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Effect of freezing on IgG and coagulation protein levels in equine plasma: case study

.

María Isabel Calero Granda

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María Isabel Calero Granda

Calificación: **/**

Nombre del profesor, Título académico: **Eduardo Díaz, M.V, Msc., PhD**

Firma del profesor: **_____**

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Firma del estudiante: _____

Nombres y apellidos: María Isabel Calero Granda

Código: 00124449

Cédula de identidad: 1720287836

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RESUMEN

En medicina equina, los productos de plasma se han utilizado como fuente de inmunoglobulinas, agentes anti endotóxicos y para proporcionar soporte coloidal para una gran variedad de patologías. El plasma es la porción líquida de la sangre equina y está compuesto por nutrientes, gases, hormonas, minerales y proteínas. Las proteínas de coagulación presentes en el plasma son importantes en los procesos hemostáticos y se pueden medir por tiempos de protrombina. Otro componente importante del plasma es la inmunoglobulina G, que es importante en las transfusiones para potros recién nacidos. Se describen varias técnicas para recolectar plasma. Se llama plasma fresco congelado cuando se recolecta por centrifugación de sangre completa o plasmaféresis y se congela dentro de las primeras 8 horas después de la recolección. Mientras que, el plasma congelado se colecta por sedimentación de 12 a 48 h y luego por decantación de glóbulos rojos, entonces se congela. Hasta el momento, no se ha publicado información sobre los tiempos de protrombina y los niveles de IgG en plasma equino. Por lo tanto, este estudio de caso ha medido los tiempos de protrombina y los niveles de IgG en plasma fresco congelado y plasma congelado del mismo donante antes y después de la congelación. El estudio reveló una mayor disminución en los niveles de IgG del plasma congelado que en el plasma fresco congelado después de 50 días de almacenamiento a -20 ° C. Este resultado sugiere que se recomendaría plasma fresco congelado para transfusiones cuando se trata la falla de la transferencia de inmunidad pasiva. El plasma fresco congelado y el plasma congelado aumentaron los tiempos de protrombina después de la congelación y ambos estaban fuera del rango normal. Este resultado tiene una gran relevancia al hablar de transfusiones de plasma para deficiencias de factores de coagulación, ya que es evidente que los factores de coagulación se ven afectados por el almacenamiento de 50 días a -20 ° C.

Palabras clave: congelación, equino, IgG, inmunidad, protrombina, plasma, plasma congelado, plasma fresco congelado.

ABSTRACT

In equine medicine, plasma products have been used as a source of immunoglobulins, anti-endotoxin agents, and to provide colloidal support for a huge variety of pathologies. Plasma is the liquid portion of equine whole blood and is composed by nutrients, gases, hormones, minerals and proteins. Coagulation proteins present in plasma are important in hemostatic processes and can be measured by prothrombin times (PT). Another important component of plasma is immunoglobulin G which is important in transfusions for newborn foals. Various techniques are described for harvesting plasma. It is called fresh frozen plasma when it is collected by centrifugation of whole blood or plasmapheresis and is frozen within the first 8 hours after collection. Whereas frozen plasma is collected by 12- 48 h sedimentation and followed by decantation of red blood cells, then it is frozen. So far, no information about PT times and IgG levels on equine plasma have been published. Therefore, this case study has measured PT times and IgG levels on fresh frozen plasma and frozen plasma from the same donor before and after freezing. The study revealed a higher decrease on frozen plasma then in fresh frozen plasma IgG levels after 50-day storage at -20°C. This result suggests that fresh frozen plasma would be recommended for transfusions when treating failure of transfer of passive immunity. Fresh frozen plasma and frozen plasma increased PT times after freezing and both were out of the normal range. This result has a high relevance when talking about plasma transfusions for clotting factor deficiencies since it is evident that clotting factors are affected by 50-day storage at -20°C.

Key words: equine, freezing, fresh frozen plasma, frozen plasma, IgG, immunity, prothrombin, plasma.

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INTRODUCTION

In equine medicine, plasma products have been used as a source of immunoglobulins, as anti-endotoxin agents, and to provide colloidal support (Tennet-Brown, 2011). This therapy is used specially in plasma content deficiencies, such as protein losing enteropathy, septicemia, endotoxemia, failure of transfer of passive immunity in foals, pleuritis, nephropathy, disseminated intravascular coagulation (Feige, Ehrat, Kästner & Schwarzwald, 2003). They have been recognized as a valuable resource for more than a century (Ziska, Schumacher, Duran & Brock, 2012).

Plasma is the remaining liquid portion of equine whole blood after removing the cells and 90% of this non-living matrix is water. It contains nutrients, gases, hormones, minerals and proteins that are indispensable for vital functions. Proteins are the most abundant component. They regulate biochemical reactions, act as carriers for plasma constituents, provide colloid osmotic pressure, and participate in coagulation and immunity (Ziska, 2009). Coagulation proteins present in plasma are very important in hemostatic processes and screening tests such as prothrombin time (PT) are fundamental to asses it. PT is used to detect disorders involving the activity of the factors I, II, V, VII, and X of the extrinsic and common pathways (Casella, Giannetto, Fazio, Giudice & Piccione, 2009).

Another compound of plasma are globulins, a heterogenous mixture of proteins (Ziska, 2009). The most abundant immunoglobulin in plasma is the IgG and it has a relatively long half-life of three weeks. A healthy adult horse should have an IgG range of 1500 - 2000mg/dL (Tizard, 2004; Mudge & Williams,2016). There are several methods for measuring IgG. Radial immunodiffusion (RID) is the gold standard for IgG concentration measurement in horses (Lunn & Horohov, 2004). Equine IgG can also be determined by ELISA in plasma or serum. A semiquantitative, stall-side ELISA has been developed for measuring IgG in the field (Idexx SNAP Foal IgG Test Kit, Greensboro, NC). Another method used is Turbidimetric immunoassays (TIA), based on a specific antigen-antibody reaction in the presence of excess antibody concentration, enabling accurate quantitation of circulating IgG concentrations. This method has been proved to give results highly correlated to RID (McCue, 2007).

Various techniques have been used over the past years to harvest plasma from equine donors (Ziska, Schumacher, Duran & Brock, 2012). Plasma can be considered fresh frozen plasma when it is collected by centrifugation of whole blood or plasmapheresis and must be frozen

within 8 hours of collection to preserve coagulation factors II, VII, IX, X, XI, and XII (Tennet-Brown, 2011). To reduce costs, some hospitals do gravity sedimentation of whole blood in the refrigerator (~ 4^{o}C) for 12- 48 hours, followed by the decantation of plasma; this plasma is termed frozen plasma because it is frozen 8 hours after collection (Wilson, *et.al*, 2009). This plasma has been proved to preserve VII, VIII, IX, XI, XII and antithrombin, but factor X has been shown to be decreased (Mudge & Williams, 2016). Both techniques result in plasma containing small amounts of erythrocytes, which might be enough to cause sensitization on the recipient horse. Also, as it is an open collecting method, bacterial contamination can occur (Tennet-Brown, 2011). On the other hand, plasmapheresis appears to be the most convenient method for plasma extraction where the donor is part of a closed loop with blood withdrawn continuously and immediately centrifuged (Feige, Ehrat, Kästner & Schwarzwald, 2003).

There are several clinical uses for plasma. Hyperimmune plasma for example, which is produced by donors with a high concentration of circulating immunoglobulins against specific pathogens, is usually fresh frozen plasma (Tennet-Brown, 2011; Mudge & Williams, 2016) and can be used to help fighting specific pathogens when transfused. For instance, plasma transfusion is often used in foals as a treatment for failure of passive transfer (FPT), as prophylaxis to *Rhodococcus equi* or as part of the treatment of critically ill foals (Hollis, Wilkins, Tennet-Brown, Palmer, & Boston, 2016). Since mares have a diffuse epitheliochorial placenta type, the transplacental transfer of maternal antibodies *in utero* is not possible (Erhard, Luft, Remler & Strangassinger , 2001). Therefore, newborn foals critically depend on the ingestion of colostrum until they can make their own immunoglobulins. Failure of passive transfer in foals can be rated according to their IgG concentrations once they have passed their first 24 hours. Complete failure of passive transfer in foals is defined as an IgG concentration lower than 400 mg/dl. A partial FPT refers to foals with an IgG concentration that ranges between 400 and 800 mg/dl. Adequate levels of IgG are considered over 800 mg/dl (McCue, 2007; Tennet-Brown, 2011; Mudge & Williams, 2016). It also provides colloid osmotic pressure due to its protein content. Furthermore, it is an economical option in foals but not in adults because of the large volumes needed (Ziska, 2009). This kind of treatment is also recommended for Intravascular Disseminated Coagulation (IDC) which is secondary to a massive inflammation, representing an imbalance between procoagulant and fibrinolytic systems (Mudge & Williams, 2016).

There is no published data about IgG levels and PT times on stored equine plasma. Therefore, the purpose of the current study is to evaluate the effect of freezing on plasma quality by measuring IgG levels and prothrombin times in fresh frozen plasma and frozen plasma from the same donor before and after freezing.

MATERIALS & METHODS

A 12-year-old Draft gelding horse was used as donor; the horse had passed a clinical inspection, had all the vaccines up to date and was negative to Coggins test for equine infectious anemia. Also, PCV (Packed Cell Volume) and total proteins were measured to determine if it was an ideal donor. His PCV was 40% and total proteins 6,4.

For whole blood collection donor was restrained in a stock. A 13G 1 ½ inch needle was placed on the left jugular vein. The puncture site was previously clipped and sterile prepped (Feige, Ehrat, Kästner & Schwarzwald, 2003). Blood was collected on PCV blood collection bags (TERUMO, 2019) containing CPDA- 1 (citrate phosphate dextrose adenine) as anticoagulant and stabilizer which is the suggested anticoagulant for blood storage (Mudge & Williams, 2016). During extraction the bag was laid over a scale to guarantee 450 ml of blood were extracted. The blood bag was gently waved to ensure anticoagulant and blood was mixed. Experimental fresh frozen plasma was achieved by the extraction of a 10 ml sample of blood mixed with the anticoagulant, and then it was centrifuged for ten minutes at 4000 G for 5 minutes (Feige, Ehrat, Kästner & Schwarzwald, 2003; Mudge & Williams, 2016). Plasma was extracted from the tube and divided into two tubes. One tube was used for testing prothrombin time and IgG levels by turbidimetry technique within the first hour after extraction. The tube was transported in refrigeration to maintain coagulation factors. The other tube was frozen for 50 days at -20°C.

The blood bag was stored at 4°C for 48 hours to allow erythrocyte sedimentation for frozen plasma. After 48 hours erythrocytes were decanted from the blood bag, leaving just plasma on the bag. Two 10 ml sample were extracted in a non-anticoagulant tube. One was used to measure prothrombin time and for measuring IgG. Plasma bag and the remaining tube were frozen for 50 days.

On day 50 fresh frozen plasma tube was defrosted in a warm water-bath not exceeding 37.8°C, then it was submitted for prothrombin time testing and IgG turbidimetry testing. The same was done with the frozen plasma tube on its day 50.

RESULTS

Table 1 shows the results obtained on IgG levels and PT times on fresh frozen plasma and frozen plasma before freezing and 50 days after freezing. For fresh frozen plasma IgG levels before freezing were 1683 mg/dL and prothrombin time was 11.1 seconds. Whereas, for frozen plasma initial IgG levels were 1647 mg/dL and prothrombin time was 11.4 seconds. After 50 days of freezing the results obtained for IgG and prothrombin levels for fresh frozen plasma were 1557 mg/dL and 14.1 seconds respectively. While, for frozen plasma IgG was 1185 mg/dL and PT was 14.4 seconds.

Plasma Sample	**IgG before freezing (mg/dL)**	**PT before freezing (s)**	**IgG after freezing (mg/dL)**	**PT after freezing (s)**
Fresh Frozen Plama	1683	11.1	1557	14.1
Frozen Plasma	1647	11.5	1185	14.4
Difference	36	0.4	372	0.3

Table 1. Effect of freezing on frozen plasma and fresh frozen plasma IgG and PT levels

Figure 1shows the decrease of IgG concentration on both samples after 50 days of freezing. Fresh frozen plasma IgG decreased 126 mg/dL after 50 days of freezing, which means it decreased an 8%. On the other hand, frozen plasma IgG decreased 462 mg/dL, which means it decreased 28.05% after 50 days of freezing.

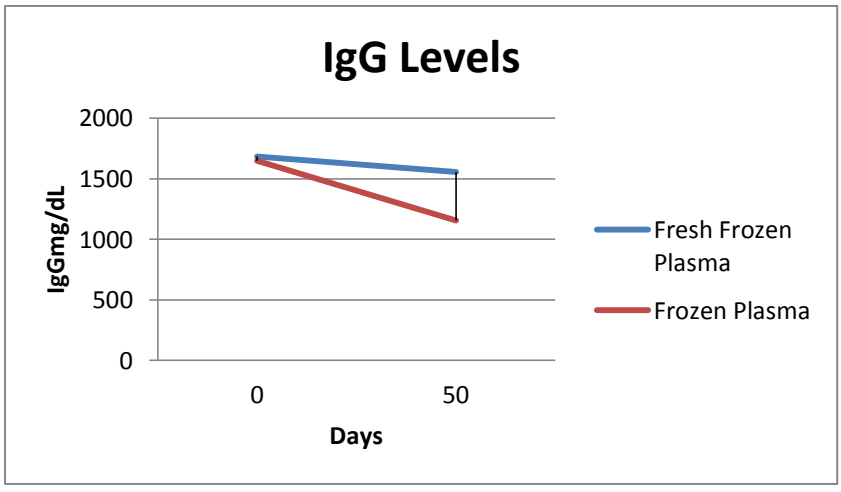

Figure 1. IgG variation before and after freezing on frozen plasma and fresh frozen plasma

Prothrombin time results on fresh frozen plasma were 11.1 seconds and after 50 days of freezing increased to 14.1 seconds. On the other hand, frozen plasma were 11.5 seconds before freezing, this increased to 14.4 seconds after 50 days of freezing.

DISCUSION

This study measured IgG levels and PT times before freezing and after 50 days of storage at - 20°C, as the these are elements of this product with clinical importance, Plasma administration is an important factor on treatment for critically ill horses. It is used for treating failure of transfer of passive immunity, clotting factor deficiencies, hypoalbuminemia, and disseminated intravascular coagulation in horses (Tennet-Brown, 2011; Wilson, et.al, 2009). For this reason, plasma is available in many practices, some of them have commercial fresh frozen plasma and some other practices have their own frozen plasma (Casella, Giannetto, Fazio, Giudice & Piccione, 2009).

The result of this study reveals that IgG levels of fresh frozen plasma decreased an 8% after freezing. Despite having a decrease on IgG levels after freezing, these were maintained within the normal range of 1500 – 2000mg/dL established in the literature (Tizard, 2004). On the other hand, frozen plasma IgG levels decrease 24% after freezing which shows that frozen plasma levels can drop under the normal range after freezing. These results demonstrate that IgG levels are affected by 50-day storage at -20°C. The difference of 36 mg/dL on IgG levels before freezing between fresh frozen plasma and frozen plasma suggests that 48h of storage at 4°C and decantation decreased IgG levels on frozen plasma. The difference on the results between fresh frozen plasma and frozen plasma can be supported by the fact IgG has a half-life of three weeks (Tizard, 2004; Mudge & Williams,2016). However, the values ranking this difference are among the normal values.

Even though our results showed that prothrombin times were not preserved within the reference range after freezing on either fresh frozen plasma or frozen plasma, a difference between fresh frozen plasma and frozen plasma was shown on prothrombin times. Before freezing, fresh frozen plasma PT times were lower than frozen plasma PT times, both however, were within the reference range. This demonstrates that 48 h storage at 4°C does not make a considerable alteration on PT times. However, it could be inferred that coagulation factors are affected by 48h of storage and decantation before freezing (Wilson, et.al, 2009). On the other hand, once both samples were defrosted after 50 days, PT times were considerably increased and actually out of the normal range. It is important to note that before and after freezing PT times were longer in frozen plasma. This suggests that the storage of plasma for 48 hours has no influence on PT times. Whereas the storage for 50 days at -20°C does have an effect increasing PT times. Considering that PT detects disorders

on the activity of the factors I, II, V, VII, and X of the extrinsic and common pathways (Casella, Giannetto, Fazio, Giudice & Piccione, 2009), the present study suggests that this factors are not considerably affected by 48h storage at 4°C. On the other hand, it suggests that I, II, V, VII, and X clotting factors are considerably affected by 50-day storage at -20°C on both fresh frozen plasma and frozen plasma.

In summary, the results of this study indicate a decrease on fresh frozen plasma and frozen plasma IgG levels after 50-day storage at -20°C. This decrease was more evident on frozen plasma with a 28% decrease comparing with the IgG levels of the same sample before freezing. The comparison with the 8% IgG decrease on fresh frozen plasma suggests that that 48h of storage at 4°C and decantation affects IgG levels. This result has a clinical importance as plasma is used for treating failure of transfer of passive immunity and IgG levels of the transfused plasma should be within the normal range. It could be concluded that fresh frozen plasma would be the recommended one for this purpose. Fresh frozen plasma and frozen plasma had similar PT times, both within the normal range before freezing. After freezing both increased and were out of the normal range. This result has a high relevance when talking about plasma transfusions for clotting factor deficiencies as the mentioned clotting factors will be considerably affected. More studies should be done to assess for how long plasma can be stored at -20°C without altering its clotting factors. For reaching more reliable results another study with more samples will be required for reaching consistent results.

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