Comparison of human growth factors between serum and plasma by flow cytometric bead-based immunoassay

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ABSTRACT

Growth factors are important for cell proliferation and sometimes served as the tumor prognostic biomarkers. Several growth factors are stored in platelets (platelet growth factors) and are important for the initiation of wound healing process. The study was to determine platelet growth factors releasing into serum and plasma with time by flow cytometric bead-based immunoassay. Thirteen growth factors from twenty specimens were compared between serum (clot activator tube) and plasma (citrate tube), at 15 minutes and 24 hours. The results showed that three platelet growth factors (PDGF-AA, PDGF-BB, EGF) were higher concentrations in serum than in plasma and one platelet factor (HGF) was lower concentration in serum than in plasma. In addition, one red blood cell growth factor (EPO) was higher concentration in serum than in plasma. The other growth factors were no significant difference between serum and plasma at both time. In conclusion, the several growth factors showed the different levels between serum and plasma, and also change with time. Therefore, the type and time of blood specimen must be considered for the growth factor evaluation.

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

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Introduction

Growth factors are a naturally occurring substance capable of stimulating cell proliferation, hematopoiesis, differentiation and regulate many cellular processes. The platelets contain several growth factors and cytokines (more than 300 types) (James, Bradshaw, 1984), the important ones are Platelet-derived growth factor (PDGF), Transforming growth factor (TGF), Vascular endothelial growth factor (VEGF), Epidermal growth factor (EGF) and Fibroblast growth factor (FGF) (Sunitha Raja, Munirathnam Naidu, 2008). The other growth factors are also found in the blood, such as Erythropoietin (EPO) mainly derived from kidneys, Angiopoietin mainly derived from endothelial cells. In contrast with, white blood cell growth factors that are seldom found in blood, for examples Granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM–CSF) and Stem cell factor (SCF) (Bradshaw, Cavanaugh, 1991).

The blood collecting tube without anticoagulants as clot activator tube (contained clot activator) renders the aqueous component called “serum”, while blood collecting tubes with anticoagulants as citrate tube renders the aqueous component called “plasma”. The previous studies compared the effect of various anticoagulants to platelets and the type of anticoagulant showed the different degree of platelet activation (Do Amaral et al., 2016; Macey et al., 2002). The citrate anticoagulant was preferred by most investigators in the platelet studies because of less platelet activation and well preserved platelet function (Golanski et al., 1996; Lei et al., 2009). However, the difference of growth factors between serum and plasma has not been fully elucidated. Some previous research raised the issue of cancer prognosis by PDGF and VEGF, one study used serum while the other used plasma (Madsen et al., 2012; Leitzel et al., 1991). Therefore, the effect of blood collecting tube and the type of specimen (serum vs plasma) against growth factors are required further study.

This study was compared the growth factors in serum and plasma at two time points. The finding from the study could lead to the appropriate specimen and time to measure the growth factors. Finally, the study might guide the type of blood component, in order to obtain the maximum of growth factors for the application in orthopedic and musculoskeletal injurie (Willits et al., 2013).

Objectives of the study

To compare growth factor levels in blood between serum and plasma at two time points

Materials and Methods

Study design and population

The study was conducted with the informed consent of volunteer subjects and approved by the Committee on Human Rights Related to Research Involving Human Subject, Faculty of Medicine Ramathibodi Hospital, Mahidol University, based on the Declaration of Helsinki ID 06-59-49. Total twenty blood specimens were obtained from five subjects. Each subjects was composed of serum (clot activator tube) and plasma (citrate tube) at 15 mins. and 24 hrs (one collection) in **[Fig. 1]**. The blood was obtained from the healthy volunteers accordding to the blood donors criteria, who aged between 20 and 30 years (all specimen obtained from women), without any known blood

dyscrasia, no taking any medicines as aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), anti-platelet and anti-coagulant drugs during the preceding two weeks.

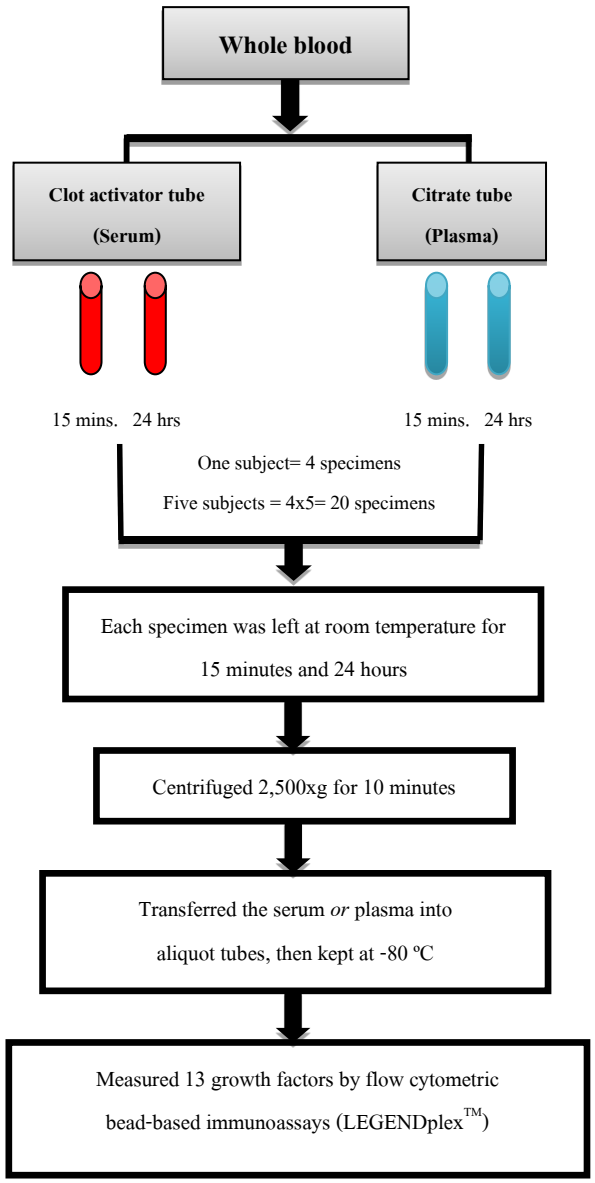

Figure 1. The experimental design for the growth factor evaluation

The specimen collection

To the determine the appropriate time for growth factor comparison, blood from the healthy volunteer was drawn into two blood collecting tubes, clot activator tube 6 ml (micronized silica particles IMPROVACUTER®) and citrate tube 4.5 ml (3.2% sodium citrate IMPROVACUTER®). The specimens were left at room temperature and observed the clot formation. The clot was fully retracted at 24 hrs, therefore the appropriate time for growth factor comparison will be at 15 mins. (initial collection after centrifugation) and at 24 hrs (full retraction of clot).

The whole blood specimens were drawn from five healthy volunteers into the collecting tubes. One subjectwas composed of four specimens (two clot activator tubes and two citrate tubes). Total twenty specimens were obtained to complete the study in **[Fig.1]**. The whole blood specimens were separated into serum and plasma by centrifuged at 2,500xg for 10 minutes, then kept at -80 °c before growth factors measurement.

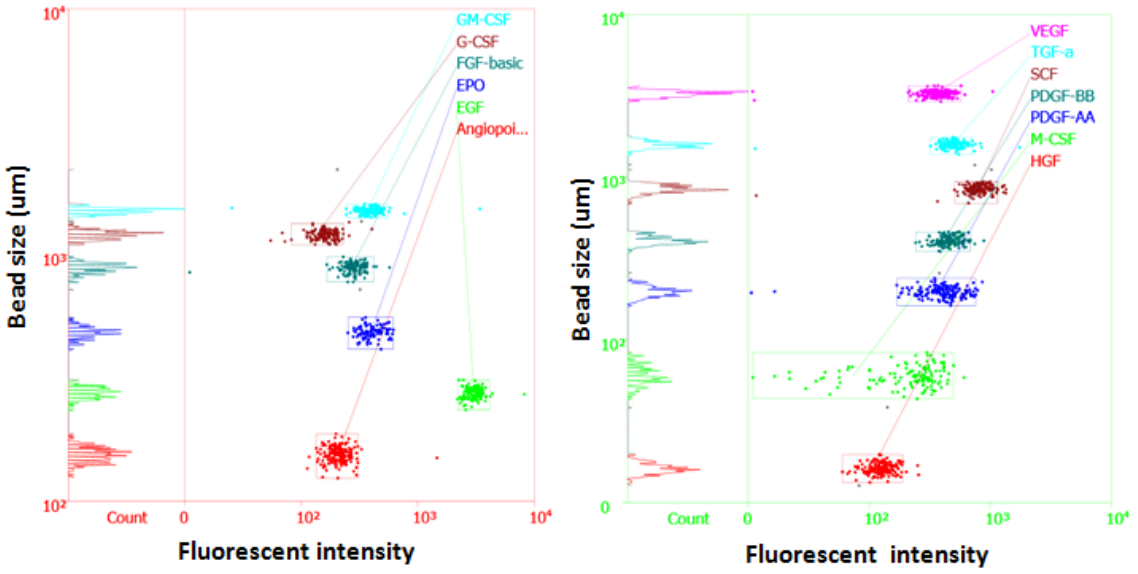

Figure 2. Flow cytometric bead-based immunoassay for the measurement of growth factor concentrations

Growth factors measurement

All serum and plasma were measured for thirteen growth factors; Angiopoietin, Endothelial growth factor (EGF), Erythropoietin (EPO), Fibroblast growth factor-basic (FGF-basic), Granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM–CSF), Hepatocyte growth factor (HGF), Macrophage colony-stimulatingfactor (M-CSF), Platelet-derived growth factor-AA (PDGF-AA), Platelet-derived growth factor-BB (PDGF-BB), Stem cell factor (SCF), Transforming growth factor-alpha (TGF-alpha) and Vascular endothelial growth factor (VEGF), by flow cytometric bead-based immunoassay "LEGENDplex™ Multi-Analyte Flow Assay Kit" (Cat. No. 740180, Human Growth Factor Panel (13-plex)). The steps of growth factor analysis were following the microtubes protocol and the data were analyzed by "LEGENDplex™ Data Analysis Software" in **[Fig.2]**.

Statistical Analyses

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 between serum and plasma were analyzed by Mann-Whitney U test and the comparison of growth factor concentrations at 15 mins. and 24 hrs were analyzed by Wilcoxon test. P-value less than 0.05 was considered the significant difference in **[Fig.3 and 4]**.

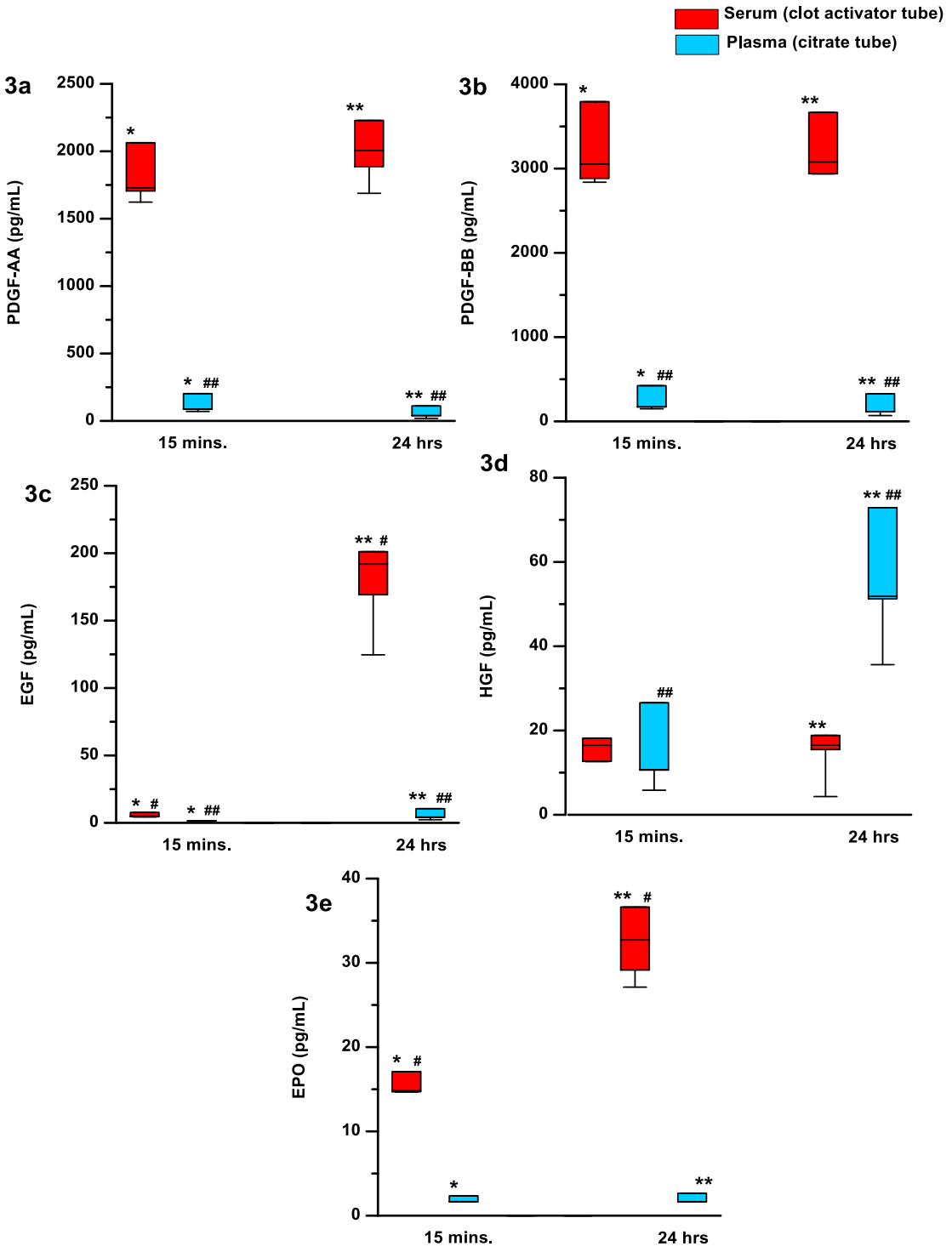

Figure 3. The growth factors that showed the significant differences between serum (clot activator tube) and plasma (citrate tube) at 15 mins. and 24 hrs: **3a**; PDGF-AA, **3b**; PDGF-BB, **3c**; EGF, **3d**; HGF, **3e**; EPO.

*, **Comparison between serum and plasma at the same time with statistical significance ($p < 0.05$)

#, ##Comparison between the same type of specimens at 15 mins. and 24 hrs with statistical significance ($p < 0.05$)

Results

There were four platelet growth factors (PDGF-AA, PDGF-BB, EGF, HGF) and one red blood cell growth factor (EPO) that showed the significant difference between serum and plasma in **[Fig.3]**.

PDGF-AA and PDGF-BB showed the highest concentration in serum when comparing with the other growth factors. PDGF-BB were higher concentration than PDGF-AA in both serum and plasma (around 1.5x). At both time the concentration of PDGF-AA and PDGF-BB were obviously different between serum and plasma (around 10x). Moreover, PDGF-AA and PDGF-BB slightly decreased with time in plasma but did not change in serum in **[Fig.3a and 3b]**.

EGF showed the increasing trend with time in both serum and plasma but more profound in serum. At both time the concentrations were higher in serum than in plasma and obviously higher in serum at 24 hrs (around 10x) in **[Fig.3c]**.

HGF increased with time in plasma but did not change in serum. The concentration between serum and plasma was not different at 15 mins. but was different at 24 hrs in **[Fig.3d]**.

EPO increased with time in serum but did not change in plasma. The concentrations were higher in serum than in plasma at both time and obviously higher in serum at 24 hrs (around 8x) in **[Fig.3e]**.

The remained growth factors, Angiopoietin, FGF-basic, G-CSF, GM-CSF, M-CSF, SCF, TGF-alpha and VEGF, were no significant difference between serum and plasma and did not differ with time in **[Fig.4]**.

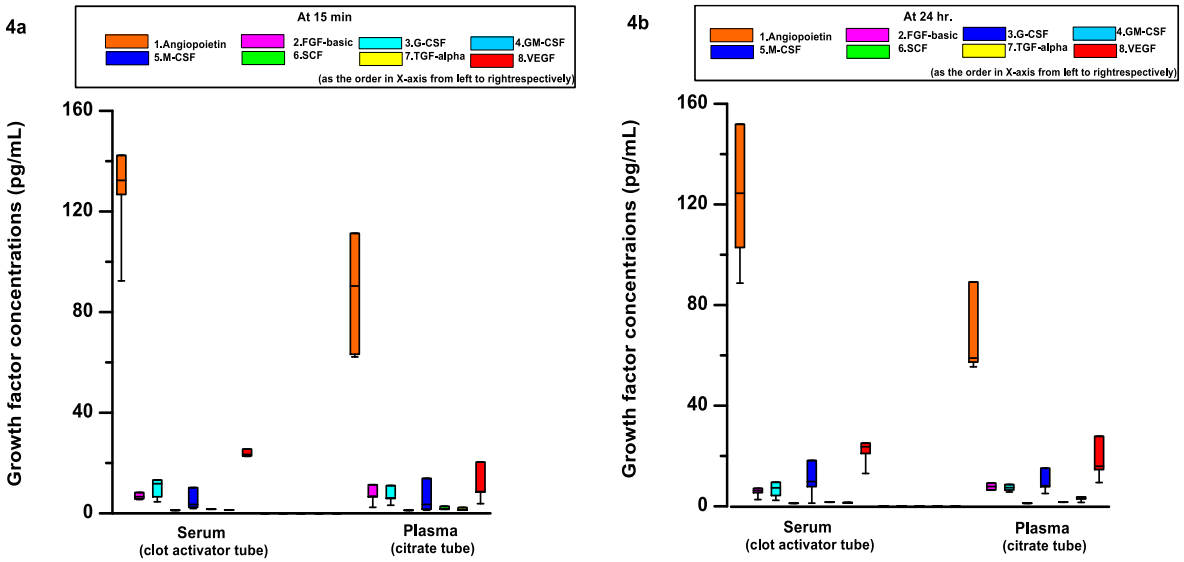

Figure 4. The growth factor concentrations (Angiopoietin, FGF-basic, G-CSF, GM-CSF, M-CSF, SCF, TGF-alpha and VEGF) that showed "no" significant difference between serum (clot activator tube) and plasma (citrate tube): **4a**; at 15 mins., **4b**; at 24 hrs.

Discussion

PDGF is the growth factor that is found abundant in alpha granules of platelets and will release after the activation, the three common isoforms in platelets are PDGF (AA, AB, BB) (Bennett et al., 2003). PDGF molecules are also important chemotactic and mitogenic factors with regard to the inflammatory cells (Everts et al., 2006; Molloy et al., 2003) in order to initiation of wound healing process after blood vessel injury. From the results PDGF-AA and PDGF-BB showed the highest concentration among the other growth factors (in **[Fig.3]**) because both were mainly stored and released into the serum by platelets during the activation. While the other growth factors in blood were majorly derived from the other cells or other organ tissues (James, Bradshaw, 1984). In addition, PDGF-BB was higher concentration than PDGF-AA because platelets contained more PDGF-BB than PDGF-AA.

The clot activator tube contained the micronized silica particles that enhanced the clot formation and also induced the activation of platelets, therefore the platelet growth factors were released into the serum compartment of clot activator tubes. The PDGF-AA and PDGF-BB initially showed the peak level at 15 mins. (in serum, not in plasma), indicated rapidly releasing into the serums in **[Fig.3a and 3b]**. The results were similar as the previous study (Huang et al., 1983), that the concentration of PDGF are higher in serum than in plasma. The slightly decreasing of PDGF-AA and PDGF-BB in plasma at 24 hrs maybe caused by the degradation with time or the consumption by circulating blood cells.

EGF is another platelet growth factor that is stored and released after platelet activation. EGF is important in the chronic wound healing, endothelial cells and keratinocytes (Bennett et al., 2003). EGF was gradually released with time in contrast with PDGF-AA and PDGF-BB that were suddenly released at 15 mins. in **[Fig.3a, 3b and 3c]**. Delayed degranulation of EGF from platelets was concordance with the normal physiologic function of EGF to promote the late wound healing process and wound epithelization (Everts et al., 2006). The previous study (Lev-Ran et al., 1990) also showed the higher concentration of EGF in serum than in plasma.

HGF are produced by several types of epithelium such as kidney, lung, liver and tumor including platelets (Matsumoto, Nakamura, 1991), therefore the level found in blood could be primarily originated from the other sources, but not limited to only platelets. Then the results at 15 mins. showed no difference between serum and plasma in **[Fig.3d]**, however at 24 hrs the plasma level was higher than the serum level in **[Fig.3d]**, the reason for such a result was not fully elucidated. The possible explanation might be that the HGF from platelets was trapped in the clot or consumed by the circulating blood cells during the clot formation.

PDGF, EGF, VEGF, HGF, TGF-alpha and FGF-basic are the platelet growth factors. Normally 70% of growth factors in platelet are released within 10 mins. and nearly 100% are released within 1 hr after the activation (Brass, 2010). The results from the study implied that PDGF-AA and PDGF-BB were rapidly released within first 10 mins. while EGF was slowly released within 1 hr.

VEGF, TGF-alpha and FGF-basic are also the platelet growth factors, but the results showed no difference between serum and plasma at both time, in **[Fig.4]**. Because VEGF, TGF-alpha and FGF-basic could be derived from the various cells in the body (macrophages, endothelial, keratinocytes, and fibroblast cells) but not majorly derived

from the platelets(McInnes et al., 1998; Hoeben et al., 2004). Then, the levels were depended on individual condition of each person, but not related directly to the type and time of specimens.

EPO is a red blood cell growth factor, mainly synthesized by fibroblasts in the renal cortex (Jelkmann, 2011). EPO is important for red blood cell production, proliferation and differentiation of the erythroid progenitor cells (Lacombe, Mayeux, 1999). In theory, the level of EPO should not differ between serum and plasma because EPO is not the platelet growth factors, and not found in other circulating blood cells. But the results showed that EPO was higher in serum than in plasma and much more prominent at 24 hrs in **[Fig.3e]**. The possible explanations were that EPO could be derived from the residual factors in some blood cells (before releasing into serum during clot formation).

G-CSF, GM-CSF and M-CSF are white blood cell growth factors. Their major functions are stimulating the bone marrow to produce granulocytes and stem cells as well as promoting white blood cell proliferation and differentiation (Lieschke , Burgess 1992). G-CSF, GM-CSF and M-CSF are produced by endothelium, macrophages and a number of other immune cells, and there are presence in blood circulation (Xu et al., 2000). The above cells are rarely found in blood circulation, therefore the results showed no difference between serum and plasma at both time in **[Fig.4]**.

Angiopoietin and SCF are produced by endothelium and fibroblasts. Angiopoietin is the main regulator of vessel formation by controlling vascular permeability, inflammation and remodeling (Isidori et al., 2016). SCF is essential hematopoietic cytokine that interacts with other cytokines to preserve the viability of hematopoietic stem and progenitor cells (Hassan, Zander, 1996). Angiopoietin and SCF are not derived mainly from the circulating blood cells, therefore the results showed no difference between serum and plasma at both time in **[Fig.4]**.

Conclusions

The several growth factors showed the different levels between serum and plasma, and also change with time. For using the growth factors as the prognostic biomarker in cancers, the specific type of specimen and the time of measurement must be defined in order to obtain the correct value. Furthermore, the results from the study emphasized the important of the type of blood components and collecting time for the application of growth factors in orthopedic and musculoskeletal injuries.

Acknowledgements

This research project is supported by Faculty of Medicine Ramathibodi Hospital Mahidol University.

The laboratory equipment and flow cytometric analysis were provided by The Clinical Immunology Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital Mahidol University.

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