

## ผลของสารกันเลือดแข็งและเวลา ก่อนวิเคราะห์ต่อการตรวจวัดสารเร่งการเติบโตในชีรั่มและพลาสma

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### บทคัดย่อ

สารเร่งการเติบโตในเลือดซึ่งมีความสำคัญต่อกระบวนการสมานแผลส่วนใหญ่มีมาจากการเกล็ดเลือด ปัจจุบันยังไม่มีแนวทางสำหรับการตรวจวัดสารเร่งการเติบโตในเลือดและชนิดของสารกันเลือดแข็งที่เหมาะสม จึงเป็นที่มาของการศึกษาในครั้งนี้ โดยได้เก็บและวิเคราะห์เลือด 100 ตัวอย่าง (10 ชุดตัวอย่าง) ซึ่งเก็บในหลอดเลือดต่างชนิดกัน ได้แก่ clot activator, plain, citrate, heparin และ ethylenediaminetetraacetic acid (EDTA) ชีรั่มหรือพลาสma ที่ได้ใช้สำหรับวัดความเข้มข้นของสารเร่งการเติบโตที่เวลา 15 นาที 4, 8 และ 24 ชั่วโมงตามลำดับ ผลการทดลองพบว่า สารเร่งการเติบโตจากเกล็ดเลือด 3 ชนิด คือ platelet-derived growth factor (PDGF)-AA, PDGF-BB และ epidermal growth factor (EGF) และ สารเร่งการเติบโต erythropoietin มีความเข้มข้นสูงขึ้นในชีรั่มเมื่อเวลาผ่านไปแต่ไม่พบรการเปลี่ยนแปลงในพลาสma ในขณะที่สารเร่งการเติบโตชนิดอื่นไม่พบรการเปลี่ยนแปลงของความเข้มข้น นอกจากนี้ความเข้มข้นของสารเร่งการเติบโตจากเกล็ดเลือด 3 ชนิด คือ PDGF-AA, PDGF-BB และ EGF ในชีรั่มมีความสัมพันธ์ระดับปานกลางกับจำนวนเกล็ดเลือด โดยสรุปชนิดของสารกันเลือดแข็ง เวลา ก่อนการวิเคราะห์ และจำนวนเกล็ดเลือด ส่งผลต่อความเข้มข้นของสารเร่งการเติบโตในเลือดบางชนิดได้

**คำสำคัญ:** สารเร่งการเติบโต สารเร่งการเติบโตจากเกล็ดเลือด สารกันเลือดแข็ง ชีรั่มและพลาสma

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## Effect of Anticoagulants and Pre-analytical Time on Serum and Plasma Growth Factor Analysis

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### Abstract

Growth factors in blood are important for wound healing and contributed mainly from platelets. Currently, there is no consensus about measurement method and suitable anticoagulant for growth factor analysis. One hundred blood samples (ten collections) were drawn into various blood collecting tubes including clot activator, plain, citrate, heparin, and ethylenediaminetetraacetic acid (EDTA). Serum or plasma obtained at 15 minutes, 4, 8 and 24 hours were analyzed for the presence of growth factors. Three platelet growth factors, [platelet-derived growth factor (PDGF)-AA, PDGF-BB and epidermal growth factor (EGF)], and erythropoietin were increased in the concentration with time in serum but unchanged in plasma. Other growth factors showed no difference between serum and plasma. Furthermore, serum PDGF-AA, PDGF-BB, and EGF showed moderate correlation with platelet count. In conclusion, types of anticoagulant, time before analysis and platelet count affected the concentration of some growth factors in blood.

**Keywords:** Growth factors, Platelet growth factors, Anticoagulants, Serum and plasma

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## Introduction

Growth factors are substances that stimulate cell proliferation, hematopoiesis and differentiation. The platelets contain a wealth of growth factors and cytokines more than 300 types<sup>(1)</sup> and several growth factors in blood are mainly contributed from platelets. The common platelet growth factors are platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF). Activated platelets are enable to secrete substances from their granules including growth factors in alpha granules. The role of these growth factors is to initiate inflammation and promote wound healing.<sup>(2,3)</sup>

Concentration of growth factors (PDGF and VEGF) in blood is correlated with stages of cancers.<sup>(4, 5)</sup> However, previous studies showed that types of anticoagulant affected the concentration of growth factors<sup>(6-8)</sup> and platelet count also affected the concentration of platelet growth factors in blood.<sup>(9, 10)</sup>

Samples for blood growth factor measurement are composed of two main types. The sample from tube without anticoagulant (as clot activator and plain tubes) gives aqueous component called “serum”, while the sample from tube with anticoagulant (as citrate, heparin, EDTA tubes) gives aqueous component called “plasma”.

The suitable sample for blood growth factor measurement has not been resolved and the discrepancy of blood growth factor concentration between serum and plasma has not been explored. Therefore, we studied the effect of anticoagulant (serum and plasma samples), time before analysis and platelet count on the concentration of growth factors in blood.

## Materials and methods

### Sample collection

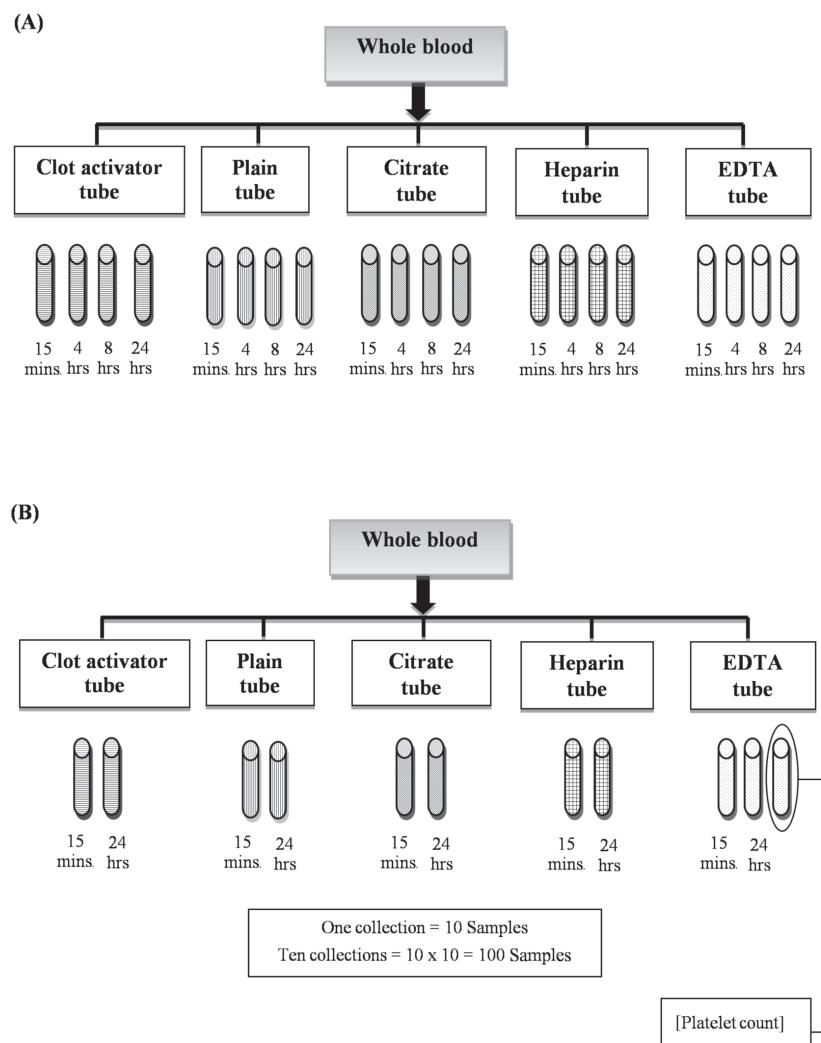
One hundred and twenty samples were obtained from two healthy volunteers (a 38-year-old man and a 28-year-old woman) with no blood dyscrasia (normal complete blood count and coagulogram), no chronic infection (as HIV, HBV and HCV infections) and no current medication. Twenty samples were used in four time points evaluation and one hundred samples were used in two time points evaluation. The study was conducted with the informed consents of subjects and was approved by the Committee on Human Rights pertaining to research involving human subject, Faculty of Medicine, Ramathibodi Hospital, Mahidol University (ID 06-59-49 8).

### Four time points evaluation

Twenty samples of whole blood from the woman volunteer were drawn at the same time into four clot activator tubes, four plain tubes, four citrate tubes, four heparin tubes, and four EDTA tubes. Leaving the samples at room

temperature (25 °C) for 15 minutes, 4, 8, and 24 hours, following by centrifuging at 2,500xg at 25 °C for 10 minutes to obtain serum or plasma (each time point composed of five

samples; clot activator, plain, citrate, heparin and EDTA tubes). All serum or plasma samples were kept at -80 °C before growth factors measurement (Fig. 1A).



**Fig. 1** Sample collection for thirteen growth factor measurement: (A) Four time points evaluation; the results shown in Fig. 2, (B) Two time points evaluation; the results shown in Fig. 3, and platelet count; the results shown in Fig. 4.

### Two time points evaluation

For one collection, ten samples of whole blood were drawn at the same time into two clot activator tubes, two plain tubes, two citrate tubes, two heparin tubes and two EDTA tubes. Five collections were obtained at two weeks apart from each volunteer. Therefore, total ten collections (one hundred samples) were obtained from two volunteers. In each collection, an additional EDTA tube was obtained for platelet count. Leaving the samples of each collection at room temperature for 15 minutes and 24 hours, following by centrifuging at 2,500xg at 25 °C for 10 minutes to obtain serum or plasma samples (each time point was composed of five samples; clot activator, plain, citrate, heparin, and EDTA tubes). All serum or plasma samples were kept at -80 °C before growth factors measurement (Fig. 1B).

### Thirteen growth factors measurement

The LEGENDplex™ Human Growth Factor Panel bead-based immunoassay is multiplex sandwich immunoassay (Multi-Analyte Flow Assay Kit" (Cat. No. 740180, Human Growth Factor Panel13-plex)). The kit is designed to detect and quantify thirteen human growth factors by flow cytometer, consisting of angiopoietin, EGF (epidermal growth factor), EPO (erythropoietin), FGF-basic (fibroblast growth factor-basic), G-CSF (granulocyte-colony stimulating factor), GM-CSF (granulocyte-macrophage colony-

stimulating factor), HGF (hepatocyte growth factor), M-CSF (macrophage colony-stimulating factor), PDGF-AA (platelet derived growth factor-AA), PDGF-BB (platelet derived growth factor-BB), SCF (stem cell factor), TGF- $\alpha$  (transforming growth factor-alpha) and VEGF (vascular endothelial growth factor).

The frozen samples were thawed completely at room temperature, mixed and centrifuged at 2,500xg for 10 minutes. The supernatants were used for growth factors measurement by flow cytometric bead-based immunoassay. The data were obtained and analyzed by LEGENDplex™ Data Analysis Software (the detail of growth factor measurement and data analysis can be found at [www.biologend.com](http://www.biologend.com)).

### Platelet count

An additional EDTA tube from two time points evaluation was measured for platelet count by automated cell analyzer (XE-2100; Sysmex) (Fig. 1B).

### Statistical analysis

For statistical analysis, the SPSS version 16.0 software (SPSS) was used. The data were represented in median and range. The comparison of growth factor concentrations among five blood collecting tubes at each time point was analyzed by Kruskal Wallis H test. The comparison of growth factor concentrations from the same type of blood collecting tubes was analyzed by Wilcoxon test. *p*-value less

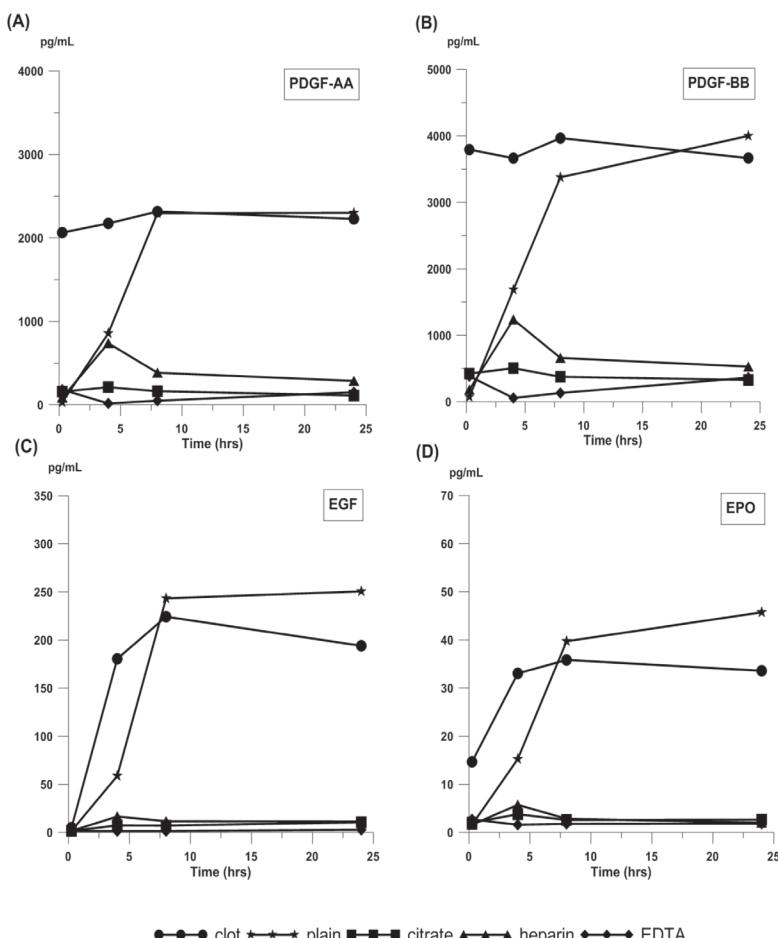
than 0.01 was considered statistical significance. The correlation between the platelet count and growth factor concentration was analyzed by Pearson correlation score (r score).

## Results

### Four time points evaluation and Two time points evaluation

Thirteen growth factors were measured, but only four growth factors of PDGF-AA,

PDGF-BB, EGF, and EPO showed dynamic change with time in four time points evaluation (15 minutes, 4, 8 and 24 hours) (Fig. 2) and also showed significant difference in two time points evaluation (15 minutes and 24 hours) (Fig. 3). Other growth factors of angiopoietin, FGF- $\beta$ , G-CSF, GM-CSF, M-CSF, SCF, TGF- $\alpha$ , HGF, and VEGF showed no dynamic change with time. G-CSF, GM-CSF, and SCF were very low in concentration to be



**Fig. 2** Four time points evaluation of growth factors from clot activator, plain, citrate, heparin, and EDTA tubes at 15 minutes, 4, 8 and 24 hours: (A) PDGF-AA, (B) PDGF-BB, (C) EGF, (D) EPO

quantified. (results not shown)

Four time points evaluation of PDGF-AA and PDGF-BB concentrations were much higher than those of the other growth factors. The concentration of PDGF-BB was higher than that of PDGF-AA both in serum and plasma. The serum concentration from plain tube gradually increased with time and peaked at 24 hours, while the serum concentration from clot activator rapidly increased and peaked at 8 hours. The plasma concentration of citrate, heparin, and EDTA tubes did not change with time (Figs. 2a, 2b). Two time points evaluation of PDGF-AA and PDGF-BB concentrations showed significant difference between serum and plasma. However, the serum concentration from plain tube at 15 minutes was similar with that of plasma and only showed significant difference at 24 hours. The plasma concentration from citrate, EDTA, and heparin tubes were not significantly different at both time points (Figs. 3a, 3b).

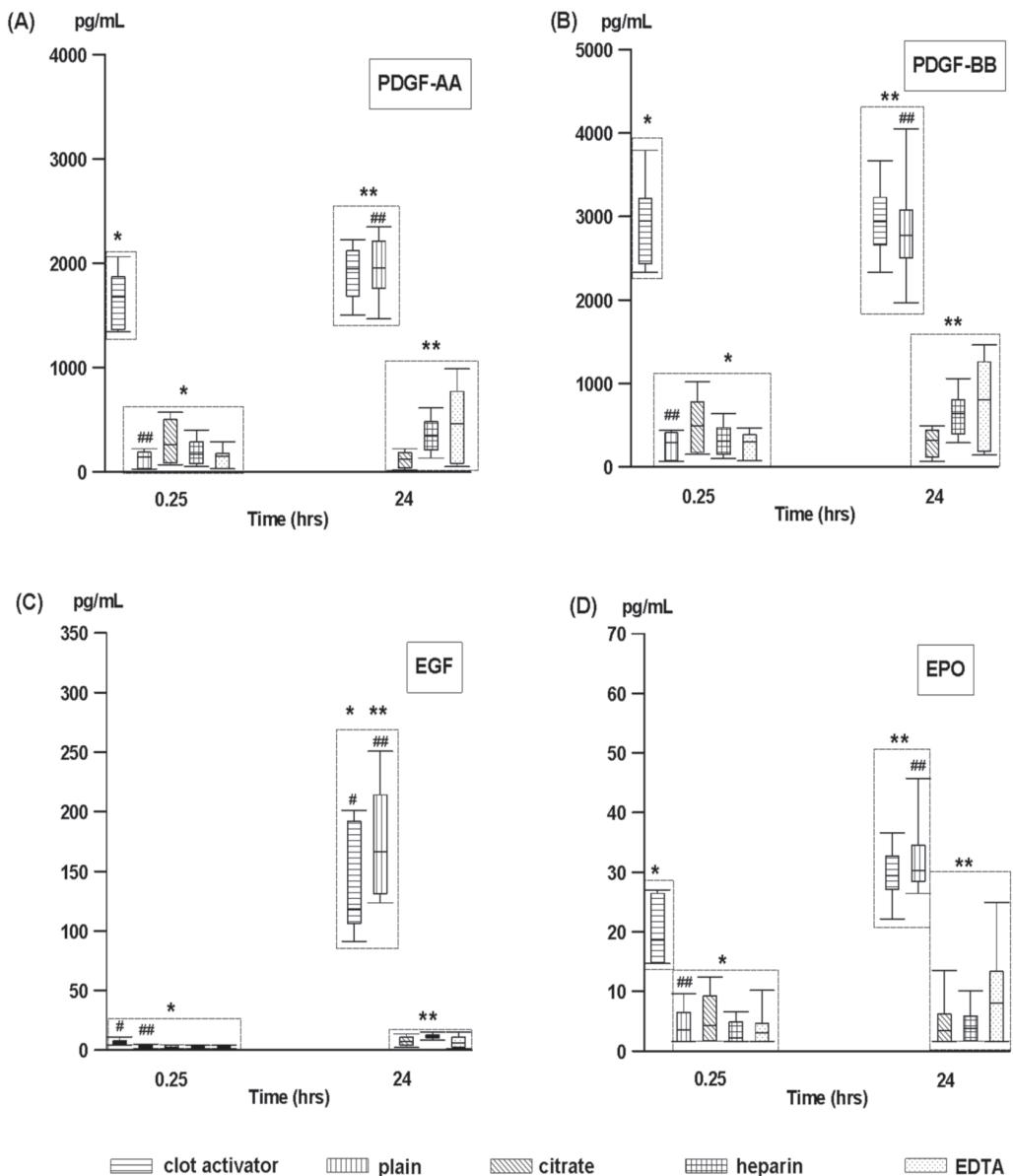
Four time points evaluation of EGF, the serum concentration from clot activator tube gradually increased with time and peaked at 8 hours, while the serum concentration from plain tube peaked at 24 hours. The plasma concentration, however, showed no change with time (Fig. 2c). Two time points evaluation of EGF at 15 minutes, both serum and plasma concentrations were low (less than 50 pg/mL) and comparable with each other. At 24 hours, the serum concentration was higher and showed significant difference against that at 15 minutes,

while the plasma concentration did not change without any significant difference (Fig. 3c).

Four time points evaluation of EPO, the serum concentration from clot activator tubes gradually increased with time and peaked at 8 hours, while the serum concentration from plain tubes peaked at 24 hours. The plasma concentration from citrate, EDTA and heparin tubes showed no change with time (Fig. 2d). Two time points evaluation of EPO showed significant difference between serum and plasma. However, the serum concentration from plain tube at 15 minutes was comparable with that from plasma, and only showed significant difference at 24 hours. The plasma concentrations from citrate, EDTA, and heparin tubes were not significantly different at both time points (Fig. 3d). Other growth factors of angiopoietin, FGF-basic, G-CSF, GM-CSF, M-CSF, SCF, TGF- $\alpha$ , HGF, and VEGF showed no difference between serum and plasma at both time points (results not shown).

#### Correlations between platelet count and platelet growth factors

Platelets release a variety of growth factors such as PDGF, EGF, TGF- $\alpha$ , VEGF, FGF, and HGF. In this study, only three platelet growth factors (PDGF-AA, PDGF-BB, and EGF) have reached high enough concentration for correlation study against platelet count. The concentration of the rest of platelet growth factors (TGF- $\alpha$ , FGF- $\beta$ , VEGF, and HGF) were too low to be quantitated for correlation study.



**Fig. 3** Two time points evaluation of growth factors from clot activator, plain, citrate, heparin, and EDTA tubes at 15 minutes and 24 hrs: (A) PDGF-AA, (B) PDGF-BB, (C) EGF, (D) EPO

\* statistical significance ( $p < 0.05$ ) at 15 minutes and \*\* statistical significance ( $p < 0.01$ ) at 24 hrs among five blood collecting tubes by Kruskal Wallis H test (no statistical difference among blood collecting tubes in each box)

# statistical significance ( $p < 0.05$ ) of clot activator tube and ## statistical significance ( $p < 0.01$ ) of plain tube at 15 minutes and 24 hrs by Wilcoxon test.

At 15 minutes, PDGF-AA and PDGF-BB from clot activator tubes showed moderate correlation against platelet count. The *r* values were 0.42 and 0.45, respectively (Figs. 4a, 4c). PDGF-AA and PDGF-BB concentrations from plain tube were too low to be quantitated for correlation study. EGF from clot activator tubes and plain tubes were also too low to be quantitated (Fig. 4e).

At 24 hours, PDGF-AA, PDGF-BB, and EGF from both clot activator tubes and plain tubes reached the peak concentration, therefore, we combined the results from both tubes for correlation study. PDGF-AA showed weak correlation against platelet count (*r* value = 0.11) (Fig. 4b) while PDGF-BB showed strong correlation against platelet count (*r* value = 0.55) (Fig. 4d). EGF also showed strong correlation against platelet count (*r* value = 0.62) (Fig. 4f).

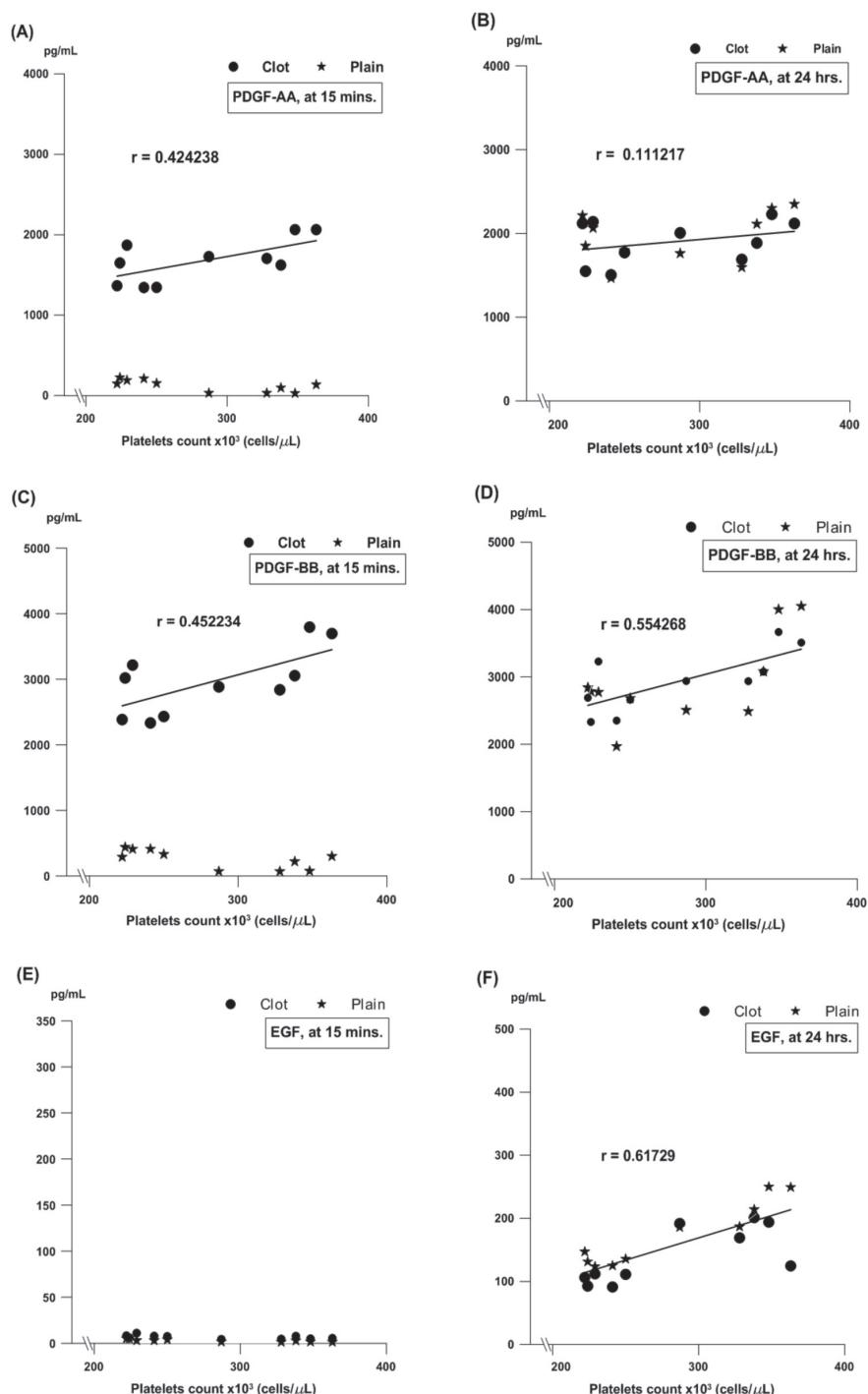
## Discussion

PDGF-AA and PDGF-BB are growth factors of the same family found mainly in platelets.<sup>(11)</sup> Normally, the concentration of PDGF is high, particularly in activated platelets where they release various bioactive molecules from their granules.<sup>(1)</sup> This is consistent with our results showing that serum concentration of PDGF from clot activator tubes was initially high at 15 minutes. Clot activator tube contains micronized silica particles that can induce rapid clot formation and platelet activation-degranulation. Therefore, at 15 minutes,

PDGF-AA and PDGF-BB concentrations were high in clot activator tubes, but not in plain tubes. In contrast, plasma concentrations have shown no significant difference. This is due to anticoagulants (citrate, heparin, and EDTA) inhibit coagulation proteins and also inhibit platelet activation resulting in little or no platelet growth factors released into plasma.

EGF is another platelet growth factor that is released after activation, but the serum concentration from clot activator tube at 15 minutes was low. We hypothesized that EGF was slowly released from alpha granules, while PDGF was suddenly released. Normal physiologic function of EGF is to promote late phase of wound healing and wound epithelialization.<sup>(2)</sup> Previous study also described that EGF was gradually released from platelets when the clot retraction was in progress *in vitro*.<sup>(12)</sup> PDGF-AA and PDGF-BB are important for early phase of wound healing and are suddenly released after activation.<sup>(13)</sup> Therefore, the serum concentration of EGF was peaked after PDGF, but both showed the peak at 24 hrs. The plasma concentration of EGF showed no significant difference because of no platelet activation.

EPO is a red blood cell growth factor mainly produced from fibroblasts in a renal cortex<sup>(14)</sup> and not a growth factor that mainly derived from blood cells (also platelets). The results showed the serum concentration of EPO increased with time and contradicted with the theory that EPO originated mainly from renal



**Fig. 4** Correlations between platelet count and platelet growth factors in serum from clot activator and plain tubes: (A) PDGF-AA at 15 minutes, (B) PDGF-AA at 24 hours, (C) PDGF-BB at 15 minutes, (D) PDGF-BB at 24 hours, (E) EGF at 15 minutes, (F) EGF at 24 hours.

but not blood cells. We hypothesize that the low level and high variation (wide SD range) of EPO concentration in serum was the explanation, and further study is necessary.

VEGF, HGF, TGF- $\alpha$  and FGF- $\beta$  are also the platelet growth factors, but the results showed no differences between serum and plasma at any time point. The explanation was that VEGF, HGF, TGF- $\alpha$ , and FGF- $\beta$  are derived mainly from various cells in the body and not majorly derived from platelets.<sup>(15, 16)</sup>

G-CSF, GM-CSF, and M-CSF are white blood cell growth factors that produced and acting locally in bone marrow.<sup>(17, 18)</sup> These cytokines are rarely found in blood, therefore the results showed very low concentration and no difference between serum and plasma at any time point.

Angiopoietin and SCF are produced by endothelium and fibroblasts.<sup>(19, 20)</sup> Angiopoietin and SCF are not derived mainly from the circulating blood cells, therefore the results showed no difference between serum and plasma at any time point.

The growth factors that showed higher concentration in serum than plasma were platelet derived-growth factors (PDGF-AA, PDGF-BB, and EGF, except EPO). Moreover, the concentration of these growth factors correlated with platelet count. Therefore, we hypothesized that these growth factors were

released from platelets during clot formation in serum, but not in plasma.

In conclusion, the measurement of growth factors in blood, three platelet growth factors (PDGF-AA, PDGF-BB, and EGF) and one red cell growth factor (EPO) showed higher concentration in serum than in plasma. Moreover, the study demonstrated moderate to strong correlation between three platelet growth factors (PDGF-AA, PDGF-BB, and EGF) against platelet count, indicated the releasing of these growth factors from platelets. The growth factors in blood showed different concentrations between serum and plasma, and also showed dynamic change with time. Therefore, the type of sample and the time before analysis should be concerned for appropriate interpretation of blood growth factor level.

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## Conflict of interest

The authors declare that they have no conflicts of interest.

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