Original Article

Circulating thrombopoietin levels in normal healthy blood donors and in aplastic anemia patients in relation to disease severity

Abhay Singh, Anupam Verma, Soniya Nityanand[1], Rajendra Chaudhary, Priti Elhence

Departments of Transfusion Medicine, [1]Hematology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India

Abstract:

Background: Thrombopoietin (TPO) is the key hematopoietic growth factor regulating the production of platelets from bone marrow megakaryocytes and maintaining platelet hemostasis. This study was done to find any relationship between the levels of thrombopoietin and the severity of disease in patients with aplastic anemia. **Materials and Methods:** Serum samples were collected from 52 patients with a confirmed diagnosis of aplastic anemia and 45 normal healthy blood donors of both sexes over a period of 2 years, and TPO was estimated by using commercially available TPO-specific-enzyme-linked immunosorbent assay. **Results:** The median TPO level of 1190 pg/ml (range 625-7651 pg/ml) in aplastic anemia patients was significantly higher than the median TPO level of 121.1 pg/ml (81.25-237.7 pg/ml) in normal healthy blood donors ($P = 0.000$). No significant difference was observed in TPO levels of male and female patients ($P = 0.453$). The median TPO concentrations observed in very severe aplastic anemia, severe aplastic anemia, and nonsevere aplastic anemia were 2765 pg/ml (range 625-6451 pg/ml), 1190 pg/ml (range 672.1-7651 pg/ml), and 1111.5 pg/ml (range 761.1-2289.2 pg/ml), respectively. TPO in patients of very severe aplastic anemia was significantly higher than patients of nonsevere aplastic anemia ($P = 0.043$), with no significant relation among rest of the groups. **Discussion:** TPO levels in aplastic anemia patients were significantly higher than in healthy blood donors; however, in aplastic anemia patients TPO levels were significantly higher only in patients with very severe disease.

Key words:

Aplastic anemia, blood donor, megakaryocytes, thrombopoietin

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Correspondence to:
Dr. Anupam Verma,
Department of Transfusion Medicine, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India.
E-mail: aver2211@gmail.com

Introduction

Thrombopoietin (TPO), a hematopoietic growth factor is involved in regulation of platelet production by bone marrow. Elevated levels of these hematopoietic growth factors have been observed in several bone marrow failure diseases such as aplastic anemia (AA) that is characterized by pancytopenia and bone marrow hypocellularity.[1,2] It is a well-demonstrated fact that TPO is involved in stimulating the growth of committed megakaryocyte progenitors, with progressive maturation of megakaryocytes, and proplatelet formation.[3]

Several aspects of relationship between platelet concentration and serum TPO levels have been studied but with no conclusive evidence, with some studies indicating the platelets to contain TPO receptors that efficiently bind and remove TPO from circulation,[4] hence, suggesting that circulating levels of TPO are inversely related to platelet mass. Several others opine that TPO is constantly produced *in vivo*, primarily in the liver and kidneys[5] and that the circulating TPO levels are regulated by platelet concentration.[6] Even reports stating that megakaryocyte mass inside bone marrow may regulate circulating TPO levels are also available[7] Recent addition being the exploration of hypothesis that TPO is under circadian control.[8]

The circulatory level of TPO is expected to vary with blood platelet counts; therefore, it is prudent to study factors involved in platelet kinetics in patients with extremely low levels of platelet counts. There is probably no data about the same from Indian subcontinent. Hence, a pilot study was undertaken at our center to estimate the TPO levels in AA patients with varying severity of thrombocytopenia and to ascertain any relation with severity of disease.

Materials and Methods

This two year prospective study was conducted at a tertiary care teaching hospital in north India. The study was approved by the institute's research and ethics committee and was performed after obtaining informed consent from all subjects.

Subjects and Samples

Study population consisted of 52 patients of confirmed diagnosis of AA pertaining to guidelines for diagnostic criteria[9] and 45 healthy blood donors

who served as controls. Criteria for the selection of whole blood donors were in accordance with the Drugs and Cosmetics Act, Government of India.[10] As mentioned by Camitta *et al.*[11] and Bacigalupo *et al.*[12] AA was considered to be severe if bone marrow examination revealed cellularity of < 25% or 25% to 30% with less than 30% of residual hematopoietic cells along with two out of any of the following three features:

1. Neutrophils < 0.5×10^9 /l;
2. Platelets < 20×10^9 /l;
3. Reticulocytes < 20×10^9 /l.

Very severe AA was considered to have similar features as that of severe AA except neutrophils < 0.2×10^9/l. AA patients not fulfilling the criteria for severe or very severe type were graded as nonsevere AA. The demographic and clinical details of patients and donors were recorded from blood donor cards, case files and computer-based hospital information system.

At the time of inclusion in the study, 2 ml of blood sample was collected in Ethylenediaminetetra-acetic acid (EDTA) vials and 5 ml blood sample in plain glass tube. EDTA sample was used for determining platelet counts by automated cell counter (KX 21, Sysmex Corporation, Japan). The clotted sample was centrifuged at 2,000 rpm for 5 min and supernatant serum was stored at –20°C until thawed for TPO testing by enzyme linked immunosorbent assay (ELISA).

Thrombopoietin testing

The test for detecting TPO was done by using DuoSet ELISA Developmental System human TPO (R&D system, catalogue number DY288, Minneapolis, USA) containing the basic components required for the development of sandwich ELISA, as per manufacturer's instructions. Briefly, the capture antibody (mouse antihuman TPO) solution was diluted to the strength of 1.0 µg/ml by reconstituting it with phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl. 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 with pH of 7.2-7.4. The ELISA plate was prepared by pipetting 100 µl of reconstituted capture antibody solution per well and overnight incubation at room temperature (RT). After washing with wash buffer, the plate was blocked by adding 300 µl of block buffer containing 1% bovine serum albumin (BSA) in PBS with 0.05% NaN_3, and incubated at RT for 1 h. The plate was ready to use after second wash with wash buffer. Sample measuring 100 µl was added per well and incubated at RT for 2 h. After washing, 100 µl of detection antibody (biotinylated goat antihuman TPO) reconstituted to the strength of 400 ng/ml in reagent diluent containing 0.1% BSA, 0.05% Tween 20 in Tris buffered saline (20 mM Trizma Base, 150 mM NaCl) was added per well and incubated for 2 h at RT. The color was developed by adding streptavidin horseradish peroxidase and tetra methyl benzidine as substrate solution. The reaction was stopped by adding 50 µl of acid and absorbance was recorded at 450 nm. The lower limit of the sensitivity of the assay was calculated by plotting a standard curve against various sample concentration of the standard solution provided and was found to be 23.1 pg/ml.

Data were maintained on SPSS version 16 (IBM corporation, USA) and Mann-Whitney U test was applied to explore the TPO levels in AA patients on the basis of severity. Linear regression analysis was done and significance was estimated by calculating analysis of variance (ANOVA). A *P* value of less than 0.05 was considered to be significant.

Results

Out of 52 patients, 11 were of very severe aplastic anemia (VSAA), 23 were of severe aplastic anemia (SAA), and 18 were of nonsevere aplastic anemia (NSAA), respectively. Characteristics of patients and normal healthy blood donors are summarized in Table 1. The median age of all patients taken together was 29 years and for donors it was 34 years. Notably, the median TPO level of 1 190 pg/ml (range 625-7 651 pg/ml) in AA patients was significantly higher than the median TPO level of 121.1 pg/ml (range 81.25-237.7 pg/ml) in normal blood donors ($P = 0.00$). The median TPO concentrations observed in VSAA, SAA, and NSAA were 2 765 pg/ml (range 625-6 451 pg/ml), 1 190 pg/ml (range 672.1-7,651 pg/ml) and 1 111.5 pg/ml (range 761.1-2 289.2 pg/ml). TPO in patients of VSAA was significantly higher than patients of NSAA ($P = 0.043$), with no significant relation among rest of the groups [Figure 1].

As expected, the median platelet counts of 18×10^9/l (range 7×10^9/l to 36×10^9/l) in AA patients was significantly lower than the median platelet counts of 193×10^9/l (range 130×10^9/l to 332×10^9/l) in normal healthy blood donors ($P = 0.000$). The median platelet counts observed in VSAA, SAA, and NSAA were 14×10^9/l (range 7×10^9/l to 34×10^9/l), 18×10^9/l (range 9×10^9/l to 24×10^9/l), and

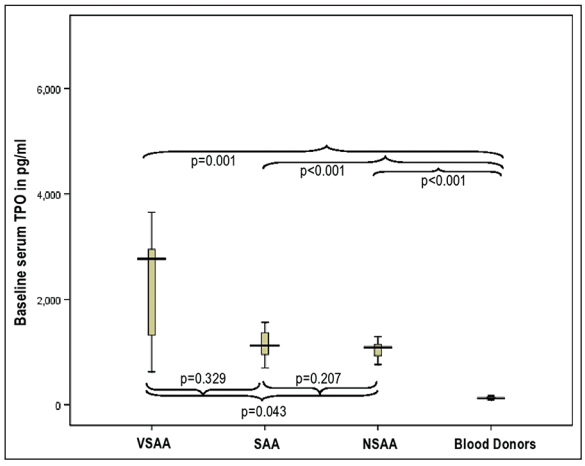

Figure 1: Comparison of serum thrombopoietin among patients of varying severity of aplastic anemia and normal healthy blood donors

Table 1: Characteristics of aplastic anemia patients and normal healthy blood donors

Subject characteristic	Very severe AA	Severe AA	Non severe AA	Blood donors
Number	11	23	18	45
Median age in years (range)	19 (11-58)	29 (11-75)	30 (12-54)	33 (23-46)
Gender (M/F)	10/1	19/4	15/3	40/5
Median TPO in pg/ml (range)	2,765 (625-6 541)	1,190 (672.1-7 651)	1,111.5 (761.1-2 289.2)	121.1 (81.25-237.7)
Median platelet count × 10^9/l (range)	14 (7-34)	18 (9-24)	27 (14-36)	193 (130-332)

27×10^9/l (14×10^9/L to 36×10^9/l), respectively. Platelet counts showed a significant decreasing trend with severity of aplastic anemia except between VSAA and SAA ($P = 0.458$) [Figure 2].

Linear regression analysis with 95% confidence interval, between serum TPO levels and platelet counts in AA patients and normal healthy blood donors, showed no clinically significant correlation ($r^2 = 0.002$), and ANOVA P value = 0.731 and 0.777, respectively [Figures 3 and 4].

Also no significant relation was observed between TPO values in males and females both in AA patients and blood donors ($P = 0.453$).

Discussion

In this study, we estimated serum TPO in 52 AA patients and compared it to that of 45 normal healthy blood donors. We recorded a 10-fold increase in serum TPO levels in AA patients as compared to normal healthy blood donors, which is in concordance to the studies done by Schrezenmeier *et al.*[13] and Jing *et al.*,[14] showing 25-fold and 14-fold increase in German and Chinese population, respectively. Recently in Japan, Abe *et al.*[15] have also documented elevated levels of serum TPO in AA patients. There appears to be great variations in serum TPO in AA patients based on geographic distribution, the reasons for the same are still not elucidated. To the best of our knowledge, ours is the first study exploring the TPO levels in AA patients and in healthy volunteers in Indian population.

Numerous theories have been postulated regarding regulation of TPO in humans but with no conclusive evidence. Kojima *et al.*[16] opined that regulation of TPO in humans is a multifactorial response including up regulation due to feed back mechanism, decreased utilization, and decreased elimination. It was also thought that serum TPO regulation is very closely linked to megakaryocyte mass in bone marrow and that a critical amount of megakaryocyte mass was required to set up negative feedback[7] but Marsh and co-workers[17] found high TPO levels in their study on AA patients who had either reduced or absent bone marrow megakaryocytes challenging the role of megakaryocytes in regulation of TPO. Also, studies showing both megakaryocyte and platelet expressing TPO receptors[18,19] highlighted the fact that there was an inverse relation between circulating platelet and serum TPO.[20,21] Recently Sheding *et al.*[22] have reported that circulating platelets regulates serum TPO levels by binding, internalization and degradation of endogenously produced TPO, which has been previously ruled out by Kojima *et al.*[16] as they did not find any significant reduction in serum TPO levels even after platelet transfusion. The evidences in support for regulation of serum TPO in patients of thrombocytopenia are still insufficient. We observed high levels of serum TPO in AA patients in our study but we were unable to demonstrate any significant correlation between circulating platelet counts and levels of serum TPO in AA patients with varying severity. These findings suggest that some other factors may contribute to regulation of TPO levels.

Similar to the findings of Kojima *et al.*[16] we observed significantly elevated levels of serum TPO in patients of VSAA as compared to NSAA patients, while Marsh *et al.*[17] failed to observed this effect that may be due to lesser number of VSAA patients in their study. On the other hand, we did not observe any significant difference in TPO levels between SAA and NSAA though the trend was higher for SAA patients. Also, we were not able to find any significant correlation between serum TPO levels and

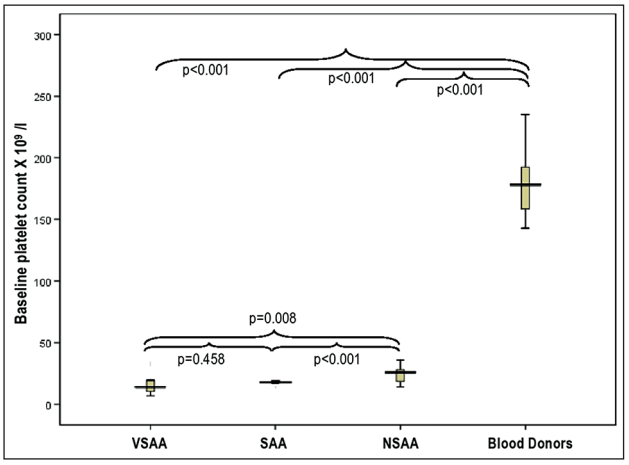

Figure 2: Comparison of circulating platelet counts among patients of varying severity of aplastic anemia and normal healthy blood donors

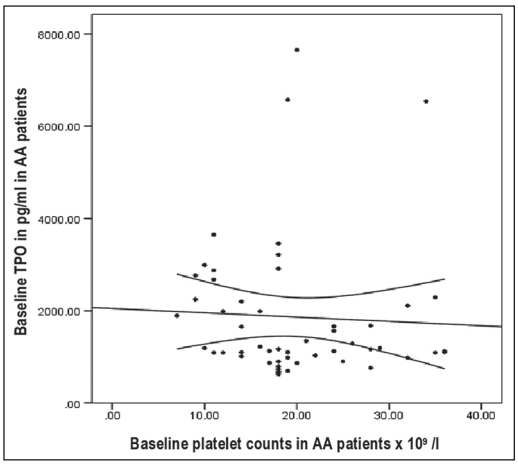

Figure 3: Linear regression analysis with 95% confidence interval indicated by upper and lower curved lines, with each dot representing aplastic anemia patients with their platelet counts corresponding to *x*-axis and TPO levels to *y*-axis

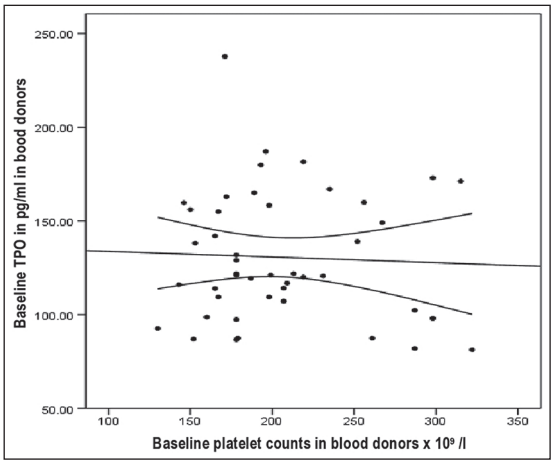

Figure 4: Linear regression analysis with 95% confidence interval indicated by upper and lower curved lines, with each dot representing normal healthy blood donor with their platelet counts corresponding to *x*-axis and TPO levels to *y*-axis

circulating platelet counts that are in line with the observations made by Schrezenmeier *et al.*[13] and Marsh *et al.*[17] Thus, in our study where patients who already had been transfused with blood/component before they were referred to our hospital did not show any correlation. However, Kojima *et al.*[16] did observe a significant correlation between serum TPO and circulating platelets only in the patients who had not received any platelet transfusions. Supporting the observation of Schrezenmeier *et al.*[13] on TPO levels in healthy controls, we also observed no significant correlation between serum TPO levels and circulating platelet counts in normal healthy blood donors.

Thus, this study has provided a glimpse into baseline levels of TPO in AA patients and normal healthy blood donors in Indian population. Our data provide further evidence that supports the concept that measuring TPO level may play some role in classifying the severity of disease in AA and consequently its management that varies according to the severity level. Hence, we recommend measuring serum TPO level as a useful tool in diagnosing thrombocytopenia in AA and other thrombocytopenic disorders. Similarly, evaluation of serum TPO in plateletpheresis donors may help in elucidating the reasons of low platelet counts in such donors who are rendered unfit to donate platelets.

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