# Effects of Recombinant Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor on Platelet Aggregation in Healthy Volunteers

## Rekombinant Granülosit Koloni-Stimüle Edici Faktör ve Granülosit-Makrofaj Koloni-Stimüle Edici Faktörün Sağlıklı Gönüllülerde Platelet Agregasyonu Üzerine Etkileri

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Geliş Tarihi/*Received:* 24.02.2015
Kabul Tarihi/*Accepted:* 24.04.2015

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**ABSTRACT Objective:** Recombinant human granulocyte colony-stimulating factor (G-CSF) is increasingly used for stem cell mobilization in healthy donors for allogeneic stem cell transplantation. However, a possible association between thrombosis and G-CSF administration has been reported. This study was performed to investigate the in vitro effects of G-CSF and granulocyte-macrophage-colony-stimulating factor (GM-CSF) on platelet aggregation in healthy volunteers. **Material and Methods:** Platelet aggregation was induced by adenosinediphosphate (ADP) and collagen in platelet rich plasma (PRP) and whole blood (WB) samples from 26 healthy volunteers (20 volunteers for G-CSF, and 6 volunteers for GM-CSF study). Three concentrations of G-CSF solution (10, 50 and 100 ng/mL) and GM-CSF (5, 10 and 20 ng/mL) were prepared. Each concentration of G-CSF and GM-CSF solutions and the control diluent were incubated with PRP and WB samples. After incubation, aggregation responses were evaluated with ADP (1 μM and 5 μM) and collagen (1 μg/mL) in PRP and WB samples. **Results:** We found that G-CSF increased ADP and collagen induced platelet aggregation in PRP and ADP induced platelet aggregation rate in WB. GM-CSF didn't affect ADP and collagen induced platelet aggregation rate in whole blood and platelet-rich plasma in vitro. **Conclusion:** This study showed that G-CSF administration may lead to platelet hyperaggregability. The enhancing effect of G-CSF on platelet aggregation should be taken into consideration in clinical usage.

**Key Words:** Platelet aggregation; granulocyte colony-stimulating factor; granulocyte-macrophage colony-stimulating factor

**ÖZET Amaç:** Rekombinant insan granülosit koloni uyarıcı faktör (G-CSF), allojeneik kök hücre transplantasyonunda sağlıklı donörlerde kök hücre mobilizasyonu için giderek artan oranlarda kullanılmaktadır. Bununla birlikte, G-CSF kullanımı ile tromboz birlikteliğine ait raporlar bildirilmektedir. Bu çalışmada, ile in vitro olarak G-CSF ve granülosit makrofaj koloni uyarıcı faktör (GM-CSF)'ün sağlıklı gönüllülerde platelet agregasyonu üzerine etkileri incelenmiştir. **Gereç ve Yöntemler:** Yirmi altı (20 gönüllü G-CSF, 6 gönüllü GM-CSF) sağlıklı gönüllüden alınan tam kan (TK) ve plateletten zengin plazma (PZP) örneklerinde adenozindifosfat (ADP) ve kollajen ile platelet agregasyonu indüklendi. Üç konsantrasyonda G-CSF solüsyonu (10, 50,100 ng/mL) ve GM-CSF solüsyonu (5, 10, 20 ng/mL) hazırlandı. Her bir konsantrasyondaki G-CSF ve GM-CSF solüsyonu ve kontrol dilüenti PZP ve TK örnekleri ile inkübe edildi. İnkübasyondan sonra agregasyon yanıtları PZP ve TK örneklerinde ADP (1 μM ve 5 μM) ve kollajen (1 μg/mL) ile ölçüldü. **Bulgular:** Biz, G-CSF'nin PZP'de ADP ve kollajen ile indüklenmiş, TK'da ADP ile indüklenmiş platelet agregasyonunu artırdığını gösterdik. GM-CSF, TK ve PZP'de, in vitro, ADP ve kollajen ile indüklenmiş platelet agregasyonunu etkilemedi. **Sonuç:** Bu çalışma, G-CSF uygulamasının platelet hiperagregabilitesine yol açabileceğini göstermiştir. Klinik kullanımda G-CSF'nin platelet agregasyonunu artırıcı etkisi gözönüne alınmalıdır.

**Anahtar Kelimeler:** Trombosit kümelenmesi; granülosit koloni uyarıcı faktör; granülosit makrofaj koloni uyarıcı faktör

doi: 10.5336/medsci.2015-44486



**Turkiye Klinikleri J Med Sci 2015;35(2):112-7**

Recombinant human granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have come into use for years. Today, principal use of these cytokines is myelosupression after chemotherapy inducing aplasia, after allogeneic or autologous bone marrow transplantation and mobilization of $CD34^+$ progenitor cells.[1,2] On the other hand, increasing use of these cytokines is complicated by increasing side effects, such as arterial thrombosis and venous thromboembolism.[3,4]

Specific G-CSF receptors on platelets have been demonstrated by Shimoda and co-workers.[5] Some studies found increased collagen and adenosine diphosphate (ADP) induced platelet aggregation after ex vivo incubation with G-CSF, and in vivo.[6-8] Moreover, Spiel et al. have demonstrated that G-CSF increases collagen-ADP induced platelet plug formation in the platelet function analyzer.[9] However, LeBlanc et al. found reduced platelet aggregation in allogeneic blood stem cell donors treated with G-CSF.[10] Based on these trials, no firm conclusion can be drawn at present on the effect of G-CSF on platelet function. In addition, data on the effects of GM-CSF on platelet function are very limited.

In this study, we aimed to investigate the influence of G-CSF and GM-CSF on platelet aggregation using collagen and ADP in platelet-rich plasma (PRP) and whole blood (WB) of healthy volunteers in vitro. Because platelet aggregation studies in WB would reflect the in vivo situation as closely as possible.

## MATERIAL AND METHODS

This study was performed with 26 healthy volunteers. Twenty of them with a mean age of 23.9±2.6 years (10 were male, and 10 were female) were included in G-CSF group. The others with a mean age of 25.5±1.2 years (23-31 years, 5 were male, and 1 was female) were included in GM-CSF group. No medications (especially anti-aggregant drugs) were taken for at least 10 days prior to the study. Written informed consents were obtained from the participants in both groups, and the study was approved by the local ethics committee.

### G-CSF AND GM-CSF PREPARATIONS

Filgrastim (Neupogen, Thousand Oaks, CA, Amgen) was used as G-CSF and molgramostim (Leucomax, Sandoz/Schering Plough) as GM-CSF. We used 10 ng/mL G-CSF concentrations in PRP studies because it has been demonstrated that ADP induces platelet aggregation mostly at 10 ng/ml G-CSF concentrations.[5] We prepared also higher G-CSF concentrations (10, 50, and 100 ng/ml) in WB studies because of the existence G-CSF receptors on the cells other than platelets.[11,12] Anyway, it was demonstrated that G-CSF concentrations in the serum was 49 ng/mL in adults and 117 ng/mL in children after subcutaneous G-CSF administrations.[13]

We used 1 and 5 ng/mL GM-CSF concentrations in PRP studies because of the demonstration of 1-5 ng/mL GM-CSF serum level after subcutaneous GM-CSF administrations and used higher GM-CSF concentrations (5, 10, 20 ng/mL) in WB studies because of the existence GM-CSF receptors on the cells other than platelets.[14] For these reasons we prepared 5% DW solutions of the G-CSF and GM-CSF to include 10, 50, and 100 ng/mL G-CSF concentrations in WB samples, 10 ng/mL G-CSF concentrations in PRP samples and 5, 10, and 20 ng/mL GM-CSF concentrations in WB samples and 1, and 5 ng/mL GM-CSF concentrations in PRP samples.

### PLATELET AGGREGATION STUDIES

Aggregation studies were performed using Whole Blood Aggregometer Model 560 (Chrono-log Corporation Havertown, PA, USA) maintaining the cuvette temperature at 37°C. Two Omniscribe Chart Recorders (Coulter Electronics Ltd. Luton, England) were used to register aggregation curves. One of them was used for impedance channel and the other for optical channel. Collagen (1 mg/ml, Chrono Par. No. 385, Chrono-Log Corporation, Havertown, PA, USA) and ADP (1 mM in PRP, and 5 mM in WB, Chrono Par. No: 384, Chrono-Log Corporation, Havertown, PA, USA) were used as agonists.

The blood samples were drawn from antecubital vein without venous occlusion into plastic syringes containing 1/10 volume of 3.8% trisodium citrate. Platelet counts were determined with an automated cell counter (Medonic CA 610 Cell Ana-

lyzer, Sweden). Platelet-rich plasma (PRP) and platelet-poor plasma were prepared by centrifuging as described previously.[15] Optical method was used in PRP studies. Above-mentioned cytokine solutions were added to PRP samples. Before beginning the aggregation studies, PRP samples were incubated with these solutions for 15 minutes.[5] Impedance method was used in WB studies. %35-45 hematocrit levels were used in WB studies and in order to adjust this level, isotonic saline was added into the samples when needed. Similarly, above-mentioned cytokine solutions were added to WB samples and incubated for 15 minutes before the beginning of aggregation studies. The aggregation curves before addition of cytokine solutions into samples were compared with the aggregation curves after the addition of cytokine solutions into the samples. Maximum aggregation value was in terms of "ohm" in WB studies and "percent" in PRP studies. Time needed for maximum aggregation was as "seconds" in both samples. Studies were performed within 2 hours after the samples were obtained.

### STATISTICAL ANALYSIS

Results are expressed as means±standard error of the mean (SEM) depending on the distribution of data. n equals to the number of patients. Differences between the values before and after G-CSF and GM-CSF administrations were evaluated by using "paired-t test" and "Wilcoxon-t test" respectively. p values of <0.05 were considered statistically significant.

## RESULTS

### AGGREGATION STUDIES IN PLATELET-RICH PLASMA

In the G-CSF group at 1 μM concentration of ADP and 1 mg/mL concentration of collagen, the mean maximum aggregation value and maximum aggregation time with G-CSF were significantly higher than the without G-CSF values ($p<0.05$) (Table 1). G-CSF statistically significantly increased ADP and collagen induced platelet aggregation at 10 ng/mL G-CSF concentration.

In the GM-CSF group at 1 μM concentration of ADP and 1 mg/mL concentration of collagen, the mean maximum aggregation value and maximum aggregation time with GM-CSF were not significantly different than the without GM-CSF values ($p>0.05$) (Table 1). GM-CSF didn't show on increased ADP and collagen induced platelet aggregation at 1 and 5 ng/mL GM-CSF concentrations.

### AGGREGATION STUDIES IN WHOLE BLOOD

In the G-CSF group at 5 μM concentration of ADP, the mean maximum aggregation value and maximum aggregation time with G-CSF were signifi-

**TABLE 1:** Platelet aggregation studies with ADP and collagen in platelet rich plasma.

| | ADP (1 μM) Maximum Aggregation | | Collagen (1 μg/mL) Maximum Aggregation | |
|---|---|---|---|---|
| **G-CSF group (n=20)** | **Value (%)** | **Time (second)** | **Value (%)** | **Time (second)** |
| Before G-CSF | 21.1±2 | 38.4±1.9 | 68.3±2.8 | 217.2±17 |
| After G-CSF (10 ng/mL) | 31.6±2.3 | 44.7±2.7 | 82.8±2.9 | 254±14.6 |
| p value | | | | |
| Before G-CSF vs After G-CSF | <0.01 | <0.01 | <0.01 | <0.01 |
| **GM-CSF group (n=6)** | | | | |
| Before GM-CSF | 30.9±10.9 | 125±37.6 | 75.1±9.3 | 273±54 |
| After GM-CSF (1 ng/mL) | 33.4±9.5 | 138.3±34.1 | 76.5±7.3 | 258±42.5 |
| p value | | | | |
| Before GM-CSF vs After GM-CSF1 | >0.05 | >0.05 | >0.05 | >0.05 |
| After GM-CSF (5 ng/mL) | 38.8±10 | 158.3±45 | 73.9±4.7 | 250±32.6 |
| p value | | | | |
| Before GM-CSF vs After GM-CSF5 | >0.05 | >0.05 | >0.05 | >0.05 |

Data w ere presented as the mean±SD.

G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage-colony-stimulating factor; ADP: Adenosinediphosphate.

cantly higher than the without G-CSF values ($p<0.05$) (Table 2). G-CSF statistically significantly increased ADP induced platelet aggregation at 10, 50 and 100 ng/mL G-CSF concentrations. However, at 1 mg/mL concentration of collagen the mean maximum aggregation value and maximum aggregation time with G-CSF were not significantly different than the without G-CSF values ($p>0.05$) (Table 2). We couldn't find any relationship between G-CSF and collagen induced platelet aggregation in WB studies.

In the GM-CSF group at 5 μM concentration of ADP and 1 mg/mL concentration of collagen, the mean maximum aggregation value and maximum aggregation time with GM-CSF were not significantly different than the without GM-CSF values ($p>0.05$) (Table 2). GM-CSF didn't increase ADP and collagen induced platelet aggregation at 5,10 and 20 ng/mL GM-CSF concentrations.

## DISCUSSION

This study investigated the influence of G-CSF and GM-CSF on platelet aggregation using collagen and ADP in PRP and WB of healthy volunteers in vitro, and we found that G-CSF increased ADP and collagen induced platelet aggregation in PRP and ADP induced platelet aggregation rate in WB. In addition, we found that GM-CSF didn't affect ADP and collagen induced platelet aggregation rate in PRP and WB in vitro.

There is considerable evidence that thrombosis is a common complication of malignancy, and G-CSF is widely used for the treatment of chemotherapy induced neutropenia.[16] In a meta-analysis of studies investigating the use of rhG-CSF with chemotherapy, Barbui et al. reported that 1.2% of cancer patients experienced thrombotic complications.[17]

**TABLE 2:** Platelet aggregation studies with ADP and collagen in whole blood.

| | ADP (5 μM) | | Collagen (1 μg/mL) | |
|---|---|---|---|---|
| | Maximum Aggregation | | Maximum Aggregation | |
| **G-CSF group (n=20)** | **Value (ohm)** | **Time (second)** | **Value (ohm)** | **Time (second)** |
| Before G-CSF | 10.1±0.7 | 340±25.4 | 16.1±0.9 | 550±28 |
| After G-CSF (10 ng/mL) | 14±1.1 | 426±34.7 | 14.4±1.0 | 570±33.4 |
| p value | | | | |
| Before G-CSF vs After G-CSF10 | <0.01 | <0.01 | >0.05 | >0.05 |
| After G-CSF (50 ng/mL) | 14.5±0.9 | 390±22 | 16.3±1.1 | 525±32.5 |
| p value | | | | |
| p Before G-CSF vs After G-CSF50 | <0.01 | <0.05 | >0.05 | >0.05 |
| After G-CSF (100 ng/mL) | 17.3±0.8 | 444±33 | 16.3±1.1 | 563±36 |
| p value | | | | |
| Before G-CSF vs After G-CSF100 | <0.01 | <0.01 | >0.05 | >0.05 |
| **GM-CSF group (n=6)** | | | | |
| Before GM-CSF | 7.7±1.4 | 418±54 | 11.5±1.7 | 465±37.9 |
| After GM-CSF (5 ng/mL) | 8.7±1.7 | 343±35 | 14±1.6 | 485±23.8 |
| p value | | | | |
| Before GM-CSF vs After GM-CSF5 | >0.05 | >0.05 | >0.05 | >0.05 |
| After GM-CSF (10 ng/mL) | 12±0.9 | 425±47.5 | 15.1±1.8 | 550±33.6 |
| p value | | | | |
| Before GM-CSF vs After GM-CSF10 | >0.05 | >0.05 | >0.05 | >0.05 |
| After GM-CSF (20 ng/mL) | 12.2±1.6 | 365±47.5 | 14±1.7 | 485±44.2 |
| p value | | | | |
| Before GM-CSF vs After GM-CSF20 | >0.05 | >0.05 | >0.05 | >0.05 |

Data w ere presented as the mean±SD.

G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage-colony-stimulating factor; ADP: Adenosinediphosphate.

G-CSF receptors are present on the surface of platelets, and these receptors appear to be functional in vitro and in vivo.[5,6] The number of platelet G-CSF receptors was found to be less than those on granulocytes (41±7 vs. 412±158) but almost equally active in terms of binding affinity (300±150 pM vs. 350±90 pM).[5] Avenarius et al. have demonstrated the effects of G-CSF on platelets at first, and these researchers have found that G-CSF increased collagen and epinephrine induced platelet aggregation of 20 healthy volunteers in vitro.[6] Subsequently, Shimoda et al. studied 4 healthy volunteers and found that statistically significantly increased ADP induced platelet aggregation in vivo.[18] Harada et al. and Kuroiwa et al. were also found increased ADP and collagen induced platelet aggregation compared to control group in in vivo studies performed in 5 and 10 healthy volunteers respectively ($p<0.01$).[19,20] All of these studies have been made at PRP. Recent study have demonstrated that even application of single and low dose G-CSF (5 mg/kg bodyweight) has a major impact on shear-dependent platelet plug formation.[9]

Spiel et al. speculated that bone marrow-derived factors may be responsible for in vivo platelet aggregation.[8] Polymorph nuclear neutrophil-derived proteases like elastase and cathepsine G are both significantly increased during G-CSF treatment and they have potent platelet activation properties.[21,22] Some cases of acute arterial thrombosis occurring in healthy donors, possibly due to G-CSF, have been published.[23] During the collection of stem cells with G-CSF for myocardial neovascularization from 15 coronary artery disease patients, 2 patients developed myocardial infarction and G-CSF was suspected from this situation.[24] Kang et al. have found high rate of stent restenosis in myocardial infarction patients to promote angiogenesis with intracoronary infusion of peripheral blood stem-cells mobilized with G-CSF contrary to cell infusion and control group patients.[25] All these data indicate that G-CSF leads to a hypercoagulatory state that is especially pronounced in the arterial system. The lack of efficacy of G-CSF for cardiac repair after acute myocardial infarction might be related to the untoward prothrombotic side effects of this medication.[26] However, there are also conflicting reports. Rivera et al. reported that G-CSF didn't affect the coagulation cascade on the platelet activation systems in vivo.[27] LeBlanc et al. have found decreased ADP induced platelet aggregation after G-CSF administrations in vivo.[10]

Most of these studies have been performed on platelet-rich plasma. We studied in whole blood in addition to platelet-rich plasma and we found that G-CSF increased ADP and collagen induced platelet aggregation in platelet-rich plasma and ADP induced platelet aggregation in whole blood. Kaptan et al. have studied platelet aggregation in whole blood at 10 healthy volunteers in vitro and found ADP (5, 10 μM) and collagen (2, 5 μg/ml) induced platelet aggregation and significant relationship between G-CSF concentrations (1, 10 and 100 ng/mL) and augmentation of platelet aggregation response.[7] We used lower collagen dose (1 μg/mL) in this study than Kaptan et al. used. One μg/mL-collagen dose may be inadequate to induce platelet aggregation in WB studies because of the WB contains G-CSF receptor bearing cells other than platelets.[11,12] It was reported that 5 μg/ml collagen dose is a potent agonist dose for platelet aggregation.[28] We didn't show any correlation between the degree of increased platelet aggregation and the increased G-CSF concentration contrary to Kaptan et al. found. The cause of this condition may be our start the study with 10 ng/ml G-CSF concentrations and sufficient of this dose to induce of all platelet G-CSF receptors. Because, Shimoda et al. have shown that 10 ng/mL G-CSF concentration attained maximum ADP induced platelet aggregation in vitro.[5]

GM-CSF receptors have been found on megakaryocytes but not on platelets.[29] Shimoda et al. and Avenarius et al. couldn't find any effect on platelet aggregation after the use of GM-CSF.[5,6] In spite of few cases in our study, we couldn't find any effect on platelet aggregation after the addition of GM-CSF to platelet-rich plasma and whole blood at levels achieved with clinical applications. Likewise, Tomer et al. have found no platelet activation and aggregation in in vivo studies performed on Rhesus monkeys.[30] However, venous thromboembolism such as pulmonary thromboembolism, vena

cava inferior thrombosis, thrombosis in the site of venous catheter had been reported after the use of GM-CSF.[2,31] Further large-scale studies must be performed to investigate the platelet aggregation effects or other hematological effects of GM-CSF.

In conclusion, this study showed that G-CSF administration may lead to platelet hyperaggregability. The enhancing effect of G-CSF on platelet aggregation should be taken into consideration in clinical usage.

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