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Original Article

# The Effect of Incubation Time on the Activity and Stability of Factor VIII during the Preparation Process

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## ABSTRACT

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**Background and Aims:** Hemophilia is a rare autoimmune disorder caused by autoantibodies directed in the majority of the cases against clotting factor VIII (FVIII). FVIII is extracted from human plasma or engineered from mammalian cell cultures using recombinant DNA technology. In Iran, most of the used FVIII is prepared from human plasma in Iranian Blood Transfusion Organization. It was seen important to estimate its stability and activity from bleeding time until product preparation.

**Material and Methods:** In the analytical study, 60 healthy male donors (20-50 years old), 15 donors from each blood group, were selected after obtaining informed consent. Donors' blood was collected in QUADRI-PACKs and centrifuged after keeping at 24°C for 2 hours. The separated plasma was divided into three groups and incubated in the lab (22°C) for 0, 90, and 180 minutes, respectively. Then, samples were stored at -20°C for one month. Afterward, the plasma was thawed, and FVIII activity was assayed.

**Results:** The activity of FVIII significantly ($p<0.05$) reduced by delay in freezing; after the time of 0 min: 134.84%±42, after 90 min: 126.88%±38, after 180 min: 120.22%±34. At all incubation times, the highest and the lowest FVIII activity were observed in A and O blood groups, respectively ($p<0.05$). FVIII activity was increased along with increasing age up to 35-40, but it decreased in subjects of 40-50 years old. These experiments confirmed that the longer the delay in freezing fresh frozen plasma, the greater the decrease in FVIII stability.

**Conclusions:**. According to the results of this study, the best blood donors for FVIII product are those with blood group A in the age range of 40-35 years.

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## Introduction

FVIII is a plasma metal ion-dependent protein that its deficiency associates with hemophilia A [1]. The gene for FVIII is located on the X chromosome (Xq28). It is synthesized as a multi-domain, a single-chain molecule with a molecular mass of approximately 300 kDa. FVIII functions as a cofactor for the serine protease, factor IXa, in the anionic phospho-lipid surface-dependent conversion of factor X to Xa [2].

The basic treatment to prevent bleeding in people with hemophilia A is factor replacement therapy. This is the infusion of FVIII to control bleeding. It comes from two sources: human plasma or DNA technology called recombinant FVIII. Fresh frozen plasma (FFP) was the first manner of treatment for hemophilia A, but it contained only low amounts of FVII, and large volumes of FFP were needed to inject to stop bleeding episodes. In the mid-1960s, FFP was allowed to thaw in the cold. The precipitated plasma, with more FVIII in a smaller volume, could be stored in frozen form as "cryoprecipitate" [3, 4].

By the late 1960s, FVIII was separated from pooled plasma by developing methods and lyophilized in packaged bottles in an accurate dose. This lets the patients with hemophilia A to home treatment [3]. By the early 1980s, it was found that deadly blood-borne viruses, including hepatitis viruses and human immunodeficiency viruses, could be potentially transmitted by human blood and its derived products. Viral inactivation methods and methods used to screen viruses in blood donation greatly improved the safety of plasma-derived products; yet there were still concerns about this [5]. In 1984, recombinant human FVIII was produced by gene cloning method [6]. Although the risk of pathogen transmission may be minimized by recombinant factor VIII, some study reported that the patients' immune system would be more stimulated by recombinant form [7]. Plasma-derived FVIII concentrates have von willebrand factor (VWF), which would mask the epitope sites on the FVIII molecule or would prevent FVIII endocytosis by dendritic cells [7-9].

Although recombinant FVIII concentrates are readily accessible, in a developing country such as Iran, cryoprecipitate is still an important plasma product to provide a concentrated form of factor VIII. Because of the low half-life of the enzyme which is about 8-12 hours, it is important to optimize the steps of cryoprecipitate production [10]. Among the most critical factors affecting yield are storage time of whole blood and procedures for freezing, thawing, and reconstitution [11]. The most variable factor that may affect the activity of FVIII is the time and temperature between donation and the start of the freezing process before providing cryoprecipitate. Also, it was hypothesized that the activity of the FVIII might be different among different blood groups. Considering three different incubation times before FFP preparation, the activity and stability of FVIII



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were investigated among different blood groups.

## Materials and Methods

This study was conducted in Alborz Blood Transfusion Organization. Sixty male blood donors (age: 30-50 years), fifteen from each blood groups, were selected following health examinations. The study was approved by the Ethics Committee of Alborz Blood Transfusion Organization. The aim of the study and methodology were explained to all the participants, and they signed written informed consent. People with any kind of medication and high blood pressure and those who had problems with blood clotting were prevented from entering into the study. Also, the partial thromboplastin time test performed for all participants who did not have the above-mentioned problems to ensure they had not any problem in the intrinsic pathway of blood coagulation. The acceptable partial thromboplastin time test was in the range of 35-40 sec. The blood group of the participants was determined by HiPer® Blood Grouping kit.

**Blood collection**

Sixty units of whole blood (fifteen units from each blood groups A, B, AB, O) were collected (450±45 mL) from random donors in QUADRI-PACK and conserved with citrate-phosphate-dextrose adenine. QUADRI-PACK is a storage set used for red blood cells that divides the original red blood cells unit into four total bags. Immediately after blood collection, blood units were incubated at 20-24˚C for 2 hours. Then, the whole blood was centrifuged 3600g for 9 min. For each unit, the plasma was separated from precipitated cells into one bag. After weighing the separated plasma, it was divided into three equal volumes, weighed again. After sealing each of the three bags, one bag was immediately stored at -20˚C, and the second and third bags were stored at -20˚C after 90 and 180 min, respectively, for one month.

**Measurement of FVIII activity**

FFPs were thawed in a water bath at 37°C before measurement of FVIII activity. The activity of FVIII was measured in duplicate using one-stage clotting assay with reagents from Diagnostica Stago and STA instrument. FVIII activity was expressed as a percentage of the reference plasma, which had an assigned value of 100% [12].

**Statistical analysis**

SPSS statistics software was used to perform only statistical operations. FVIII activities were expressed as the mean±standard deviation for the three times of plasma preparation. The normality of the data was determined. ANOVA and Tukey's test (pair-wise comparisons) were used to determine differences between individual groups. P values less than 0.05 were considered statistically significant.

## Results

After each FFP preparation, it was divided into three equal volumes as mentioned before section. One of three volumes was immediate transmitted into -20˚C. The second and third volumes were stored at -20˚C after 90 min. and 180 min. staying in lab temperature (22˚C), respectively. The immediately time was



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considered as the first time, and 90 min. and 180 min. were considered as the second and third times, respectively. The FVIII activities (mean±SD) with the 95% confidence interval in three lab incubation times were as 134.84%±42, 126.88%±38 for the first, second, and third times respectively. 120.22%±34. Hence, FVIII activity was decreased significantly ($p<0.05$) by delay in FFP freezing after preparation.

**Comparison of FVIII activity in blood groups**

The mean FVIII activity (%) was examined for the four ABO blood groups (Table 1). One-way ANOVA analysis showed significant differences among blood groups ($p<0.001$) and also among defined times for each group ($P<0.001$) in FVIII activity. Subjects with blood group A had a significantly higher FVIII activity than others ($p<0.05$) as followed by B, AB, and O blood groups. Although, FVIII activity in B blood group was significantly higher than AB and O blood groups ($p<0.05$), the difference among AB and O blood groups did not show any statistical significance ($p>0.05$). All blood groups showed a significant decrease in the FVIII activity with increasing delay in FFP freezing after preparation.

**Comparison of FVIII activity between different age groups**

Figure 1 shows the level of FVIII activity in different age groups of each ABO group. The correlations between FVIII activity and age were checked using regression analysis. Our results revealed a linear and positive correlation ($r=0.9$, $p<0.001$) between FVIII activity and age up to 35-40 years; however, a negative correlation was observed ($r= -0.54$, $p<0.05$) by increasing age (Fig. 2).

**Table1.** Comparison of FVIII activity among four ABO blood groups during three examination times

| Blood group | Activity (%) 1th time | Activity (%) 2nd time | Activity (%) 3rd time | P-value |
|---|---|---|---|---|
| **A** | 149.92±38 | 142.57±42 | 135.14±39 | p<0.05 |
| **AB** | 128.11±46 | 118.96±45 | 109.41±41 | p<0.05 |
| **B** | 138.10±44 | 131±42 | 128.49±41 | p<0.05 |
| **O** | 123.26 ±34 | 115±40 | 107.86±42 | p<0.05 |
| **p-value** | p<0.001 | p<0.001 | p<0.001 | |

Values are presented as means ± SD.

## Discussion

FVIII is one of the clotting factors processed at the Blood Transfusion Center for hemophilia A patients. Although concentrated and lyophilized FVIII and its recombinant form have been available for many years, cryoprecipitate prepared from FFP have, to date, been used in some countries such as Iran. To optimize its production, it is essential to know the effect of various factors such as delay times on the FVIII activity during its preparation. The half-life of FVIII is short and about 8-12 hours.





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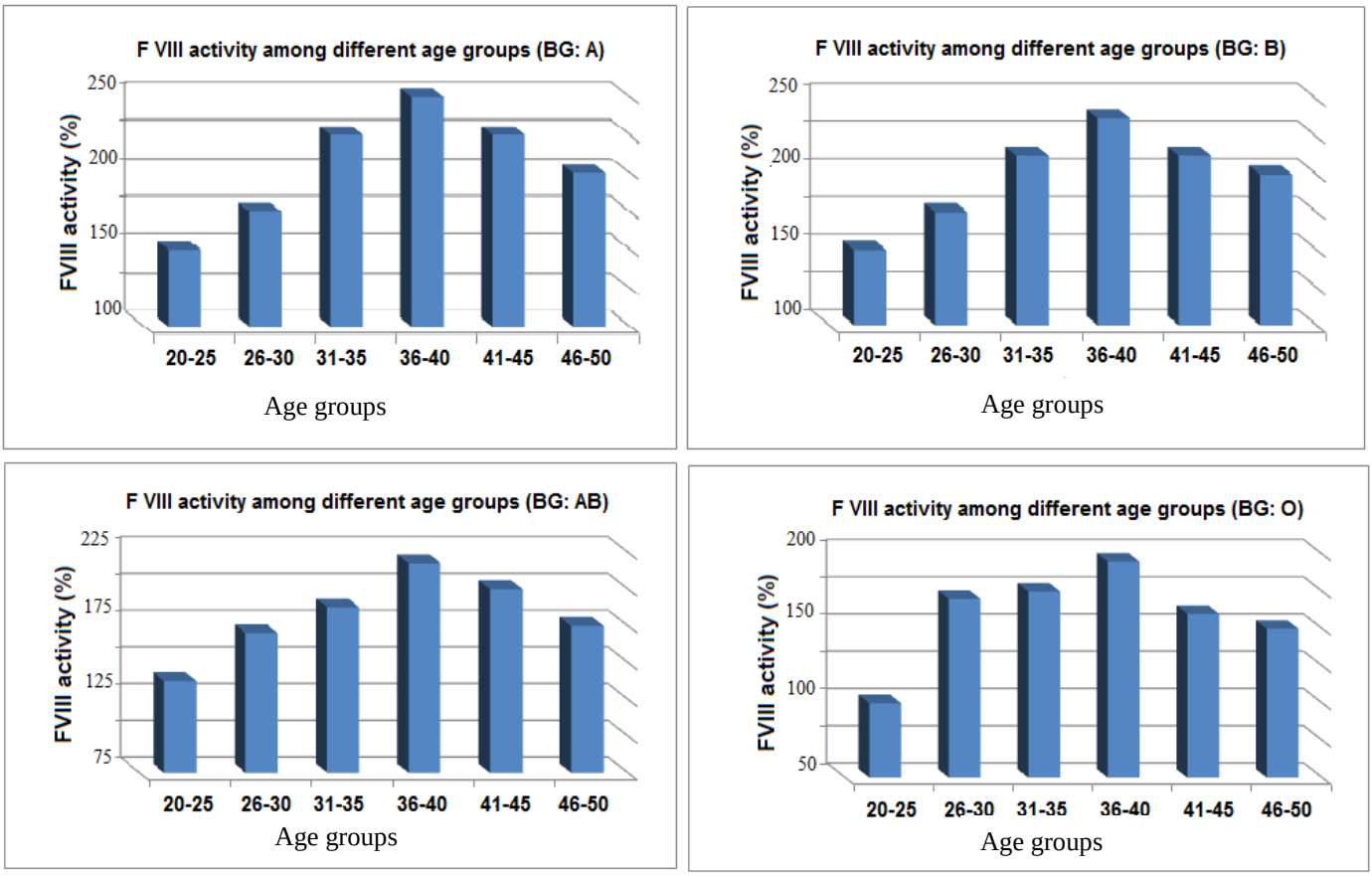


**Fig. 1.** Comparison of FVIII activity among different age groups. BG=Blood groups

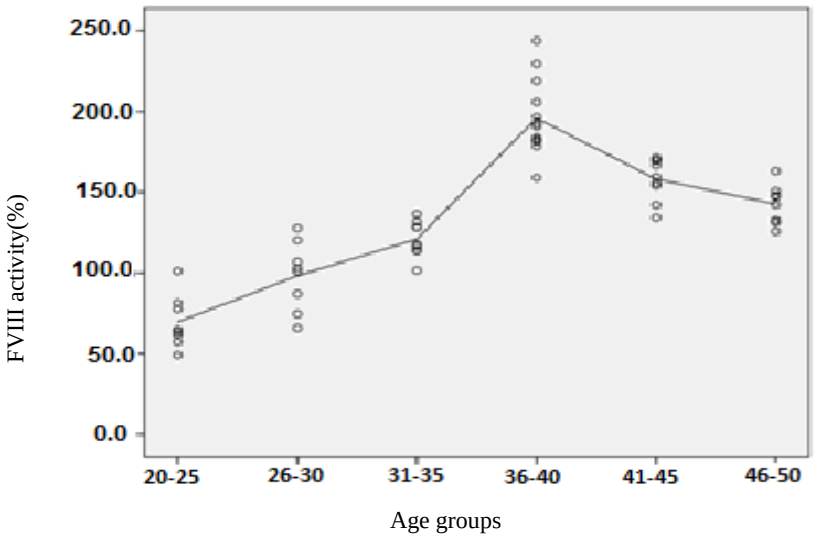


**Fig. 2.** Correlations between FVIII activity and age groups

Like most of the proteins, it is more stable at low temperature and gradually loses its activity out of the refrigerator and rapidly in high temperature. In this study, the initial incubation time (2 h) and temperature (22-24˚C) after blood donation and before centrifuge of whole blood were considered according to the routine program performed in



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Blood Transfusion Center in Alborz province. The results of the study showed that 90 and 180 min delay in FFP freezing significantly decreases FVIII activity in compassion to immediate FFP freezing. Carlebjörk et al. reported that in plasma FVIII was stable for at least two h at room temperature [13]. Smith et al. showed that the percentage of FVIII activity significantly decreases by holding plasma at 1-6°C for 2, 8, 15 hrs, respectively, before freezing [14]. Swärd-Nilsson et al. found that storage at room temperature for six h causes a small but statistically significant decrease in FVIII. They concluded that for an optimal yield of FVIII, freezing should start within four h after plasma donation. Our results demonstrated that FFP freezing should be done within two h after plasma donation [15].

VWF is the specific carrier of factor VIII in plasma and protects it from proteolytic degradation, prolonging its half-life in circulation and efficiently localizing it at the site of vascular injury. According to the previous studies, there is a close relationship between plasma levels of Von Willebrand and factor VIII [16]. It is a large multimeric glycoprotein that its plasma levels differ among people. The variability in its plasma levels depends on some factors such as age, race, ABO and Lewis blood groups, epinephrine, inflammatory mediators, and endocrine hormones [16, 17]. Some studies reported that group O and group AB subjects have the lowest and the highest plasma Von Willebrand levels, respectively [17, 18]. Other studies, based on genotype found that genotype OO individuals have the lowest plasma VWF levels and heterozygous individuals for the O allele (genotypes AO, BO) possess significantly lower plasma VWF levels than those not carrying an O allele (genotypes AA, AB, BB) [16, 19]. According to our results, the order of increase in FVIII activity was from blood group O with the lowest, then AB, B and with highest in blood group A. Song et al. found FVIII activity as the lowest in subjects with blood group O and the highest in those with either B or AB. They described the variations in results likely due to intrinsic genetic variability and environmental factors [20]. Also, the results of FVIII activity and its relationship with blood group partially accord with the results of VWF. As Smith et al. suggested, the transport and chaperoning function of VWF for FVIII may be responsible for the association between ABO and FVIII activity [21].

In this study, the relationship between FVIII activity and age groups was also evaluated among the blood groups. Wang et al. reported that FVIII and VWF levels show significant and positive relationships with age [22]. Cohen et al. revealed a linear increase in VWF and FVIII with increasing age [23]. Our results identified that FVIII activity increased along with increasing age up to 35-40, but it decreases in subjects of 40-50 years old. The reduction in FVIII activity was not statistically important in the subjects who were over 40 years old. Since the age defined for blood donation is up to 50 years, the changes in FVIII were evaluated up to middle age. Little is known about the mechanisms that control





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the changes in VWF and FVIII levels with age. Aging is characterized by the accumulation of damage and other harmful changes, leading to decrease in some protein activity, although an increase in their gene expression may be observed [24]. In spite of these interpretations, the evaluation of such changes in FVIII activity with increase in age need more molecular and biochemical studies.

## Conclusion

Given that FVIII is one of unstable blood clotting factors and as a quality index for FFP and cryoprecipitate, it is necessary to optimize FFP production conditions to prevent a decrease in FVIII activity. According to the results of this study, the best time of plasma freezing is immediately after FFP production, and the best blood group and age for FVIII product are blood group A and the age group 35-40.

## Conflict of Interest

The authors have no conflict of interest.

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