases of pseudothrombocytopenia.

Fig.1: Showing small, medium and large platelet clumps.

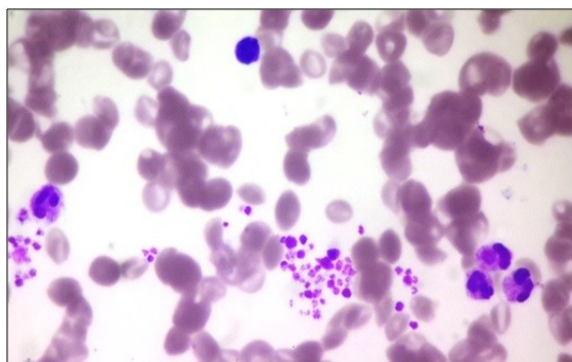
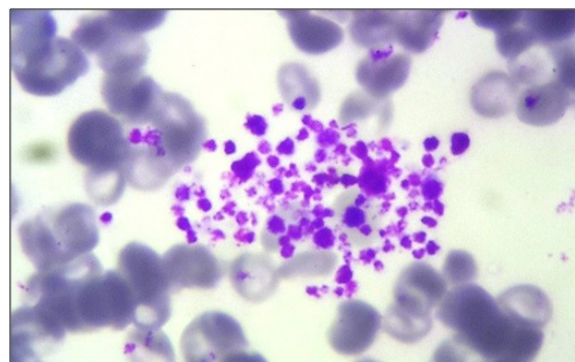


Fig.2: Showing presence of large platelet clump.



The present study also evaluated for the role of any specific drug intake in cases of Pseudothrombocytopenia. The cases of inflammatory conditions (which were maximum in the present study), and surgical conditions showed to be treated with antibiotics, where as the cases of obstetrics and gynaecology (which were second in the number) were treated with haematinics and calcium preparations. In other conditions, also, the antibiotics, antipyretics and oral hypoglycaemic drugs were supplemented. However, the study did not reveal any specific drug intake, to be associated with pseudothrombocytopenia.

4. Discussion

The present study assessed 103 cases of suspected EDTA-dependent Pseudothrombocytopenia (EDTA-PTCP) for its correctness using manual platelet count as the gold standard, and comparing the platelet counts in two different anticoagulants (EDTA and CPT) at different times from the time of collection of blood sample.

Most of the studies have tried different other anticoagulants to get correct platelet count in cases of EDTA-PTCP. Platelet count obtained in normal samples of CPT - anticoagulated blood may be more accurate than sub-optimal blood collection techniques in which traumatic venipuncture or delayed mixing with EDTA often might induce spontaneous platelet clumping in vitro^{17,18}. Use of tri-sodium citrate did not alter cell counting and sizing for 30 minutes after sampling. Pyridoxal 5'-phosphate was added in order to prevent platelet aggregation as it exhibits remarkable anti-aggregant and disaggregant effect in vitro. The pH was brought to neutrality by adding Tris to the CPT mixture^{16,17}. In CPT-anticoagulated specimens the signals and instrumental flags of platelet clumping were absent, and the platelet number correlated very well with a microscopic count from a finger stick drawn into Unopette. The complete blood count was very similar in "normal" hematological specimens either collected in K3.EDTA or in CPT¹⁶.

Harrison *et al*²⁰ investigated platelet function after 5-minutes pre-incubation of citrated whole blood with pyridoxal 5'-phosphate, using a novel system for rapid and accurate determination of platelet function in vitro. After the preincubation with pyridoxal 5'-phosphate, platelet aggregation was triggered by membranes coated with collagen and ADP or collagen and ephinephrine. As a result, a large delayed closure time of capillary aperture by platelet aggregates was recorded.

Lippi and Facchinetti¹⁹ reviewed the literature about instrumental flags and accurate platelet count, avoiding platelet clumping. Author's suggested that, the samples with instrumental flags for platelet clumps in EDTA-anticoagulated samples should be reanalyzed after blood collection by employing alternative anticoagulants, preferably those suitable for CBC. If platelet clumps are known or suspected, however, blood collection and CBC should be resolutely and immediately performed after collection of the blood with alternative anticoagulation by CPT.

Thus, in routine hematological practice CPT can be an alternative anticoagulant to K3.EDTA, most suitable for automated complete blood count and useful in avoiding EDTA-induced platelet clumping¹⁶. CPT - anticoagulant was therefore used to compare the automated platelet counts in two anticoagulants Manual platelet count has been used as the gold standard to obtain correct platelet count in cases of EDTA-PTCP, similar to the present study^{8,16}.

Hyun-Sook Chi⁸ reported frequency of EDTA-PTCP cases in the range of 0.07 to 0.11%. The findings reported by other workers are illustrated in table 4. The present finding of 0.07% cases of EDTA-PTCP of total haemograms is comparable with that of Hyun-Sook Chi⁸ and Sakurai *et al*²³.

In the present study, EDTA-PTCP accounted for 4.9% of all cases of thrombocytopenia. Very few studies have reported the incidence of EDTA-PTCP amongst the cases of thrombocytopenia. Silvestri *et al*²⁴ observed 7.5 to 15% cases of thrombocytopenia were in fact cases of EDTA-PTCP. Similarly, Lichtman *et al*² also noted EDTA-PTCP to account for 15-30% of all cases of thrombocytopenia. The present finding is lower than that of these workers.

Male to female ratio in cases of EDTA-PTCP observed by different studies are: Shreiner and Bell¹ – 2:1, Onder *et al*⁶ – 1: 2, Casonato *et al*⁷ – 1:3 and the present findings are similar to that of the later two studies.

The mean age of the patients in present study was found to be 36.78 ± 20.33 years with the range of 3-85 years. Casonato *et al*⁷ observed the mean age of 41.1 and 43.9 years respectively, in males and females. The mean age and the age range observed by Bizarro⁹ and Wilkes *et al*²⁵ are 55.7 years (6 months to 88 years) and 44 years (19-62 years) respectively. The present findings are more or less similar with that of these workers and the EDTA-PTCP can occur in any age group.

Distribution of the cases according to different disease categories in the present study is shown in table 1. Though present study had more number of cases of inflammatory disorders, obstetrics and gynaecological conditions and surgical cases, the phenomenon of EDTA-PTCP was observed in almost all disease categories including neoplastic, degenerative, metabolic and autoimmune diseases and was noted in diseases of almost all the organs and tissues of the body and was not specific for any particular disease category. Similar findings have been noted by other workers also. Casonato *et al*⁷ found EDTA-PTCP in cases of Hypertension, Diabetes Mellitus, Gall stones, Hyperthyroidism, Nephrolithiasis, Cystourethritis,

ulcerative colitis, Gastroduodenal ulcer, Hemiplegia, Ovariectomy, Marfan's syndrome, Buerger's disease and Tubercular Pleuritis. Bizarro⁹ also observed EDTA-PTCP in variety of non-autoimmune and autoimmune disorders.

EDTA-PTCP occurs not only in disease conditions, but also in healthy disease free states. The incidence of EDTA-PTCP in healthy persons observed by Casonato *et al*⁷ is 54.5 % (18 of 33) and by Bizarro⁹ is 48.2% (54 of 112). The present finding of EDTA-PTCP in healthy individuals is much lower than that observed by these workers, probably because the present study has shown the obstetrical cases under the disease category.

In the present study, comparison of platelet count in EDTA anticoagulated blood at different time interval showed significant difference between initial platelet count and that at 0-30 minutes (Table 2). Similarly, the comparison of initial platelet count in EDTA with platelet counts in CPT anticoagulated blood at different time intervals showed significant difference between initial platelet count in EDTA anticoagulated blood with platelet count in CPT anticoagulated blood at 0-30 minutes (Table 2).

The lower mean platelet count in initial EDTA blood sample on which EDTA-PTCP was suspected is probably because these samples were different and collected by the clinical residents and at different time. The samples which we personally collected and used for cell counts at 0-30 minutes did not show significant difference of cell counts. However, the counts in EDTA anticoagulated blood were still significantly lower than that in manual counts and with CPT anticoagulated blood.

The mechanism of PTCP is complex⁸. IgG class of antibodies are much more involved in platelet clumping as compared with IgM and IgA⁸. The antibodies are directed against ubiquitous antigens, as this phenomenon is easily reproduced by incubating patient's plasma or serum with EDTA anticoagulated blood from normal subjects [9]. The target structure is cryptic epitopes that are normally hidden in platelet membrane glycoprotein (GP) IIb/IIIa complex. Since GP IIb/IIIa requires the presence of calcium to maintain its heterodimeric structure, EDTA can dissociate GP IIb/IIIa by its chelating effect, resulting in exposure of epitopes on GP IIb²⁶. This alteration in conformation is also associated with temperature²⁷. New anticoagulant-antiaggregant mixture, called as CPT mixture, prevented EDTA induced platelet clumping and was found to be most suitable for automated complete blood count in routine hematological practice, as an alternative anticoagulant to K3-EDTA¹⁶.

Onder *et al*⁶ have also graded the platelet clumps from grade 0 to 4+ according to its size in ten high power fields (400x magnification) but in wet films. Hyun-Sook Chi⁸ showed that platelet clumps are readily detectable due to presence of specific warning flags and typical scatterogram patterns produced by modern blood counters. When the clumps are small and their size is equal to or slightly larger than a leucocyte, no instrumental signal are present, and the clumps may be counted by automated instruments as white cells; in these cases a false increase in white cells count is recorded. However, the PTCP can rapidly be confirmed by the microscopic findings of platelet agglutinates on blood smear in these cases⁸.

Present study showed the number of small clumps to be more than the number of medium sized clumps which in turn were found to be more than the number of large clumps except in the range of $71-90 \times 10^9/l$ platelet count where the number of medium sized clumps was lowest (Table 3). However, there was no significant correlation between platelet clump counts and the initial platelet count in cases of EDTA-PTCP.

Platelet clumping can result in inaccurate platelet concentrations which easily lead to misdiagnose pseudothrombocytopenia as true thrombocytopenia if analyzed with hematology analyzer only¹³. In case of low platelet counts, peripheral blood smear examination and observation of platelet clump is recommended with manual platelet count, although it is labor - consuming and time – costing¹³.

Occasional cases of EDTA – PTCP have been described following the administration of valproic acid²⁸, olanzapine²⁶ or levofloxacin²⁹. Berkman *et al*⁵ described a case of EDTA-PTCP following administration of oral anticoagulant (Warfarin) therapy.

In order to assess any particular drug intake to be related with pseudothrombocytopenia, we noted the drugs given to these patients. The cases of inflammatory conditions and surgical cases which formed majority of the cases of EDTA-PTCP were treated with antibiotics, whereas the obstetrics and gynaecological cases were treated with haematinics and calcium preparations. In other conditions also, the antibiotics, antipyretics, and oral hypoglycaemic drugs were supplemented. However the present study did not find any specific drug intake to be associated with pseudothrombocytopenia.

The present study also noted, many of the patients have received the various antibiotics prior to the detection of EDTA-PTCP but, whether this has resulted in development of pseudothrombocytopenia is not certain; and there is no clear evidence that associates the presence of EDTA-PTCP with specific diseases or the use of specific drugs and it is seen in both healthy and diseased states^{8,22}.

Table 4: Percentage of EDTA-PTCP cases reported by other workers

Author's	Percentage of EDTA-PTCP cases
Hyun-Sook Chi ⁸	0.07 to 0.11%
Lichtman <i>et al</i> ²	0.9 to 0.21%
Bizarro ⁹	0.11%
Bartels <i>et al</i> ¹	0.1%
Mori <i>et al</i> ²²	0.1%
Mant <i>et al</i> ³	0.3%
Manthorpe <i>et al</i> ⁴	1.2%
Lippi <i>et al</i> ¹⁶	2%
Sakurai <i>et al</i> ²³	0.03 to 1.9%

5. Conclusions

- i. In all the cases of thrombocytopenia showed by automated blood cell counters, the examination of peripheral blood film is must for the presence of platelet clumps to detect the cases of EDTA- dependent Pseudothrombocytopenia.
- ii. To get correct platelet count in these cases, the manual platelet count is the 'gold standard'. However, CPT anticoagulated blood also shows comparable platelet counts similar to the manual platelet counts. Therefore, CPT is the better alternative anticoagulant for EDTA in cases of EDTA – dependent Pseudothrombocytopenia for automated platelet counts.
- iii. Recognition of EDTA-PTCP will prevent needless evaluations of thrombocytopenia and related therapeutic decisions.

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