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Reference IntervalsDeterminationforvon Willebrand Factor Activity Using Innovance® VWF AC Reagent in Healthy Adults of Indonesia

Yona Mimanda[1], Elizabeth Sidhartha[2], Anton Sumarpo[2], Rahayuningsih Dharma[3]

[1]Department of Clinical Pathology, Faculty of Medicine and Health Sciences, SyarifHidayatullah State Islamic University, Jakarta, Indonesia
[2]Scientific Affairs, PT Sysmex Indonesia, Jakarta, Indonesia
[3]Department of Clinical Pathology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

ABSTRACT— Von Willebrand Factor (vWF) is a multimeric glycoprotein that functions as bridge between platelets and subendothelial tissue, also acts as a carrier of factor VIII. Meanwhile,vWD, a hereditary deficiency of vWF is the most frequent congenital human bleeding disorder that can be classified as type I, II, and III. It has been known that vWF concentration is affected by ABO blood group.The aim of this study is to determine reference value of vWF activities in Indonesian peoples. A cross-sectional study was conducted with 60 subjectsof each O blood group and non-O blood group. The activities of vWF were determined by immune-turbidimetric method using Innovance® VWF Ac Reagent and Sysmex CS-2100i coagulometer. The measurement principle was an increased turbidity due to agglutination after the addition of polystyrene particle coated with anti GP1b and recombinant GP1b (two gain-of-function mutations included) that bound to the antibody as well as to the vWF of the sample. The reference value of vWF activities in O blood group was 24.2%-125.4% while in non-O blood group was 37.7% - 166.1%. There was a significant difference between vWF activities in the two groups ($p<0.005$).Taken together, our study concluded that the vWF activities of blood group was significantly lower than non-O blood group.

KEYWORDS: ABO blood group, inherited bleeding disorders, von Willebrand disease, von Willebrand factor

INTRODUCTION

Von Willebrand disease (vWD) is a congenital bleeding disorder caused by a deficiency or dysfunction of von Willebrand factor (vWF). Moreover, vWD is the most common inherited hemostasis disorder, with a prevalence of 1% of the world's population. It covers various symptoms of bleeding, with most cases are bleeding from the nose, menorrhagia, bleeding after tooth extraction, ecchymosis, and gum bleeding. [1,2]

Von Willebrand factor is a large multimeric glycoprotein that plays a role in primary and secondary hemostasis. In primary hemostasis, vWF assists the adhesion and aggregation of platelets to the damaged capillary subendothelial. In addition, vWF also functions as a protein carrying factor VIII to the wounded area and protects factor VIII from the degradation process. [2,3]

There are several conditions that can increase vWF antigen levels, including inflammation, liver disease, increasing age, pregnancy, use of anti-coagulants, NSAIDs, use of oral contraceptives, stimulation of adrenaline, malignancy, stress, and strenuous physical activity. Besides, another important factor is the ABO blood group system. It was stated that vWF is one of the non-erythrocytic proteins that express ABO antigen. Several studies were constantly found that vWF levels were lower in O blood group than in non-O blood group. [2]

Von Willebranddisease is diagnosed not only based on the level of vWF, but also based on its activity, especially for von Willebrand disease type 2. Lately, vWF activity determination using the ristocetin cofactor has become a reference method for assessing VWF activity, which was initially performed using an aggregometer. However, this method has several disadvantages, such as a high coefficient of variation (around 20-30%) and less sensitivity, especially at low vWF levels. In addition, it also requires longer processing time, sufficient technical expertise and it is highly prone to subjectivity. [2] As a result, many studies were conducted to develop new determination methods. One of them is the examination using the Innovance® VWF Ac reagent thatcan be done automatically using the Sysmex CS-2100i coagulometer to diminish various subjectivity factors.

Our study aims to determine the reference intervals ofvWF activity in O and non-O blood group by using the Innovance® VWF Ac reagent together with the Sysmex CS-2100i coagulometer based on immunoturbidimetric principle.[4] This value can be used as a comparative data on clinical results and as a reference material for further research.

MATERIALS AND METHODS

Study design and period

A cross sectional study was conducted at the Department of Clinical Pathology, Faculty of Medicine, Universitas Indonesia from December 2014 to February 2015.

Ethical considerations

The study protocol was reviewed and approved by The Ethical Committee of Faculty of Medicine, Universitas Indonesia with letter of approval no. 133/UN2.F1/ETIK/2015.

Population and sample size

The participants were 60 people with O blood type and 60 patients with non-O blood type healthy Indonesian adults blood types who underwent medical check-up (MCU) at Dr. CiptoMangunkusumo General Hospital, Jakarta, Indonesia and met the inclusion criteria.

Inclusion criteria

The inclusion criteria were healthy men and women aged 18 - 60 years without any bleeding record or family history of bleeding disorders, fever, and recent NSAID or antihistamine therapy. The female research subjects were not pregnant, menstruating, and using oral contraceptives. All subjects were non-smokers, not using oral anti-coagulant drugs, heparin, desmopressin, epinephrine, and had not done strenuous physical activity prior to blood collection. In addition, hematology laboratory tests (hemoglobin, hematocrit, leucocyte count, platelet count and erythrocyte sedimentation rate), liver function (AST and ALT levels), and renal function (urea and creatinine levels) were within normal range. Moreover, a signed informed consent was also collected from the participants.

Evaluation procedures

2.7 mL of venous blood which was put into a vacuum tube containing 0.3 mL of 0.109 M sodium citrate buffer. Then, the tube was immediately turned over at least 4 times, and centrifuged for 15 minutes at 1500 g to obtain platelet-poor plasma (PPP). The plasma was stable at 15ºC - 25ºC for 24 hours. The Innovance® VWF Ac reagent consists of Reagent I that contains polystyrene microparticles that bind to the monoclonal antibody GPIb. Reagent II contains blocking agent, detergent and polyvinylpyrrolidone, while Reagent III

contains recombinant fragments of GPIb.

At first, the Sysmex CS-2100i coagulometer was turned on for 30 minutes for the heating process. When ready, the sample tubes were placed on the sample rack with the adjusted tube position so the barcode label faced its scanner. For samples without barcode, the sample ID data and inspection options were inputted on the menu screen according to the location of the shelf. When the 'ready' sign appeared at the top of the screen, the ‘start’ button was hit to start the examination process.

The calibration was conducted by using the human plasma standard (SHP). Later, SHP was diluted by adding 1 ml of distilled or deionized water. The stability was checked after thawing for 4 hours at a temperature of 15-25 ºC. The normal and pathological plasma control was liquefied by adding 1 ml of distilled or deionized water. Homogeneity was ensured by rotating the tubes without stirring to prevent the formation of bubbles. Afterward, the samples were stored at 2-8ºC. After the calibration was succesfully done, the precision tests within run and between day the accuracy test were carried out using normal control and pathological control.

Measurement principle

The principle of this examination is the binding between vWF and its glycoprotein Ib (GpIb) receptor, which is also the main vWF receptor on the platelets. The recombinant GpIb in the reagent will bind to vWFthat are present in plasma sample. Sequentially, polystyrene particles coated with antibodies to GpIbbind to recombinant GpIb and the bonding with vWFwill stimulate the formation of particle agglutination. Finally, the turbidity is measured by using a turbidimeter.

Statistical analysis

The data from the within run and between day precision tests were calculated as the mean, standard deviation (SD), coefficient of variation (CV) and deviation (d). The reference value of vWF activity was processed using SPSS program (SPSS ver.20, Chicago, USA). The data were differentiated based on O and non-O (A, B, and AB) blood types. Kolmogorov-Smirnov test was performed to evaluate the normal distribution of the data. If the data are normally distributed, they will be presented as mean, otherwise, they will be presented in median.

If the two groups of data are normally distributed, the parametric test will be used, namely the unpaired t-test to find out whether there is a difference in the reference value of vWF activity based on O and non-O blood group. If one or both of the reference data distributions of the two blood groups are not normal, then the non-parametric test is used, namely the Mann-Whitney test to determine whether there is a difference between the reference value for O and non-O blood group. If the results of the comparative test between the two groups are significant, then the data analysisof O and non-O blood groups will be conducted separately. But if there is no significant difference, the data from O and non-O blood groups will be combined. A value of $p \leq 0.05$ was considered as statistically and clinically significant. If the data distribution is normal, the reference value interval is determined based on the parametric method, namely using a mean of ± 2 SD. If the distribution of reference data is not normal, then the reference interval is determined based on a non-parametric method, namely using the 2.5 to 97.5 percentile.

RESULTS

Within run precision test using normal control obtained a CV of 0.93% and SD of 0.83. Meanwhile, the

within run precision test of pathological controlproduced a CV of 0.69% and SD of 0.17 (Table 1). Between day precision test by normal control obtained a CV of 4.99%, whereas the CV was calculated to be 4.16% using pathological control (Table 2).

Table 1. Within Run Precision Tests Using Normal and Pathological Control

No	Normal Control	Pathological Control
1	88.7	24.6
2	89.9	24.4
3	89.3	24.4
4	89.7	24.6
5	87.7	24.4
6	89.6	24.4
7	88.7	24.2
8	89.9	24.3
9	89.0	24.7
10	90.7	24.7
Target	90	24
Mean	89.32	24.47
SD	0.83	0.17
CV(%)	0.93	0.69%
d (%)	(-1.44) – 0.77	0.83 – 2.91

Table 2. Between Day Precision Test Using Normal and Pathological Control

No	Normal Control	Pathological Control
1	83.8	22.4
2	75.8	23.4
3	77.2	23.5
4	76.6	23.6
5	84.6	21.6
6	84.9	23.6
7	80	22.5
8	74.4	23.7
9	81.1	23.8
10	84.0	21.5
Target	90	24
Mean	80.24	22.76
SD	4.00	0.94
CV(%)	4.99	4.16

The previously calculatedvWF activity data for O and non-O blood group were found to be normally distributed with p values of 0.052 and 0.200, respectively. Furthermore, an unpaired parametric t-test was performed to determine whether there was a difference in vWF activity for O and non-O blood groups. The results of the unpaired t-test showed that there were significant differences between the two groups of O and non-O blood types ($p < 0.005$), therefore, the reference values for the two groups were separately

determined.

As shown in table 3, we found that normal reference value of vWF activity for O blood group was 24.2% - 125.4% and for non-O blood group was 37.7% -166.1%. It is worth noting that vWF activity in O blood group was also found to be lower than non-O blood group.

Table 3. The Reference Valueof vWF level

Coagulometer	O Blood Type (%)	Non-O Blood Type (%)	*P*-value
CS2100i	24.2 - 125.4	37.7 - 166.1	< 0.005

DISCUSSION

Von Willebrand factor is produced by megakaryocytes and, in limited numbers, in the endothelium. In megakaryocytes, vWFs are formed from oligomers ranging in size from 40 kDa to multimers with a size of more than 20,000 kDa. vWF plays an important role in primary and secondary hemostasis. [1,2]

In primary hemostasis, vWF plays a role in the process of attaching platelets to damaged blood vessels, while in secondary hemostasis, vWF acts as a carrier protein for FVIII and protects it from degradation. vWF attaches to platelets via several receptors, especially glycoprotein Ib and GpIIb / IIIa. vWF also binds to a component of the subendothelial matrix, collagen. This is what ultimately attaches platelets to the damaged endothelium. The greater the size of the vWF, the stronger the platelets will stick and form a thrombus to stop bleeding. [2]

There are two forms of von Willebrand disease, i.e: deficiency or reduced level and dysfunction. Clinical symptoms of VWD include moderate to severe bleeding in the form of epictasis, menorrhagia, gum bleedingand gastrointestinal tract. This bleeding phenomenon is caused by defects in primary hemostasis and secondary hemostasis. According to the Revised Classification of vWD, the disease is divided into 6 types with basic characteristics in the form of quantitative (type 1 and type 3) or qualitative (type 2) deficiency, which can also be related to quantitative vWF deficiency. Qualitative vWF deficiency is divided into types 2A, 2B, 2M, and 2N. In type 2A, there is a decrease in function due to the absence of large multimers of vWF. Type 2B has decreased vWF function due to affinity for platelet GpIb. Type 2M decreases in vWF function due to the inactivation of the GpIb binding site on the vWF molecule, and type 2N has a decreased affinity for factor VIII. [1,2]

An important factor that affects vWF level is the ABO blood group system. A person who has O blood type has 15-25% lower vWFactivity compared to non-O blood group. The study by O'Donnel et al. explained that there is the ABH antigen structure in ABO blood group that also present in the vWF attached to the nitrogen (N) group oligosaccharide, namely in the A1 domain. A and B alleles of vWF encode the enzymes glycosyltransferase A and B that help in the formation of the oligosaccharide structure of vWF. In O blood group population, this enzyme is reduced or absent, thereforevWF oligosaccharide structure fails to be formed, resulting in a low activity of vWF. [3,7]

In our present study, weestablished reference values of vWFactivity in healthy adults of Indonesia with a

lower CV and less subjectivity due to the use of an automated coagulation analyzer, Sysmex CS-2100i.. Notably, our established reference values have a wider range when compared with the study by van der Vorm using ristocetin. [8] Consistent with the findings by Nichols et al, we also showed a significant difference of vWF activity in O blood group compared to non-O blood group, in which the former one had a lower vWF activity. [3]

CONCLUSION

Our study has established reference values of von Willebrand factor activity in healthy adults of Indonesia using the Innovance® VWF Ac reagent. The reference value for O blood type was found to be 24.2% - 125.4%, lower thanin non-O blood group (37.7% - 166.1%). Further investigations are encouraged to validate our findings in larger population.

COMPETING INTERESTS

AS and ES are currently employees of PT Sysmex Indonesia. The other authors declare that they have no competing interests.

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